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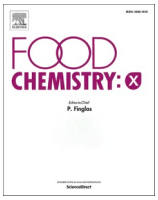

Adaptive laboratory evolution of *Lachancea thermotolerans* for enhanced production of 2-Phenylethanol and 2-Phenylethyl acetate in wine

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ABSTRACT

2-Phenylethyl alcohol (2-PE) and 2-phenylethyl acetate (2-PEA), responsible for "rose," "fruity" and "floral" aromas in wine, are derived from *L*-phenylalanine (L-Phe). *L. thermotolerans*, a common yeast used in wine fermentation, has a limited ability to utilize L-Phe, restricting 2-PE and 2-PEA production. This study aimed to evolve an L. *thermotolerans* A38 strain with enhanced L-Phe utilization through ALE, in order to increase the production of the wine aroma compounds 2-PE and 2-PEA. After 200 generations of adaptive laboratory evolution (ALE), four mutants were selected, exhibiting an 82.8 % increase in 2-PE (from 15.06 to 28.22 mg/L) and a 2.07-fold increase in 2-PEA (from 75.73 to 157.27 μg/L) in chemically defined grape juice medium (CDGJM). The evolved strains also showed improved tolerance to high sugar concentrations, SO_2, ethanol, and pH. In wine micro-vinification, they produced 14.02-fold (from 47.63 to 667.96 mg/L) more 2-PEA and 4.10-fold more 2-PE than the original strain, surpassing the commercial yeast CT10. This study demonstrates ALE as an effective strategy for boosting 2-PE and 2-PEA production in wine.

1. Introduction

Aroma is a critical sensory characteristic of wine, attracting significant attention from both experts and consumers. The "floral" attribute, plays a crucial role in evoking positive and joyful feelings among consumers. Wines with a "floral" scent are more readily accepted and preferred by consumers (Cordente et al., 2018). 2-Phenylethyl alcohol (2-PE) and its derivative 2-phenylethyl acetate (2-PEA) are characterized in wine for their "rose," "fruity," and "floral" aromas (Lyu et al., 2021). During winemaking, yeast converts *L*-phenylalanine (L-Phe) via the Ehrlich pathway, involving transamination, decarboxylation, and reduction, to produce 2-PE and 2-PEA (Zhu et al., 2024). However, L-Phe, an aromatic nitrogen source, is not ideal for microbial growth. When more accessible nitrogen sources are available, a fraction of L-Phe is degraded and enters the tricarboxylic acid cycle, inhibiting efficient 2-PE synthesis (Behringer et al., 2024; Dai et al., 2021). Study showed that compared with the control group, the contents of 2-PE and 2-PEA in wine samples supplemented with 60 mg/L L-Phe were significantly increased, and the wine samples had more floral characteristics and higher aromatic complexity (Valera et al., 2024). Although the exogenous addition of L-Phe has no significant effect on the basic physical and chemical indexes of wine (such as alcohol content and soluble solids), it will slow down the growth of yeast and is not conducive to the cost control of wine production. Therefore, breeding yeast capable of efficiently utilizing L-Phe to enhance the production of 2-PE and 2-PEA is of great practical significance for enriching the 'floral' and 'fruity' aromas in wine.

Laboratory adaptive evolution (ALE) is an effective technique for enhancing the production of desired compounds in microorganisms (Chen et al., 2024). Unlike metabolic engineering, ALE does not require consideration of the intricate and interconnected metabolic networks within cells. Instead, it involves creating suitable selection pressures that align with the desired phenotype (Shi et al., 2022). Yeast is particularly suitable for this process due to its straightforward nutritional needs and rapid reproduction. Under specific pressures, yeast can accumulate advantageous mutations through natural evolution, adaptation, and selection, allowing it to adjust better to cultivation conditions and ultimately increase the production of the desired product (Godara &

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Kao, 2021). Therefore, employing ALE directional selection on yeast capable of efficiently utilizing L-Phe is a feasible strategy to enhance the floral attributes of wine. Due to global climate change and other influences, grapes increasingly exhibit high sugar levels and low acidity, disrupting the balance of wine flavor (Gonen et al., 2024). Lactic acid, a stable organic acid, can enhance the smoothness of wine's flavor (Naselli et al., 2024). Currently, *Lachancea thermotolerans* is viewed as having the highest potential for improving acidity, as it contains highly active lactate dehydrogenase, enabling the direct conversion of pyruvate generated in the Embden-Meyerhof pathway (EMP) into lactate during wine fermentation, effectively enhancing acidity (Battjes et al., 2023; Sanchez-Suarez & Peinado, 2024; Su et al., 2024; Vicente et al., 2024). Additionally, rising temperatures impact the secondary metabolism of grapes during growth, resulting in wines with weak aromas and lacking distinctive aromatic qualities (Drappier et al., 2019). Thus, utilizing L. *thermotolerans* for focused research and breeding is the optimal solution to mitigate the adverse effects of global warming on wine production.

In our previous work, indigenous strain L. *thermotolerans* A38 was isolated from ice wine fermentation, demonstrated high lactic acid production and high fermentation capacity (Su et al., 2024). This strain has shown great potential for enhancing flavor in industrial-scale fermentation. Building on this, we selected L. *thermotolerans* A38 as the starting strain for our current study. The purpose of this study was to harvest strains that could increase the 2-PE and 2-PEA content by ALE of L. *thermotolerans* A38, and to explore its influence on the aroma of wine. This work not only offers a green and efficient method for the targeted breeding of L. *thermotolerans*, but it also provides a potential solution to mitigate the negative impact of global climate change on wine aroma, offering promising strategies for future wine production.

2. Materials and methods

2.1. Yeasts and media

Indigenous L. *thermotolerans* A38 was isolated from Merlot fermentation by our lab (Su et al., 2024). Three commercial yeast strains were obtained from the activated single colony of F15 (Laffort, France), CECA (Angel, China), and CT10 (Chr. Hansen, Denmark), and stored in 20 % glycerol at −80 °C. The yeast strains were inoculated into yeast extract peptone dextrose (YEPD) liquid culture medium (10 g yeast extract, 20 g peptone, 20 g glucose, dissolved in 1 L of purified water), and incubated at 28 °C with shaking at 200 rpm for 16–18 h before using.

The formulation of chemically defined grape juice medium (CDGJM) referred to the description of Du et al. (2022). CDGJM-Lphe was denoted CDGJM medium with L-Phe as the sole amino acid.

2.2. Adaptive laboratory evolution (ALE)

The activated L. *thermotolerans* strain was inoculated at a concentration of 1×10^6 cells/mL into 100 mL CDGJM-Lphe containing various concentrations (1, 2, 3, 4, and 5 g/L) of L-Phe. The cultures were incubated at 25 °C without shaking. Cell density at 600 nm by enzyme-labeled instrument (Synergy H1, USA) and viable cell count were recorded daily to evaluate the growth of L. *thermotolerans* at different L-Phe levels.

The ALE method described in a previous study Sun et al. (2024), with some modifications. Based on the experimental results, CDGJM-Lphe containing 2 g/L L-Phe was selected as the ALE and screening medium for subsequent experiments. The initial L. *thermotolerans* A38 strain (1×10^6 cells/mL) was inoculated (25 °C) into CDGJM-Lphe. Cell counts were recorded every 24 h using a hemocytometer with three replicates. When growth entered the stationary phase (by cell counts), the culture was transferred to fresh CDGJM-Lphe for further subculturing. Each transfer was stored in glycerol tubes at −80 °C. Every 15 days, cultures were collected for DNA extraction and sequenced at NCBI for sequence alignment to verify strain purity.

2.3. Selection of evolved clones

The cell stored in glycerol tube (−40 °C) from generations 50th (G50), 100th (G100), 150th (G150), and 200th (G200), which was activated in YEPD and cultured at 180 rpm at 28 °C for 16–18 h. They were inoculated in 200-mL steriled CDGJM at a cell concentration of 1×10^6 cells/mL and fermented at 25 °C in an incubator (Azambuja et al., 2024). The original L. *thermotolerans* A38 strain served as a control and 3 replicates were performed in each treatment. The residual sugar content was determined by DNS. After the residual sugar 3 d remained unchanged, the fermentation was terminated by adding SO_2. After clarification, the supernatant was taken to determine the content of 2-PE and 2-PEA in the fermentation liquid (as described in Section 2.4).

The evolved mixture from different generations were progressively diluted and plated onto YEPD, and incubated at 30 °C for 2 to 5 days. A plate with approximately 100 clones from the dilution series was selected, and each single colony was transferred for preservation in glycerol tubes. The colony was isolated from G50 which was named AE50-n, and the same from G100 was named AE100-n, and so on. The growth potential of evolved colonies from G50, G100, G150, and G200 was assessed. Briefly, the single colony suspension (1×10^6 cells/mL) was inoculated in CDGJM-Lphe. All treatments were incubated at 25 °C, with measurements taken every 12 h to monitor growth by assessing the absorbance at 600 nm. The original strain served as a control, with three replicates for each group.

2.4. Volatile aroma analysis

Volatile compounds were analyzed using gas chromatography combined with mass spectrometry (Thermo Fisher Scientific, USA) with modifications to the procedure described by Chen et al. (2023). The revised method included the following steps: 5 mL of wine, 1.1 g of NaCl, and 10 μL of the internal standard (4-methyl-2-pentanol, 1.034 g/L) were added to a 20 mL glass vial, which was then placed in a thermostatic autosampler tray for HS-SPME sampling. The vial was then placed in the autosampler tray for HS-SPME sampling. The samples were incubated for 20 min at 40 °C before GC–MS analysis. Volatile compounds were extracted from the vial's headspace by exposing a 2 cm DVB/CAR/PDMS (50/30 μm) fiber for 30 min at 40 °C and subsequently desorbed in splitless mode with an injector temperature of 250 °C for 5 min. The MS operated in positive electron ionization (EI) mode with a mass acquisition range of 35 to 350 *m/z*. Helium was used as the carrier gas at a flow rate of 1.0 mL/min throughout the process. Wine volatile compounds were identified by comparing retention indices with reference standards and mass spectra matched against the NIST17.0 library in Chromeleon software (Version 4.50). Quantification was performed using established calibration curves in model solutions. For volatiles without standard curves, concentrations were estimated using equations based on those with the same functional group and/or a similar number of carbon atoms. The odor activity value (OAV) of each volatile compound was calculated as the ratio of its concentration to its perception threshold (Du et al., 2022).

2.5. Conventional oenological parameter determination

The analysis glycerol, ethanol, and various organic acids (including tartaric, citric, succinic, malic, lactic, and acetic acids) was conducted using a high-performance liquid chromatography (HPLC) method developed in the laboratory (Zhang et al., 2024). The procedures included isocratic elution on an HPX-87H column with an ultraviolet detector. The flow rate was 0.6 mL/min, the mobile phase was 10 mmol/L H_2SO_4, the column temperature was 60 °C, the sample holder temperature was 10 °C, and the injection volume was 20 μL. Organic acids were detected at a UV wavelength of 210 nm. The concentrations of glycerol, and ethanol were measured using a refractive index detector and quantified using the external standard approach.

2.6. Genome sequencing of clones and wild-type starting strain L. thermotolerans A38

The original strain L. *thermotolerans* A38 and its evolved variants (AE50–51, AE100–13, AE200–27, and AE200–58) were cultured to the logarithmic phase. Genomic DNA was extracted from the yeast strains and fragmented into short DNA sequences using the NEBNext dsDNA Fragmentase enzyme (NEB, Ipswich, MA, USA). After random fragmentation, the DNA fragments underwent end repair, followed by the addition of dA tails to both ends using NEB (Ipswich, MA, USA) reagents. Sequencing adapters were then ligated to the modified DNA fragments. The adapter-ligated DNA was purified using AMPure XP magnetic beads and targeted sequences were captured using the SureSelect Human All Exon V6 Kit (Agilent Technologies, Palo Alto, CA, USA). DNA fragments within the size range of 300–400 bp were amplified by PCR.

The sequencing data were aligned to the L. *thermotolerans* CBS 6340 reference genome (GCF 000142805.1) using the Burrows-Wheeler Aligner (BWA, https://github.com/lh3/bwa), and the alignment results were processed and sorted using Samtools (https://www.htslib.org/). Coverage statistics were computed with Bedtools (https://www.htslib.org/). SNPs (single nucleotide polymorphisms) and InDels (insertions and deletions) were identified by aligning the clean reads from each sample to the reference genome using BWA with the parameters 'mem 4 -k 32 -M'. Variants were called using the Unified Genotyper tool in the Genome Analysis Toolkit (GATK), incorporating local realignment and base quality score recalibration. The SNPs and InDels were filtered using GATK's Variant Filtration tool with the following parameters: "-Window 4, -filter 'QD < 2.0 || FS > 60.0 || MQ < 40.0', -G_filter 'GQ < 20'". Variants with significant allele imbalance or sequencing errors were excluded. Structural variants (SVs), including large deletions, insertions, inversions, and translocations, were detected using Delly (https://github.com/dellytools/delly). Copy number variations (CNVs) greater than 1 kb were assessed using Cnvpytor (https://github.com/abyzovlab/CNVpytor), identifying both duplications and deletions. Functional enrichment analysis was performed on the mutated genes, followed by Gene Ontology (GO) enrichment (http://www.geneontology.org/).

2.7. Physiological property experiments of mutant strains

The physiological property of mutant strains was assessed by measuring growth density. Using a modified version of the method by Lai et al. (2022), activated yeast was inoculated into YEPD liquid medium under varying conditions with an initial concentration of 1×10^6 cells/mL. The test conditions were as follows: 1) sugar concentrations (glucose: fructose in a 1:1 ratio) at 250, 300 and 350 g/L; 2) ethanol concentrations at 8 %, 12 %, and 16 % (*v*/v); 3) SO_2 concentrations at 100, 200, and 300 mg/L; and 4) pH levels adjusted to 3.0, 3.5, and 4.0. After 24 h of incubation at 25 °C, OD600nm was measured to evaluate the tolerance of the evolved strains to sugar, ethanol, SO_2, and pH based on growth density. The original strain and commercial yeast CT10 served as controls, with three replicates performed for each condition.

2.8. Fermentation parameters in wines

Cabernet Sauvignon (CS) grapes were handpicked in 2023 from commercial vineyards located in the eastern foothills of Helan Mountain (38.18°N, 106.1°E) in China. Riesling grape juice was also obtained in 2023 from Manas County (43.48°N, 87.3°E) in China. Immediately after harvesting, the grapes were de-stemmed and crushed, with the addition of 30 mg/L sulfur dioxide. The juice was subsequently sterilized with dimethyl dicarbonate (DMDC) to eliminate wine microorganisms(Costa et al., 2008). Detailed information regarding the basic physical and chemical indicators of the grapes is provided in Table S1. Six L. *thermotolerans* strains (including the original strain, four selected mutants, and the commercial strain CT10) were co-fermented with two different commercial *S. cerevisiae* strains (F15 and CECA), respectively. Both *S. cerevisiae* and L. *thermotolerans* were inoculated at an initial concentration of 1×10^6 cells/mL in 1 L glass jars containing 600 mL of grape juice. Three biological replicates were conducted for each experimental group, with fermentations controlled at 25 °C for CS and 20 °C for RS. The residual sugar was measured daily by the dinitrosalicylic acid method (Chen et al., 2025), and fermentation was halted by the addition of SO_2 when the residual sugar fell below 4 g/L. After clarification and stabilization, the volatile compounds (as described in Section 2.5) and conventional oenological parameters (as described in Section 2.4) of the wine samples were analyzed.

2.9. Electronic nose (E-nose)

The E-nose (PEN3-Plus, Airsense, Germany) used in this study is equipped with a metal-oxide-semiconductor (MOS) sensor array consisting of 10 distinct sensors. For analysis, a 10-mL wine sample was placed into a sealed headspace bottle and maintained at 45 °C for 10 min. Subsequently, volatile compounds are aspirated into the sensor chamber at the same flow rate of 600 mL/min for 60 s. The comprehensive response of the electronic nose array is obtained from the signals of the ten sensors.

2.10. Data analysis

The data from this study were analyzed using SPSS version 26.0 (SPSS Inc., Chicago, IL, USA). Mean values and standard deviations of the raw data were calculated using Microsoft Office Excel 2021, and graphical representations were generated using GraphPad software (GraphPad Software Inc., San Diego, CA, USA) and Origin 2024b (Electronic Arts Inc., Northampton, USA). Partial Least Squares Discriminant Analysis (PLS-DA) was performed with SIMCA 14.1 (MKS Data Analytics Solutions, Sweden).

3. Results

3.1. ALE of L. thermotolerans A38

The impact of varying concentrations of L-Phe on the growth of L. *thermotolerans* A38 was investigated. As shown in Fig. 1 A and B, different levels of L-Phe significantly inhibited the growth of L. *thermotolerans* A38. In standard CDGJM, the maximum optical density (OD600 nm) recorded was 0.82 ± 0.07, with a peak viable yeast count of 8.04 ± 0.14 log10 CFU/mL. In contrast, in CDGJM where L-Phe was the sole nitrogen source, the maximum OD600 nm was 0.25 ± 0.03, and the highest viable count was 7.67 ± 0.02 log10 CFU/mL, with no significant differences observed across various concentrations. To ensure that L. *thermotolerans* A38 had an adequate nitrogen supply for growth, 2 g/L of L-Phe was selected as the stressor for prolonged ALE (Fig. 1C). After 102 days of adaptive evolution, the cell density of L. *thermotolerans* A38 in 2 g/L L-Phe ranged from 7.79 log10 cells/mL to 8.36 log10 cells/mL in the later stages (Fig. 1D).

3.2. Phenotypic characterization of evolved clones

Phenotypic characterization of evolved populations compared to wild-type by 2-PE and 2-PEA. As illustrated in Fig. 2, all evolved populations produced significantly higher levels of 2-PE and 2-PEA compared to the wild-type strain. The 2-PE concentration in G200 wine reached 19.77 ± 1.44 mg/L, a 67.7 % increase compared to the original strain (11.79 ± 1.59 mg/L) (Fig. 2A). Similarly, G150 exhibited a 2-PEA concentration of 145.19 ± 15.26 μg/L, 80.5 % higher than that of the original strain (80.44 ± 5.03 μg/L) (Fig. 2B). Based on the above results, we screened the mutant strains from each 50-generation population.

Around 50–60 individual clones were obtained from the

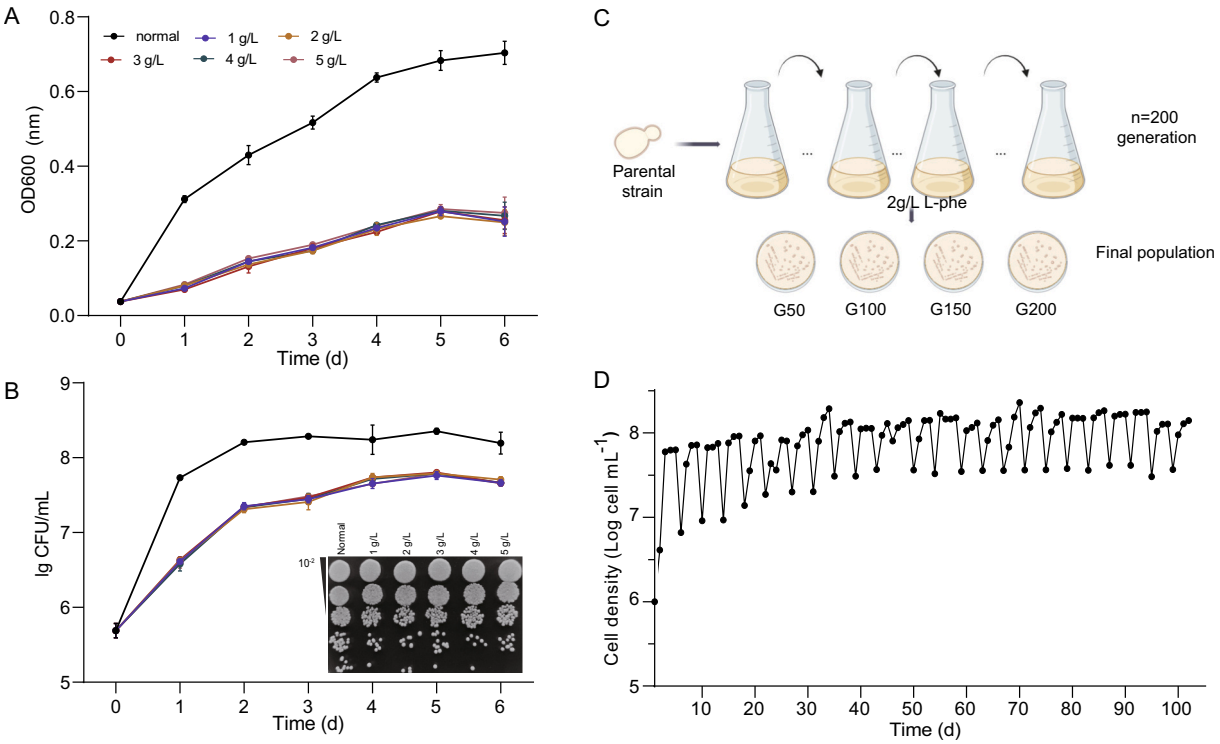

Fig. 1. Growth of L. *thermotolerans* A38 at varying levels of L-Phe: cell density measured at OD600 nm (A) and viable cell count (B). The schematic diagram of the adaptive laboratory evolution (ALE) of the original strain and growth curves of the original strain L. *thermotolerans* A38 throughout ALE (D). Error bars represent standard deviations.

evolutionary population through phenotypic analysis focused on cell density in CDJGM with 2 g/L L-Phe, in comparison to the wild-type original strains (Fig. 2C). The biomass of all clones in the presence of 2 g/L L-Phe was significantly greater than that of the original strain. Subsequently, 4 strains from every 50 generations were chosen for further testing based on their maximum OD600 (nm) values: G50 (AE50–51, AE50–57, AE0–58), G100 (AE100–13, AE100–29, AE100–55), G150 (AE150–28, AE150–31, AE150–60), and G200 (AE200–27, AE200–50, AE200–58). The highest recorded OD600 (nm) was 1.43 ± 0.02 (AE200–58), compared to the original strain's 0.75 ± 0.05 (Fig. 2C).

Wine fermentations were conducted using 12 mutant strains in standard CDGJM, with the original strain L. *thermotolerans* A38 serving as a control. The findings clarified that the levels of 2-PE in most mutant strains were significantly higher than in the original strain (Fig. 2E). The highest concentration of 2-PE was found in AE50–51 at 26.78 ± 1.31 mg/L, which was significantly more than the original strain's 15.44 ± 1.59 mg/L (an increase of 73.45 %, $P < 0.05$). There was no significant difference in 2-PE levels among AE50–51, AE200–27, and AE200–58. Additionally, all mutant strains showed significantly elevated levels of 2-PEA in the fermentation broth compared to the original strain A38, with the highest being 159.45 ± 10.99 μg/L (AE100–13), which is 2.11 times higher than the original strain's 75.53 ± 0.03 μg/L (Fig. 2D). Based on these results, we chose AE50–51, AE100–13, AE200–27, and AE200–58 for follow-up experiments.

3.3. Genomic characteristics of mutations

To investigate the genetic variations underlying evolution, we conducted a resequencing analysis of four selected mutant strains (AE50–51, AE100–13, AE200–27, and AE200–58) compared to the parental strain. We identified several potentially relevant key genes (Table 1) and annotated their functions using the NCBI database. Most of these genes are associated with metabolism and stress resistance. Notably, we found mutations in the gene related to transport proteins (KLTH0G12716g) across all evolved strains, except for AE200–27. The gene KLTH0E06292g, which is involved in intracellular protein transport, exhibited mutations in both strains evolved over 200 generations. The final step of the Ehrlich pathway involves the reduction of phenylacetaldehyde to 2-PE by alcohol dehydrogenase. We observed mutations in KLTH0E07920g, which enables NADPH dehydrogenase activity, in both AE50–1 and AE200–27 strains (Fig. 3). This may be related to the increased yield of 2-PE and its derivative 2-PEA. Additionally, we identified different mutations in stress resistance-related genes within the evolved strains, including KLTH0D05698g, KLTH0G13838g, and KLTH0D14718g. Specifically, KLTH0D05698g exhibited two mutation sites in AE50–51 and one mutation site each in AE100–13 and AE200–27. Furthermore, KLTH0C11770g which enables L-lactate dehydrogenase (cytochrome) activity, and KLTH0C11924g which involved in flocculation, were found to have deletions and SNPs in the evolved strain AE200–58. This research enhanced the understanding of the genetic basis of evolution and underscores the importance of specific genes in L-Phe metabolic pathways and stress responses, which were crucial for the development of improved strains in biotechnological applications.

3.4. Physiological performances in mutants

In order to avoid the mutant strains from being adversely affected by other characteristics while enhancing aroma production, we further analyzed its tolerance (Fig. 4). At a sugar concentration of 250 g/L, the results indicated that the cell density (OD600 nm) of the four mutant strains was significantly higher than that of the original strain (OD600 nm = 1.30 ± 0.02) and commercial yeast (OD600 nm = 1.23 ± 0.02). AE200–58 showed the highest cell density (OD600 nm = 1.43 ± 0.03). Additionally, at a sugar concentration of 300 g/L, the growth of the mutant strains was significantly better than that of the original strain ($P < 0.05$) (Fig. 4A). Furthermore, the mutant strains exhibited improved

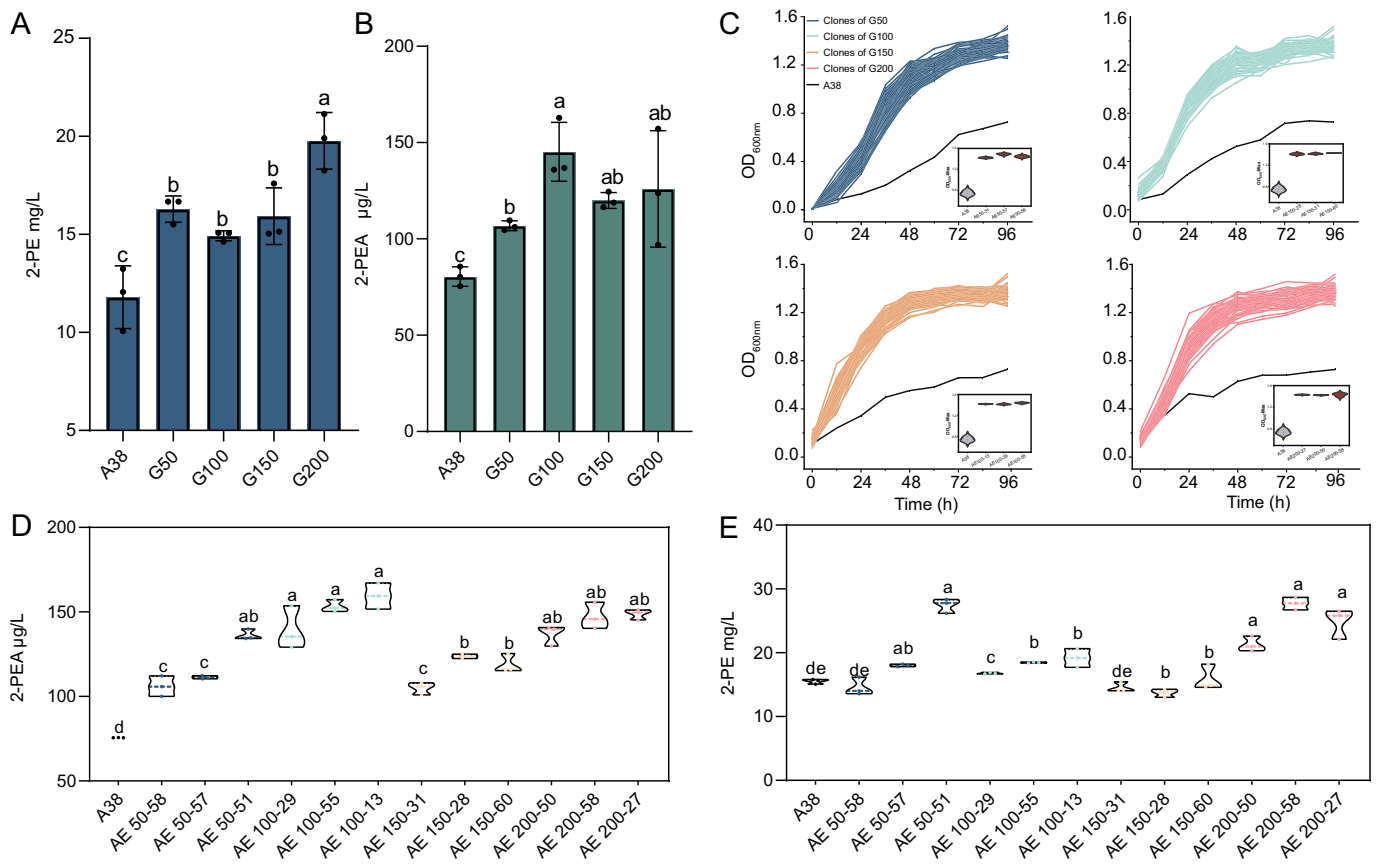

Fig. 2. Phenotypic characterization of evolved populations in normal CDJGM by the content of 2-PE (A) and 2-PEA (B). A single clonal isolate of the evolved populations was phenotypically characterized by growth curves in CDJGM with 2 g/L L-Phe. The maximum OD600(nm) values for four strains from different populations, which exhibited the highest biomass in the 2 g/L L-Phe CDJGM, are shown separately. Additionally, the levels of 2-PE (D) and 2-PEA (E) produced during wine fermentations were measured using 12 mutant strains in standard CDGJM. Error bars represent standard deviations. Distinct letters signify significant differences among the constituencies based on Tukey's test ($p < 0.05$).

growth at various ethanol concentrations (8 % and 12 % vol), with AE200–27 achieving a biomass (OD600 nm = 0.382 ± 0.02), which was 2.26 times higher than that of the original strain (OD600 nm = 0.169 ± 0.03) in 8 % vol ethanol (Fig. 4B). The mutant strains also thrived under different SO_2 levels, with their biomass significantly surpassing that of the original strains and commercial yeast CT10, particularly AE200–27 and AE200–58 (Fig. 4C). Differences in growth were also observed among various strains at different pH levels, with the mutant strains showing significantly higher biomass than the original strain at pH levels of 3, 3.5, and 4 (Fig. 4D). Therefore, the mutant strains exhibited enhanced stress resistance compared to the original strain.

3.5. Impact of evolved clones on the volatile profiles of Cabernet Sauvignon and Riesling wines

3.5.1. Effect of evolved clones on content of 2-PE and 2-PEA in different wines

The winemaking experiments using Riesling and Cabernet Sauvignon grapes showed that mutant strains significantly increased the levels of 2-PE and 2-PEA compared to the original strain and commercial L. *thermotolerans*. In Cabernet Sauvignon, the mutant strain AE200–58 significantly increased the 2-PEA content to 667.54 ± 51.9 μg/L, which was 14 times higher than the original strain's 47.63 ± 7.62 μg/L when co-fermented with CECA (Fig. 5B). This amount was double the 2-PEA produced by the commercial L. *thermotolerans* CT10 (330.77 ± 33.33 μg/L). Additionally, AE200–27 increased 2-PEA levels by 2.59 times (Fig. 5D) and 2-PE by 34.12 % compared to the original strain A38 when co-fermented with F15 (Fig. 5C). In Riesling, the levels of 2-PE in strains AE100–13, AE200–27, and AE200–58 were significantly higher than those of the original strain (Fig. 5E) ($P < 0.05$). The content of 2-PEA in Riesling wine made by AE200–58 co-fermented with CECA was 493.05 ± 137.02 μg/L, a 28.33 % increase over the original strain's 325.87 ± 45.56 μg/L, and significantly higher than that produced by commercial yeast CT10 (Fig. 5F). When co-fermented with F15, the mutant strains showed significantly higher levels of 2-PE and 2-PEA compared to the original strain, with 2-PE levels increasing by 1.68–4.10 times (Fig. 5G) and 2-PEA levels rising by up to 53.29 % (AE200–27, 178.73 ± 10.39 μg/L) (Fig. 5H). In summary, co-fermenting these mutant strains with F15 and CECA in both Riesling and Cabernet Sauvignon wines led to a notable increase in 2-PE and 2-PEA levels. Importantly, the mutant strains did not affect the overall fermentation process and were able to complete alcoholic fermentation concurrently with the original strains (Fig. S1). Organic acids, glycerol, and ethanol were analyzed at the end of the wine fermentations, with the basic chemical profiles of the final wines displayed in Table S2. In general, the amounts of alcohol in wine co-fermented with original strain was significantly higher than co-fermented with evolved strains. Compared to the wines fermented by original strain, the treatments with evolved strains significantly increased the concentration of citric acid in Riesling and Cabernet Sauvignon wines.

3.5.2. Overall effect of evolved strains on volatile profiles of wine

In the L. *thermotolerans* co-fermented with Cabernet Sauvignon wine, a total of 42 and 28 compounds with OAV > 0.1 were detected in the CECA and F15 treatments, respectively (Fig. 6). In the Cabernet Sauvignon wine co-fermented with CECA, the evolved strains exhibited

Table 1
Compared to the original strain A38, the main genes that may be involved in enhancing the production of evolved strains 2-PE and 2-PEA.

Gene sample	Mutation Type	Function
KLTH0D05698g	SNP	Cell wall components correlate with tolerance
KLTH0D14718g	Insertion	nutrient- and stress-responsive signaling pathways
KLTH0E07920g	Insertion	enables NADPH dehydrogenase activity
KLTH0F19932g	SNP	/
KLTH0G02640g	SNP	Located in mitochondrial inner membrane
KLTH0G12716g	Deletion	enables ABC-type transporter activity
KLTH0G13838g	Deletion	It is related to stress resistance, filamentous growth, etc
KLTH0C07172g	Insertion	/
KLTH0C08734g	SNP	enables peptide alpha-*N*-acetyltransferase activity
KLTH0C08976g	Insertion	enables alpha-1,2-mannosyltransferase activity
KLTH0D05236g	Deletion	Involved in regulation of DNA-templated transcription
KLTH0E00264g	SNP	Involved in phosphate ion transmembrane transport
KLTH0E03652g	Deletion	enables protein serine/threonine kinase activity
KLTH0F10648g	SNP	/
KLTH0F19954g	SNP	enables S-adenosylmethionine dependent methyltransferase activity
KLTH0G01474g	Insertion	enhancing the activity of the decapping proteins Dcp1p and Dcp2p
KLTH0G19756g	SNP	Involved in secondary metabolic process
KLTH0H10054g	SNP	enables metalloexopeptidase activity
KLTH0E06292g	Deletion	Involved in intracellular protein transport
KLTH0C01430g	Insertion	/
KLTH0C11770g	Deletion	enables L-lactate dehydrogenase (cytochrome) activity
KLTH0C11924g	SNP	Involved in flocculation

higher levels of ester and terpene compounds (e.g., ethyl butanoate, ethyl caprylate, isoamyl acetate, nerolidol, terpinen-4-ol, and nerol) compared to the starter strain L. *thermotolerans* A38. Notably, the evolved strains showed significantly higher levels of octanoic acid, trans-β-ionone, and 1-butanol. In the Cabernet Sauvignon wine co-fermented with F15, the content of 1 − Propanol, Ethyl caprylate, trans−ß − Ionone in the evolved strain wine samples was higher than in the starter strain L. *thermotolerans* A38.

In Riesling wine co-fermented of CECA and F15 with L. *thermotolerans*, 30 and 31 compounds with OAV > 0.1 were detected, respectively. In the CECA treatment, wines from evolved strains showed significantly higher levels of key compounds such as 1-hexanol, ethyl 3-hydroxybutyrate, and nerol compared to L. *thermotolerans* A38 and the commercial strain CT10. Notably, AE100–13 wines had higher levels of ester and terpene compounds than CT10. In F15-treated Riesling wine, most compounds were present at higher levels in CT10 treatments, but the evolved strains had the highest levels of 2-PE and 2-PEA. Additionally, octanoic acid, isovaleric acid, and nerol were more abundant in the evolved strains compared to L. *thermotolerans* A38. In summary, the evolved strains not only increased 2-PE and 2-PEA levels in both Cabernet Sauvignon and Riesling wines, but also enhanced the levels of ester and terpene compounds, resulting in wines with more pronounced floral and fruity aromas.

3.5.3. Aroma characterization of wines by E-nose analysis

As shown in Fig. 7, principal component 1 (F1) accounted for over 90 % of the total variance, allowing the PCA analysis to distinguish L. *thermotolerans* A38 from other evolved strains. This indicated a notable difference in aroma perception between wines produced with L. *thermotolerans* A38 and those from other treatments, while the differences among the evolved strains AE100–13, AE200–27, and AE200–58 were less pronounced (Fig. 7A). The performance of W2S and W2W sensors, which are sensitive to aromatic compounds, was further analyzed. The findings revealed that the responses from W2S and W2W sensors for wines made with the evolved strains were significantly higher than those from the original strain in Cabernet Sauvignon co-inoculated with CECA ($P < 0.05$). In groups of F15 co-inoculations, the response values from the two sensors for the evolved strains were also significantly greater than those of the original strain without significant difference, compared to commercial CT10. The responses of W2S and W2W sensors were significantly higher in Riesling wines by the evolved strains than by the original strain, regardless of commercial *S. cerevisiae* species. This indicated that Riesling and Cabernet Sauvignon wines from the evolved strains possess a more pronounced aromatic properties.

4. Discussion

Global climate change has impacted viticulture, with rising temperatures causing earlier grape ripening, higher sugar levels and lower acidity, which leading to wines with weak aromas (Gonen et al., 2024). The specie of L. *thermotolerans* with the characteristics of high lactic acid production and fermentative ability, present the potential for addressing the issue (Vicente et al., 2021). This study aimed to enhance the L-Phe utilization of indigenous L. *thermotolerans* A38 by ALE to increase aromatic compounds and mitigate effects of climate change on wine. Selecting an appropriate selective pressure is crucial for the success of ALE. L-Phe is an essential amino acid in wine and a precursor of 2-PE and 2-PEA (Zhang et al., 2020). However, L-Phe is not the preferred nitrogen source for microbial growth; when more readily utilizable nitrogen sources are available, the synthesis of 2-PE and 2-PEA is restricted (Dai et al., 2021). Therefore, L-Phe was selected as the sole nitrogen source for ALE.

Initially, the concentration selection of ALE was generated and the research indicated that different concentrations (1, 2, 3, 4 and 5 g/L) of L-Phe inhibited the growth of L. *thermotolerans* A38, with no significant

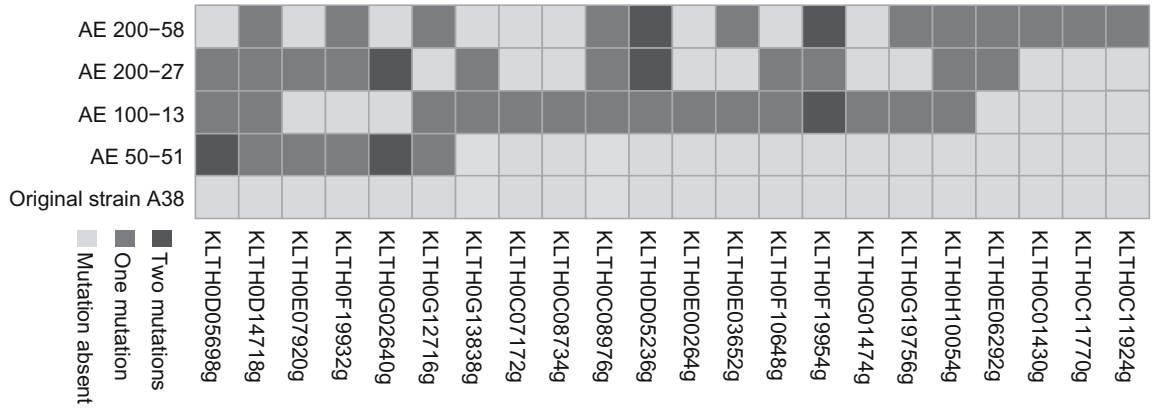

Fig. 3. Block diagram shows the mutational information of evolved strains (AE50–51, AE100–13, AE200–27, and AE200–58) compared to the original strain.

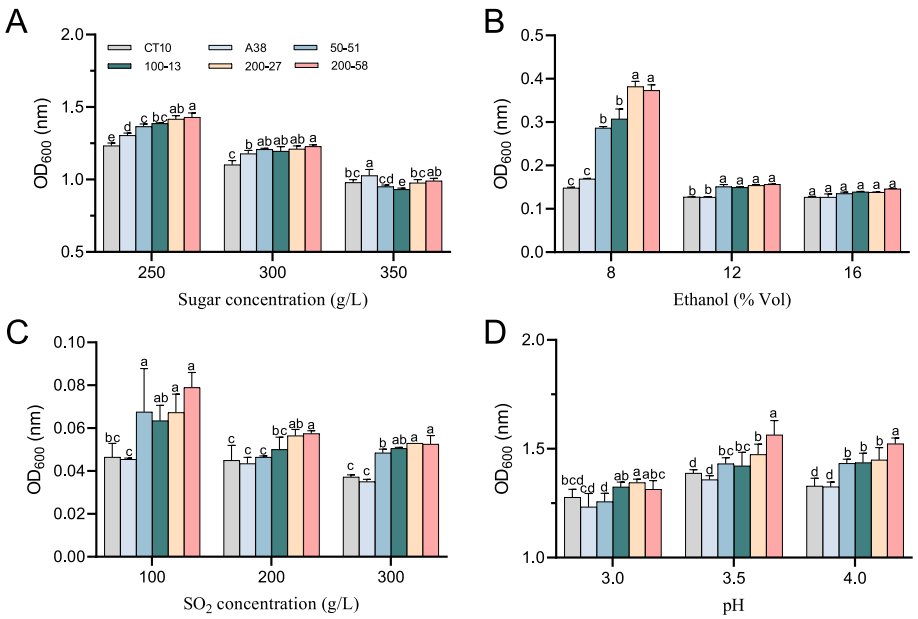

Fig. 4. Tolerance analysis of mutant strains by different concentrations of sugar (A), ethanol (B), SO_2 (C) and pH (D). Error bars represent standard deviations.

differences among the treatments. To ensure sufficient nitrogen sources during growth (Lola et al., 2023), we chose 2 g/L of L-Phe and conducted subculturing for 102 days and 200 generations (Fig. 1). The phenotypes of the evolved populations were characterized. Although the evolved mixtures from G50, G100, G150, and G200 generations exhibited significantly higher production of 2-PE and 2-PEA compared to the original strain, their growth capacities were not improved in CDGJM (Fig. 2). This may be due to the nonlinear trajectory of adaptive evolution, where the pace of fitness enhancement slows over time (Barrick et al., 2009). Subsequently, high-throughput screening on populations from each 50 generations was performed and selected 12 strains with the best growth in CDGJM-Lphe (2 g/L) from nearly 300 strains. As expected, the 12 mutant strains exhibited significantly higher production of 2-PEA (up to a 2.11-fold increase), and 9 of them showed significantly enhanced production of 2-PE ($p < 0.05$, up to a 73.45 % increase). Roy et al. (2023) found that the production of 2-PE and 2-PEA increased by 30 %–75 % after adding L-Phe to the substrate in solid-state fermentation, but a growth rate was lower than in our findings. Besides, there was no significant difference in 2-PEA content between the control group and the L-Phe-added group under the addition of 60 mg/L L-Phe in Chardonnay grape juice (Valera et al., 2024). In this study, four evolved L. *thermotolerans* strains, isolated from 200 generations of ALE processes, showed a significant 73.45 % increase in 2-PE production and a 2.11-fold increase in 2-PEA production during CDGJM fermentation. These results provide a more efficient and environmentally friendly alternative for the supplementation of exogenous L-Phe.

Here, the resequencing analysis were conducted for four evolved strains and the original strain. Genetic mutations were identified in potential relation to L-Phe transport, 2-PE synthesis, and stress resistance (Fig. 3). The gene (noted as KLTH0G12716g), enabling ABC-type transporter activity, plays a crucial role in amino acid transport (Li et al., 2012; Pan et al., 2024). Some ABC transporters generally exhibited specificity, recognizing and transporting specific amino acids or their derivatives (Kim et al., 2024). Additionally, ABC transporters regulate intracellular amino acid concentrations, ensuring appropriate amino acid supply under varying physiological conditions (Kumari et al., 2021). These transporters are not merely simple “channels” in the amino acid transport process; they also involve complex regulatory mechanisms, including interactions with other proteins, post-translational modifications, and the regulation of cellular signaling pathways (Yu et al., 2015). A four-base deletion was identified in the KLTH0G12716g gene from three evolved strains, which may be related to the transport of L-phe; however, this requires further verification. The excess NADH in yeast cells has promoted the final reduction step of the Ehrlich pathway, which is favorable for the synthesis of 2-PE (Dai et al., 2021). KLTH0E07920g (highly similar to YHR179W *OYE2*), encoding NADPH dehydrogenase activity (Zhao et al., 2017), was determined a base insertion in the mutant strains, potentially facilitating the reduction of benzaldehyde to 2-PE, thereby generating more 2-PE and its derivative 2-PEA. Genetic mutations were also identified in association with cell wall synthesis and related pathways, potentially linking with the efficient utilization of L-Phe by yeast and subsequent growth restoration. Future validation of these gene hypotheses using CRISPR-Cas9 genome editing in L. *thermotolerans* A38 was required to pinpoint key genes involved in 2-PE and 2-PEA synthesis, which would also benefit the industrial production of these compounds.

Physiological property in mutants is further analyzed. The fermentation activity and efficiency of non-*Saccharomyces* yeasts during wine fermentation are usually limited by their inability to tolerate the fermenting grape juice environment (Che et al., 2024). Therefore, four mutants (AE50–51, AE100–13, AE200–27, and AE200–58) with the strongest abilities to produce 2-PE and 2-PEA for tolerance analysis were selected. Unexpectedly, the results showed that the evolved strains exhibited higher environmental tolerance compared to the original strain (Fig. 3), allowing them to maintain better fermentation performance under a broader range of conditions. Studies have shown that ALE can improve tolerance to the target pressure and enhance tolerance to other pressures. Mavrommati et al. (2023) conducted ALE on *S. cerevisiae* using high ethanol concentration as the selective pressure. The evolved population exhibited significantly improved ethanol tolerance and enhanced tolerance to high-sugar environments. Menegon et al. (2022) research indicated that yeast strains post-ALE showed more tolerance to inhibitors in lignocellulosic hydrolysate and better stress resistance, including tolerance to environmental changes such as temperature and pH. The result may be due to mutations were discovered in genes (KLTH0G13838g, KLTH0D14718g, KLTH0D05698g and so on.) which associated with stress tolerance (Fig. 3).

The effects of mutant strains on the concentrations of 2-PE, 2-PEA, and other chemicals in two wine fermentations, are of significant interest. Study demonstrated that diammonium phosphate stimulates the gene expression of L-lactate dehydrogenase, thus providing higher specific enzyme activity in vivo and increasing L-lactic acid production

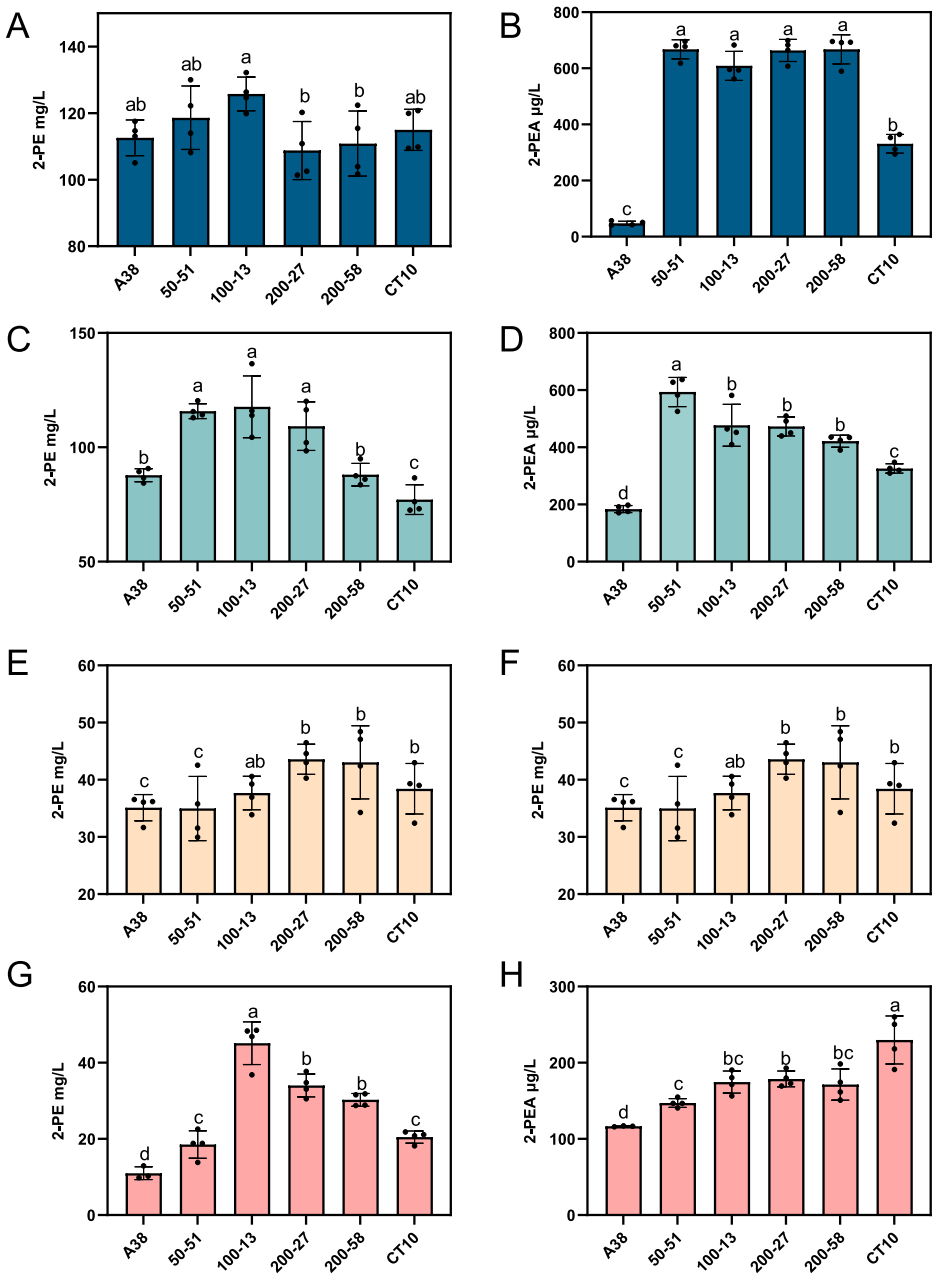

Fig. 5. Levels of 2-PE and 2-PEA in wines obtained by co-fermentation with evolved clones and commercial *S. cerevisiae*. The levels of 2-PE (A, C) and 2-PEA (B, D) in Cabernet Sauvignon co-fermented with CECA and F15, respectively. Additionally, the levels of 2-PE (E, G) and 2-PEA (F, H) in Riesling co-fermented with CECA and F15, respectively. Different letters indicate significant differences according to Tukey's test ($p < 0.05$).

(Sun et al., 2016). Initially, the gene of KLTH0C11770g (is highly similar to YML054C *CYB2*), responsible for L-lactate dehydrogenase activity (Grollman et al., 2000), was identified a base deletion, potentially resulting the enhancement of lactic acid production in CS wines (Table S2). Furthermore, previous studies indicated that floral notes are positively correlated with 2-PE and 2-PEA, characterized by rose and honey scents (Valera et al., 2024). In the study, the selected L. *thermotolerans* AE500–58 increased the 2-PEA level by 14-fold compared to the original strain in Cabernet Sauvignon wines, significantly surpassing the levels produced by the commercial CT10. The evolved L. *thermotolerans* AE200–27 enhanced the content of 2-PE by 34.12 % and 4.10-fold relative to the original strain, respectively in CS and RS wines (Fig. 5). This discrepancy may be attributed to the significant variation in phenylalanine concentrations in grapes, ranging from 3 to 138 mg/L (Bell & Henschke, 2005). The concentration of 2-PEA in most wines from the original strain (CS_{F15}: 191.56 μg/L; CS_{CECA}: 47.63 μg/L; RS_{F15}: 116.59 μg/L) was below the threshold of 250 μg/L (Du et al., 2022). The contents of 2-PE and PEA in the wine of L. *thermotolerans* co-fermented with CECA and F15 were different. Sometimes, the wine's aroma characteristics are affected by metabolites released by yeast, produced either through incomplete consumption of carbon/nitrogen sources or through specialized metabolic functions (such as enzyme activity) (Ciani & Comitini, 2015). Further analysis of other aromatic compounds in both Cabernet Sauvignon and Riesling revealed that 2-PE and 2-PEA were crucial in differentiating between the original strain, the evolved strains, and the commercial yeast CT10 (Fig. 6). These results illustrated that the evolved strains by ALE might enhance the rose and honey scents of wine.

In analysis of volatile profiles, Cabernet Sauvignon wines produced using the evolutionary strain exhibited significantly higher levels of ethyl butanoate, ethyl caprylate, isoamyl acetate, terpinen-4-ol and

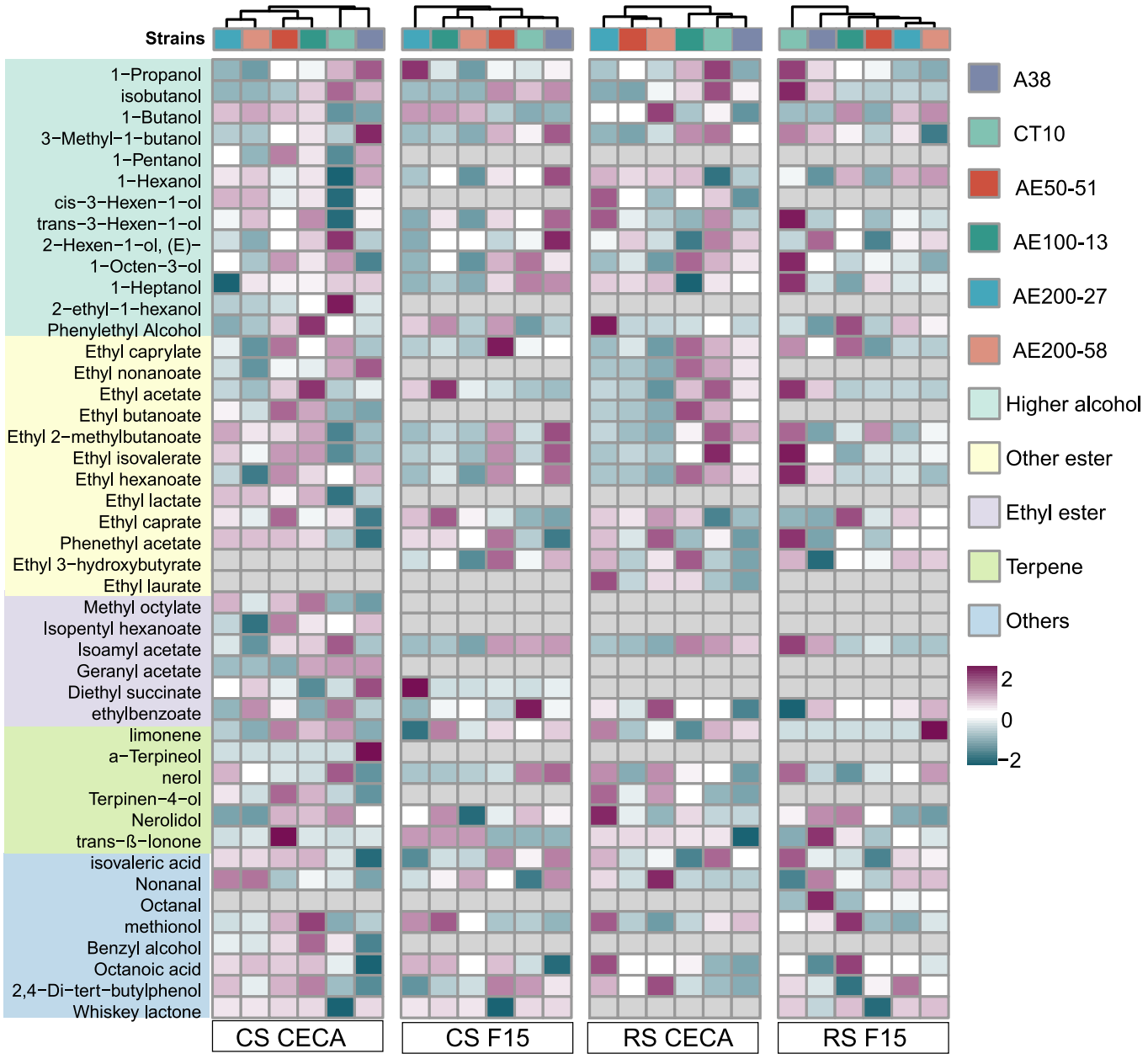

Fig. 6. Heat map of compounds with odor activity values (OAV > 0.1), the grey color indicates that the substance is OAV < 0.1 in this sample.

nerol compared to those from the original strain, potentially resulting in enhanced banana, fruit, rose, lime, and creamy roasted flavors (Yang et al., 2024). The synthesis of acetate depends on the condensation reaction of acetyl-CoA with higher alcohols catalyzed by acyltransferase (encoded by *ATF1*, *ATF2* and *AYT1* genes) (van Wyk et al., 2020). ALE may affect the metabolism of amino acids by L. *thermotolerans*, and thus affect the synthesis of higher alcohols and acetate compounds.

In Riesling wines, the evolutionary strain AE100–13 demonstrated increased levels of 1-pentanol, ethyl butanoate, and nerol, contributing to more pronounced floral and fruity aromas (Li et al., 2025). Additionally, both Cabernet Sauvignon and Riesling wines exhibit variations in the content and types of aromatic compounds when co-fermented with CECA and F15. These differences may stem from the strain specificity and metabolic interactions (Roullier-Gall et al., 2022), such as microbial cell contact, antibacterial compounds, substrate competition, and enzyme activity (Naselli et al., 2024; Ohwofasa et al., 2024).

As depicted in the Fig. 7, the *E*-nose's sensors effectively distinguished between the original strain and the evolutionary strain, in agreement with the data of volatile determinations (Fig. 7). Compared to the original strain, the evolutionary strain exhibited a higher response value on both two aroma-sensitive sensors (W2S and W2W) and suggested the elevated levels of aromatic compounds in the wine by the evolved strains. The results of volatile profile and electronic sensory evaluation highlighted that the evolved strains significantly increased the levels of 2-PE, 2-PEA, ethyl ester, and terpene compounds in Riesling and Cabernet Sauvignon wines, imparting more floral and fruity aromas (Li et al., 2025) and providing theoretical insights for the application of L. *thermotolerans* in winemaking processes. Global climate change has significantly impacted agriculture, particularly in viticulture. In most winegrowing regions around the globe, grape harvests have advanced by 2–3 weeks over the past 40 years (Ollat et al., 2016). It is accepted that climate changes-related effects on the warm viticultural areas are advanced harvest-time and temperatures, lower acidities values and high pH values, modification of varietal aroma. Wine fermentation experiments showed that evolved strains increased the content of acetate, terpenes, and other compounds, including 2-PE and 2-PEA, enhancing

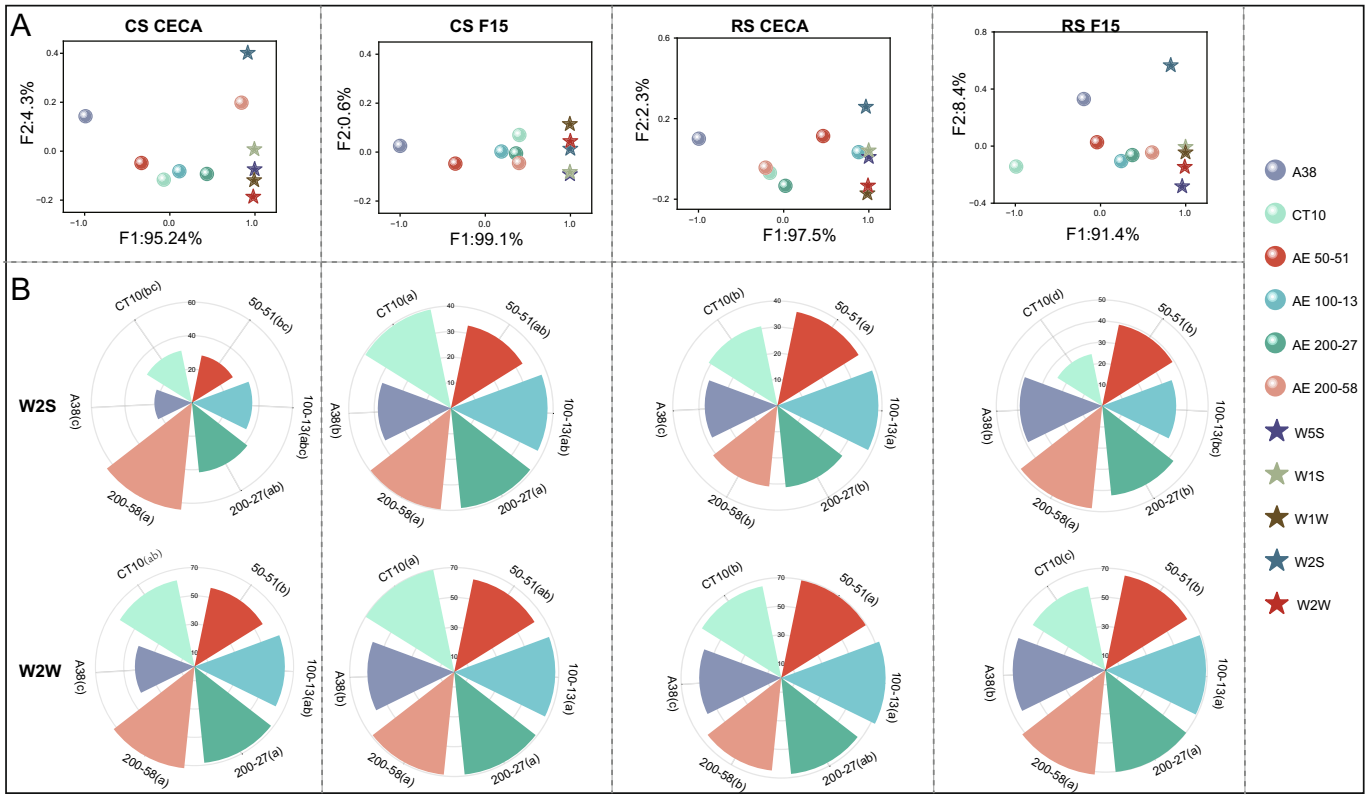

Fig. 7. The PLS-DA of *E*-nose data for Cabernet Sauvignon and Riesling wines with different strains (A), and the W2S and W2W sensor intensity for wines with different treatments (B). Different letters indicated significant differences according to Tukey's test ($p < 0.05$).

the wine's aroma. Meanwhile, the evolved strains also showed improved tolerance to high sugar concentrations, SO_2, ethanol, and pH. The results confirmed that ALE is an effective and environmentally friendly approach for sustainably enhancing the production of these compounds.

5. Conclusion

L-Phe is not the preferred nitrogen source for yeast, which affects the production of 2-PE and 2-PEA by strain L. *thermotolerans* A38. This strain underwent 200 generations of ALE in CDJGM using L-Phe as the sole nitrogen source, resulting in evolved strains (AE50–51, AE100–13, AE200–27, and AE200–58) with enhanced biosynthesis of 2-PE and 2-PEA. Four mutants were selected, exhibiting an 82.8 % increase in 2-PE (from 15.06 to 28.22 mg/L) and a 2.07-fold increase in 2-PEA (from 75.73 to 157.27 mg/L) in chemically defined grape juice medium (CDGJM). Importantly, while enhancing the target product, ALE also increases the tolerance of L. *thermotolerans* A38 to stressors such as ethanol, thereby broadening the application potential of this yeast. Genome sequencing revealed genetic variations in these evolved strains, providing valuable insights for future design and optimization of 2-PE and 2-PEA production.

CRediT authorship contribution statement

Xingmeng Lei: Writing – review & editing, Writing – original draft, Visualization, Methodology, Data curation, Conceptualization. **Yu Chen:** Software, Methodology, Data curation. **Binghong Gao:** Software, Methodology. **Xixi Zhao:** Validation, Software, Formal analysis. **Qing Sun:** Software, Data curation. **Yi Qin:** Validation, Software. **Yuyang Song:** Software. **Jiao Jiang:** Writing – review & editing, Visualization, Methodology. **Yanlin Liu:** Writing – review & editing, Visualization, Methodology, Funding acquisition.

Declaration of competing interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

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Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.fochx.2025.102483.

Data availability

No data was used for the research described in the article.

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