

# *Hanseniaspora uvarum* FS35 degrades putrescine in wine through the direct oxidative deamination pathway of copper amine oxidase 1

Bing Han<sup>a</sup>, Jie Gao<sup>a</sup>, Xiaoyu Han<sup>a</sup>, Huan Deng<sup>a</sup>, Tianyang Wu<sup>a</sup>, Chenyu Li<sup>a,b</sup>, Jicheng Zhan<sup>a,\*</sup>, Weidong Huang<sup>a,\*</sup>, Yilin You<sup>a</sup>

<sup>a</sup> College of Food Science and Nutritional Engineering, Beijing Key Laboratory of Viticulture and Enology, China Agricultural University, Tsinghua East Road 17, Haidian District, Beijing 100083, China

<sup>b</sup> Xinghua Industrial Research Centre for Food Science and Human Health, China Agricultural University, Xinghua 225700, Jiangsu, China



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## ABSTRACT

Putrescine is abundant in wine and have toxicological risks for the health of consumers. Certain microbes with oxidative deamination activity are considered to be one of the most effective ways to degrade putrescine. The characterization and possible mechanism of putrescine degradation by *Hanseniaspora uvarum* FS35 were studied in this work. *Hanseniaspora uvarum* FS35 was selected from 111 yeast strains by UPLC analysis and exhibited the ability to eliminate > 44.5 mg/L of putrescine after 12 h of culture. Transcriptome analysis showed that by adding putrescine as a nitrogen source, the gene expression level of copper amine oxidase 1 (*CuAO1*) increased, leading to a coordinated response in the oxidative deamination of putrescine to 4-amino-butanal and subsequent dehydrogenation to 4-amino-butanoate. The purified recombinant protein CuAO1 could degrade 25.8 and 21.8 mg/L of putrescine in Marselan and Cabernet Sauvignon wines, respectively. *H. uvarum* FS35 was then inoculated sequentially with *Saccharomyces cerevisiae* into Cabernet Sauvignon grape juice, and the physiochemical indexes and aroma compounds were detected by HPLC and HS-SPME/GC-MS, respectively. Wines produced from sequential inoculations showed significantly lower level of putrescine and higher amounts of glycerol, lactic acid, acetic acid, phenylethyl alcohol, ethyl acetate and β-phenylethyl acetate compared with the control fermentation of commercial *S. cerevisiae*, which proved the potential of *H. uvarum* FS35 as a promising strategy to reduce biogenic amines in wines.

## 1. Introduction

Biogenic amines (BAs) are low-molecular-weight basic nitrogenous compounds that are formed by decarboxylation of amino acids or transamination of aldehydes and ketones (Dabadé, Jacxsens, Miclotte, Abatih, Devlieghere, & De Meulenaer, 2021). Putrescine belongs to the group of aliphatic diamine and can be formed via ornithine decarboxylase pathway, agmatinase pathway, or arginine deiminase pathway (Callejón, Sendra, Ferrer, & Pardo, 2015). This amine is naturally present in plants, as well as in animals and microorganisms and plays important roles in cellular development and proliferation at low concentrations (Lorenzo et al., 2017). Both the no observed adverse effect level and oral toxicity level for putrescine is 2000 mg/L (Naila, Flint, Fletcher, Bremer, & Meerdink, 2010). Although putrescine has low toxicological activity by itself, it can enhance the toxicological effects of other amines through the inhibition of detoxifying enzymes (diamine-

oxidase and hydroxymethyl transferase), and can be detrimental to the organoleptic properties of wine, conferring putrefaction and a rotten meat smell (Alvarez, & Moreno-Arribas, 2014). The occurrence of BAs in 98 wine samples readily available to Chinese consumers were determined in our previous study, and it was found that putrescine was detected in all the samples and showed the highest concentration, reaching values of up to 31.17 mg/L in red wines and 19.43 mg/L in white wines (Han et al., 2022). However, it is difficult to establish the maximum limit for this amine in wine because of complex interactions with wine matrix. In particular, the presence of alcohol and acetaldehyde in wine can enhance the toxicological effect of putrescine due to their inhibitory effect on detoxifying enzymes in the intestinal tract of human (Callejón et al., 2015). Therefore, controlling putrescine within the limited level is critical for the quality of wine.

The investigations in recent years have focused on the possible strategies to inhibit putrescine formation in wine (Russo, Capozzi,

\* Corresponding authors.

E-mail addresses: [zhanjicheng@cau.edu.cn](mailto:zhanjicheng@cau.edu.cn) (J. Zhan), [huanggwd@263.net](mailto:huanggwd@263.net) (W. Huang), [yilinyou@cau.edu.cn](mailto:yilinyou@cau.edu.cn) (Y. You).

Spano, Corbo, Sinigaglia, & Bevilacqua, 2016). However, when putrescine is already presented in wine, or when the production of putrescine relies on indigenous microbes, the use of amine-degrading strains or amine-degrading enzymes will help to eliminate this amine (Capozzi, Fragasso, & Russo, 2020). It was reported in several studies that certain LAB and yeast have the capability to degrade putrescine during wine fermentation (Capozzi et al., 2012; García-Ruiz, González-Rompinelli, Bartolomé, & Moreno-Arribas, 2011). Additionally, flavin amine oxidases (FlavAO) and copper amine oxidases (CuAO), which differed with respect to the redox cofactor, have been applied to reduce putrescine (Callejón, Sendra, Ferrer, & Pardo, 2017). A flavin-dependent putrescine oxidase identified from *Kocuria varians* was applied to degrade putrescine in wine (Callejón et al., 2015). However, the physicochemical conditions of wine, primarily ethanol and low pH, prevent the optimal performance of most described amine oxidases (Li & Lu, 2020). Therefore, the selection of microorganisms producing BA-degrading enzymes as starters should be taken into account to solve the problem of high amine concentration in wine, so as to improve the quality, safety, and competitiveness of wine in the market.

Non-*Saccharomyces* yeasts are a heterogeneous consortium of microorganisms applied in the initial step of AF which strongly contribute to the chemical composition and the sensorial characteristics of wine (Han et al., 2021). In fact, the addition of non-*Saccharomyces* yeasts as part of mixed starter formulations, together with *S. cerevisiae*, has been recently indicated as a way to increase the aroma complexity of the wine with reducing risks for wine safety compared to uncontrolled spontaneous fermentation (Tristeza, Tufariello, Capozzi, Spano, Mita, & Grieco, 2016; Tristeza et al., 2013). It was found that putrescine was not detected in Chardonnay real wines under *H. vineae* fermentation and the histamine and tyramine were significantly decrease by other species of *Hanseniaspora* under mixed cultures (Medina et al., 2013). Similarly, the *H. uvarum* strain in co-inoculation and sequential inoculation with *S. cerevisiae* has also been found to contribute to reducing the content of BAs, and improving the overall quality of wine (Li et al., 2020). These indicate that non-*Saccharomyces* yeasts may have the potential to favor wine to be safer and more characteristic (Wei et al., 2020).

Considering the toxicological risks of BAs in wine to the health of consumers, the main purpose of this study was to describe the putrescine degradation characterization and mechanism of *H. uvarum* FS35 in wine, and the biochemical properties of recombinant CuAO1 responsible for putrescine degradation was studied. Moreover, in order to highlight its potential in winemaking, sequential fermentations with *H. uvarum* FS35 and *S. cerevisiae* were performed for the production of a typical red wine, as well as principal component analysis (PCA) was applied to underline possible correlations between samples and various wine aromas. Overall, present results suggest the use of *H. uvarum* FS35 in winemaking as a potential strategy to degrade BAs, which is of great significance for improving the safety and quality of wine.

## 2. Materials and methods

### 2.1. Chemical reagents and standards

Standards of D- (+)-glucose, D- (-)-fructose, glycerol, ethanol, organic acids, and biogenic amines were purchased from Sigma-Aldrich (Shanghai, China). Chromatographic grade reagents were provided by ThermoFisher Scientific (Shanghai, China). All other chemicals were of analytical grade and supplied by Sinopharm Chemical Reagent Co., ltd. (Beijing, China). Ultra-pure water was obtained from Milli-Q system (Millipore Corp., Shanghai, China).

### 2.2. Screening of yeasts with high BA-degrading ability

#### 2.2.1. High-throughput BCP staining method for preliminary selection

A total of 111 yeasts were used as shown in Table A.1. These strains were previously isolated in our laboratory from grape juice and wines of

different grape species and origins and properly identified by 16S rRNA partial gene sequencing as described by Li et al. (2021). These strains were kept frozen at -80 °C in a sterilized mixture of YPD medium and glycerol (50:50, v/v).

Referring to the method of Yang et al. (2020), all 111 strains were first grown in YPD broth at 28 °C for 12 h. Then a 100 µL culture was coated on YPD agar medium supplemented with amino acids (L-arginine, L-ornithine monohydrochloride, L-histidine, L-tyrosine, L-lysine, L-tryptophan and L-phenylalanine) at 0.5 % each, and pyridoxal-5-phosphate at 0.005 %. Hydrochloric acid (0.1 M or 1 M) was added to adjust a pH of the medium to 5.3 ± 0.1. Incubation was performed at 28 °C for 4 d, then the agar medium containing 0.006 % BCP was poured on each plate and set for 5 min. The strains with yellow shade were selected for the next stage trial. The medium without yeast was treated as negative control.

#### 2.2.2. Qualification of BA-degrading capacity by UPLC analysis

Based on the research of Cueva et al. (2012), a total of 69 yeast isolates were screened for their capacity to degrade BAs in assay broth consisting of must simulated medium (MSM, main components: 100 g/L glucose; 100 g/L fructose; 3 g/L tartaric acid; 0.3 g/L citric acid; 0.3 g/L L-malic acid; 0.2 g/L MgSO<sub>4</sub>·7H<sub>2</sub>O; 2 g/L KH<sub>2</sub>PO<sub>4</sub>; 0.3 g/L (NH<sub>4</sub>)<sub>2</sub>SO<sub>4</sub>; 0.6 g/L asparagine; 0.3 g/L inositol; pH 5.8) supplemented with BAs (tryptamine, 2-phenylethylamine, putrescine, cadaverine, histamine, tyramine, spermidine, and spermine) at 25 mg/L each. The assay broth (pH 5.8) was filter-sterilized (Millipore Express, 0.22 µm). Strains with an initial cell density of 6 log CFU/mL were inoculated in this assay broth. After incubation at 28 °C for 4 d, the culture was centrifuged (5000g, 5 min, 4 °C) and filtered through 0.45 µm PES filter. 1 mL of the supernatant was derivatized with dansyl chloride, separated, and analyzed with a Waters Acuity UPLC system (Waters Corp., MA, USA) as described in our previous study (Han et al., 2022). Chromatographic separation was carried on a Waters Acuity UPLC BEH C18 column (100 × 2.1 mm, 1.7 µm) connected to guard-column of the same matrix, both set at 40 °C. A binary mobile phase composed of 0.01 mol/L ammonium acetate (A) and acetonitrile (B) was used applying the following gradient program at a flow rate of 0.3 mL/min: 0–2 min 60 % A, 2–3 min 60–20 % A, 3–4 min 20–10 % A, 4–6 min 10–60 % A, achieving a complete separation in 6 min. The injection volume was 10 µL, and the pressure during the analysis was 6000 psi. Detection was performed at the wavelength of 216 nm. The data obtained was processed using Empower 2.0 software (Waters Corp., MA, USA). The medium without strain was used as negative control.

#### 2.2.3. Evaluation of BA-producing ability of selected yeasts

6 log CFU/mL of *H. uvarum* FS35, *S. cerevisiae* HL10, and *S. cerevisiae* HL17 were incubated at 28 °C for 4 d in MSM contained 0.5 % of each amino acids and 0.005 % of pyridoxal-5-phosphate as mentioned above, previously described by Li et al. (2018). The medium without yeast was treated as negative control. The capacity of isolates to produce BAs was assayed by UPLC as described in 2.2.2.

### 2.3. Characterization of putrescine degradation of *H. Uvarum* FS35

#### 2.3.1. Growth curve

6 log CFU/mL of *H. uvarum* FS35 was inoculated in yeast carbon base (YCB) medium added with 50 mg/L of putrescine at 28 °C. The medium without putrescine was used as negative control. During the incubation period, the optical density was measured at 600 nm by means of a UV spectrophotometer (Shimadzu UV-1800, Japan) at regular intervals.

#### 2.3.2. The mode of action to degrade putrescine

The cells cultured in 1 L of YCB medium supplemented with 50 mg/L of putrescine for 8 h were collected by centrifugation (5000g, 5 min, 4 °C) and washed twice with 0.1 M PBS (pH 7.4). Cells were further suspended in 0.1 M PBS (pH 7.4) and half of them were autoclaved. After

incubation with 50 mg/L of putrescine at 28 °C for 72 h, the suspension was centrifuged (10000g, 10 min, 4 °C) and the supernatant was collected. The active cells were further suspended in 0.1 M PBS (pH 7.4), sonicated in an ice bath for 20 min (sonication of 3 s, interval of 3 s) and then centrifuged at 10000g for 10 min at 4 °C to collect the supernatant. The content of putrescine in all supernatants was determined by UPLC as mentioned above.

### 2.3.3. Dynamics of putrescine degradation

Based on the research of Callejón et al. (2015), *H. uvarum* FS35 at 6 log CFU/mL was inoculated in YCB medium or MSM containing 50 mg/L of putrescine, and stirred at 28 °C for 24 h. The aliquots were taken at different time points and putrescine content was determined by UPLC as mentioned above.

### 2.3.4. Determination of monoamine oxidase (MAO), diamine oxidase (DAO) and laccase activities

The activities of MAO, DAO and laccase were determined following the corresponding instructions of assay kits (Solarbio, Beijing, China). Cells of *H. uvarum* FS35 were prepared regularly by centrifugation (5000g, 5 min, 4 °C) from 50 mL of YCB medium with 50 mg/L of putrescine addition. The cells were washed twice and re-suspended in 500 μL of 0.1 M PBS (pH 7.4). After ultrasound in an ice bath for 15 min (sonication of 3 s, interval of 3 s) and centrifugation at 10000g for 10 min at 4 °C, the cell-free supernatant (CFS) was obtained to determine the activities of MAO, DAO and laccase at the absorption of 242 nm, 500 nm, and 420 nm, respectively by a microplate reader (SPARK 10 M, TECAN, Switzerland). Control assays were carried out without putrescine.

### 2.3.5. Determination of γ-amino-butyric acid (GABA) content

The content of extracellular GABA was detected by GABA ELISA kit (Nanjing Jiancheng, Nanjing, China). The supernatant was collected periodically by centrifugation (5000g, 5 min, 4 °C) from 50 mL of cell culture with or without 50 mg/L of putrescine addition. Briefly, 50 μL of the supernatant or standard was mixed with 100 μL of HRP-conjugate reagent in micro-elisa strip plate. After incubation at 37 °C for 1 h, the mixture was washed four times and 50 μL of chromogen solution was added. Then the solution was incubated at 37 °C for 15 min protected from light and the absorbance of 450 nm was determined. 50 μL of ddH<sub>2</sub>O replaced with the supernatant was treated as blank.

### 2.3.6. Effects of wine matrix on the growth and putrescine-degrading ability of *H. uvarum* FS35

The effects of temperature and pH on the growth and putrescine degradation activity of *H. uvarum* FS35 was investigated by adding 0.5 mL of the inoculum (8 log CFU/mL) to 50 mL of MSM supplemented with 50 mg/L of putrescine, based on previous study (Zaman, Bakar, Selamat, Bakar, Ang, & Chong, 2014). The mixed solution was then incubated at 10, 20, 30, 40, or 50 °C for 48 h or adjusted to a range of pH values (3, 4, 5, 6, or 7) using 1 M HCl and 1 M NaOH, and cultured at 28 °C for 48 h. Then, the 600 nm absorbance and putrescine content of the ultimate solution were determined by UV spectrophotometer and UPLC, respectively.

To analyze the effects of wine components on the growth and putrescine-degrading ability of *H. uvarum* FS35, ethanol (0 %, 3 %, 6 %, 9 % or 12 % (v/v)), SO<sub>2</sub> (0, 30, 60, 90 or 120 mg/L), glucose (100, 200, 300, 400 or 500 g/L), arginine (0 %, 0.1 %, 0.3 %, 0.5 % or 0.7 % (w/v)), and ornithine (0 %, 0.1 %, 0.3 %, 0.5 % or 0.7 % (w/v)) were added to the mixed solution at the final concentration, respectively, based on the previous study (García-Ruiz et al., 2011). The mixture was then incubated at 28 °C for 48 h, and the 600 nm absorbance and putrescine content were measured as mentioned above.

## 2.4. Transcriptome analysis of the putrescine degradation mechanism of *H. uvarum* FS35

### 2.4.1. Transcriptome analysis

The transcriptome changes of the cells separately cultivated in YCB media (C\_8 h) and YCB media added with 50 mg/L of putrescine (T\_8 h) at 28 °C for 8 h with string were analyzed. These cells were obtained by centrifugation, washed with cold sterile normal saline, frozen in liquid nitrogen, and stored at –80 °C. According to the method of Li et al. (2020), the total RNA extraction, express chart library construction, and transcriptome sequencing of *H. uvarum* FS35 were carried out by Majorbio Bio-Pharm Technology Co., Ltd. (China). Differentially expressed genes (DEGs) were obtained on the basis of the false discovery rate (FDR) value ≤ 0.05 and |log<sub>2</sub>(Fold Change)| ≥ 1.

### 2.4.2. Quantitative real-time PCR (qRT-PCR) for validation of RNA-seq

To validate the transcriptome results, the expression changes of the selected key genes were analyzed by qRT-PCR. RNA extraction and reverse transcriptase were done using the spin column yeast total RNA purification kit (Sangon, China) and the EasyQuick RT MasterMix (CWBio, China) according to the manufacturer's instructions. Referring to the method of Li et al. (2020), the qRT-PCR was performed on a CFX96 real-time PCR detection system (Bio-Rad, CA, USA) with the commercial kit 2X M5 HiPer SYBR Premix EsTaq (with Tli RNaseH) (Mei5bio, China). *ACT1* (*actin*) was used as an internal control to normalize the PCRs, and the relative changes in the gene expression level were calculated using the 2<sup>–ΔΔCt</sup> method. The primers used in the qRT-PCR assay were synthesized by Sangon (Beijing, China) and were listed in Table A.2.

## 2.5. Expression and purification of recombinant CuAO1

The recombinant strain of GS115/pPIC9K/CuAO1 was constructed by Sangon (Beijing, China). The protein expression experiment was started by inoculating single colonies of the recombinant strain in BMGY medium (25 mL) at 28 °C and 180 rpm until an OD<sub>600</sub> reached to 2–6. Afterward, the cells were centrifuged (5000g, 5 min, 4 °C), re-suspended in BMGY medium (20 mL) and induced by 1 % methanol at 28 °C and 180 rpm for 72 h. Then, the supernatant was harvested by centrifugation at 4 °C, 12000 rpm for 10 min, and purified using Ni-NTA 6FF sefinose (TM) resin kit (Sangon, China) according to the manufacturer's instructions. The eluted fractions containing CuAO1 were pooled and analyzed using western blot. The specific activity was measured by mixing 10 μL of enzyme solution with 190 μL of a peroxidase-coupled reaction solution as described by Callejón et al. (2015). Then, the absorption of 515 nm was detected by a microplate reader (SPARK 10 M, TECAN, Switzerland), over a period of 30 min, and the initial slope of plots of OD<sub>515</sub> versus time, representing CuAO1 activity, was calculated.

## 2.6. Characterization of recombinant CuAO1

### 2.6.1. The optimal temperature and pH

The optimal temperature and pH were evaluated by incubating the enzyme mixture at temperature ranges from 4 to 85 °C, and at pH ranges from 2.5 to 8.5, respectively. The maximum enzyme activity was taken as 100 %, and relative activities were then calculated by the ratio of enzyme activity to the maximum enzyme activity (Callejón et al., 2015).

### 2.6.2. Temperature stability, ethanol and glucose tolerance

The temperature stability and ethanol tolerance were evaluated by incubating the CuAO1 at different temperatures (25–85 °C), and in different ethanol concentrations (0–15 % (v/v)) for 10 min, respectively. Similarly, the effect of glucose on CuAO1 activity was assayed at different contents (0–300 g/L) for 3 h. Then the relative activities were measured (Callejón et al., 2015).

### 2.6.3. The effects of metal ions, enzyme inhibitors, proteases, and surfactants on CuAO1 activity

The effects of metal ions on CuAO1 activity were investigated by incubating the CuAO1 with 2.5 mM of  $\text{Ca}^{2+}$ ,  $\text{Fe}^{3+}$ ,  $\text{Fe}^{2+}$ ,  $\text{Ba}^{2+}$ ,  $\text{Li}^+$ ,  $\text{Na}^+$ ,  $\text{K}^+$ ,  $\text{Ni}^{2+}$ , and  $\text{Al}^{3+}$  in the form of hydrochloride, or  $\text{Cu}^{2+}$ ,  $\text{Mg}^{2+}$ ,  $\text{Mn}^{2+}$ ,  $\text{Co}^{2+}$ , and  $\text{Zn}^{2+}$  in the form of sulfate salts for 5 min. The relative activities were measured, where 100 % of the activity was taken in absence of any metal ions (Callejón et al., 2015).

The effects of 1 mM enzyme inhibitors (bipyridyl, EDC, rasagiline, pargyline, semicarbazide, cyclopropilamine, phenanthroline, clorgiline, EDTA, dithiothreitol, and L-cysteine) on CuAO1 activity were conducted by incubating the enzyme-inhibitor mixture for 10 min. Enzyme activity in absence of inhibitor was considered as 100 % and relative activities were calculated (Callejón et al., 2015).

In order to understand the sensitivity of CuAO1 to proteases, the CuAO1 was treated with 1 mg/mL of proteinase K at 55 °C for 1 h, and 1 mg/mL of papain or trypsin at 37 °C for 1 h, respectively. Similarly, the CuAO1 was exposed to 0.01 % SDS, 0.05 % Triton-100, and 0.05 % Tween-20, respectively, for 3 h to evaluate its sensitivity to surfactants. Enzyme activity was regarded as 100 % without treatment with protease and surfactant, and relative activities were determined (Wu, Guo, Zhao, Ding, & Chen, 2018).

### 2.6.4. Kinetic parameters for putrescine

Kinetic parameters  $K_m$  and  $V_{max}$  for putrescine were determined by adding amine to the reaction mixture to a concentration between 0.05 and 5 mM. Rate constants were deduced from the slope and intercept of double-reciprocal plots of initial velocity versus putrescine concentration (Callejón et al., 2015).

### 2.6.5. Substrate specificity

To determine substrate specificity, various BAs with a concentration of 2 mM were used as substrates to test enzyme activity. Relative activity on putrescine was considered as 100 % (Callejón et al., 2015).

### 2.6.6. Evaluation of CuAO1 on putrescine degradation in wine

To investigate the ability of CuAO1 to degrade putrescine in wine, 300  $\mu\text{L}$  of enzyme solution was mixed with 2.7 mL of Marselan or Cabernet Sauvignon wine (pH adjusted to 3.5), then 50 mg/L of putrescine was added. The physicochemical parameters of Cabernet Sauvignon and Marselan wines were shown in Table A.3. Following incubation at 28 °C for 24 h, putrescine content was quantified by UPLC as described above. Samples without enzyme but cultured under the same conditions as the test samples were considered as control (Callejón et al., 2015).

### 2.7. Lab-scale vinification of *H. Uvarum* FS35

The grape juice applied to vinification was obtained from Cabernet Sauvignon grape variety (glucose 118.53 g/L; fructose 129.27 g/L; total acidity 6.63 g/L; yeast assimilable nitrogen 175.37 mg/L; pH 3.47; °Brix 21.6) of Fangshan region (2021 vintage). Referring to the method of Li et al. (2020), the grapes were destemmed, crushed, and sterilized through 0.45  $\mu\text{m}$  filter. Sulfur dioxide was added in the form of potassium sulfite, and the final concentration was 60 mg/L. Two types of fermentation were carried out: (1) pure culture with commercial *S. cerevisiae* Vintage Red (Enartis, Spain); (2) sequential fermentation (*H. uvarum* FS35 was 48 h prior to *S. cerevisiae*). All yeasts were pre-cultured in sterile grape juice at 28 °C for 24 h, and the initial inoculation concentration was 6 log CFU/mL. All fermentations were carried out at 23 °C and ended with a stable °Brix measured by a Pocket Pal-3 model refractometer (Atago, Japan). Then, the wine samples were centrifuged (5000g, 10 min, 4 °C) and the supernatants were stored at –20 °C for further analysis.

### 2.8. Determination of classical oenological parameters

The contents of sugars, glycerol, ethanol, and organic acids were determined by a high-performance liquid chromatography (HPLC) system (Waters, Dublin, Ireland) equipped with a refractive index detector (RID) and a diode array detector (DAD) as described by Han et al. (2021). Chromatographic separation was performed on a Aminex HPX-87H column (300 × 7.8 mm, 9  $\mu\text{m}$ ) with a flow rate of 0.5 mL/min by using 5 mM sulfuric acid as mobile phase. The column temperature was 55 °C and the injection volume was 10  $\mu\text{L}$ . Sugar and organic acid analysis were conducted by using RID and DAD at 210 nm, respectively. Before the injection, the samples were filtered through PES filters (0.45  $\mu\text{m}$ ). The quantification was carried out using the respective calibration curve of each compound.

### 2.9. Analysis of volatile compounds

The volatile compounds were extracted by the method of headspace solid phase microextraction (HS-SPME) (Diez-Ozaeta, Lavilla, & Amárita, 2021). Briefly, 10 mL of wine was transferred into a 20 mL headspace vial, together with 200  $\mu\text{L}$  of 2-methyl-3-heptanone internal standard (100 mg/L) and 2 g of NaCl. Samples were incubated at 80 °C for 20 min with agitation. Then, the fibre was inserted into the headspace for 20 min, and desorbed in GC injection port at 280 °C for 5 min in splitless mode.

The chromatographic analysis was performed by a Thermo 1300 gas chromatography mass spectrometry (GC-MS) equipped with a TG-5 MS capillary column (30 m × 0.25 mm × 0.25  $\mu\text{m}$ ) (Thermo, MA, USA). The temperature of GC column was initially programmed at 60 °C for 5 min, and increased at 3.5 °C/min to 100 °C for 5 min, then to 200 °C at 8 °C /min for 5 min and held at 280 °C for 15 min. High purity helium was used as carrier gas at a flow rate of 1.2 mL/min. For the MS system, the temperatures of the quadrupole and ionization source were 280 °C, and acquisitions were carried out in full scan mode. The volatile compounds were identified based on NIST 8.0 and Willey 7 libraries, and semi-quantified by calculating the relative peak area in relation to that of the internal standard.

### 2.10. Sensory evaluation

Sensory evaluation was conducted by a trained team of 12 panel members, who were trained with different wine samples for at least 3 months. According to a previously published method (Han et al., 2021), the samples were scored by 1–5 points to evaluate the aroma of Cabernet Sauvignon wines from 8 aspects (alcohol, sweetness, acidity, floral, tropical fruit, citrus fruit, herbal, spice). Each set of samples was presented to each panel member in a fixed order.

### 2.11. Statistical analysis

All experiments were performed in triplicate. Data were expressed as the mean ± standard deviation (SD) of triplicate measurements. Significance differences ( $P < 0.05$ ) were evaluated using one-way analysis of variance (ANOVA) with Duncan's test by SPSS 25.0 Software (SPSS Inc., Chicago, IL, USA). The statistical analyses of data were performed using GraphPad Prism 8.0 software (San Diego, CA, USA). The heatmap and PCA were performed using Origin 2018 software (Northampton, MA, USA).

## 3. Results and discussion

### 3.1. *H. Uvarum* FS35 was selected with high potential to degrade BAs

BCP has a yellow color under pH 5.2 and has a purple color under pH 6.8. The production of alkaline substances such as amines could increase the pH of the culture containing BCP and change its color. Thus, the

violet shade in the plate could visually reflect the unwanted BA-producing ability of strains, while the yellow shade indicated that some strains may not produce or may have the ability to degrade BAs. The coloration results were shown in Fig. 1a. The results demonstrated that 42 strains of yeast showed obvious violet, and 69 strains showed obvious yellowish color. Because the production of other alkaline/acidic substances may interfere with the chromogenic results, and the ability to degrade BAs depends not only on the species but also on the strains, BCP staining method is unable to accurately evaluate the strains with potential to degrade BAs. Therefore, in the next stage, UPLC method was used to quantitatively determine the BA-degrading activity of 69 selected strains. According to the results shown in Fig. 1b, none of the strains were able to eliminate all the BAs completely under the experimental conditions, and 13 strains exhibited high ability to degrade  $\geq 20$  mg/L of at least two amines. In general, putrescine and cadaverine were degraded to a greater extent than any other amines by all selected strains, while spermine, and spermidine were degraded to a less extent. With regards to *S. cerevisiae*, *H. uvarum*, and *S. bacillaris*, the main species involved in AF, seemed to have great potential for BA degradation, which was consistent with the previous literature (Nisiotou et al., 2018; Tristezza et al., 2016). Furthermore, the following three strains of *H. uvarum* FS35, *S. cerevisiae* HL10, and *S. cerevisiae* HL17 exhibited the highest degradation activity of total amines and the major amines (histamine, tyramine, cadaverine and putrescine) (Fig. 1c). Among them, *H. uvarum* FS35 showed better degradation ability for 2-phenylethylamine, spermine and spermidine than the other two strains and had the highest ability to degrade 79.51 % of total amines. Then, the BA-producing capacity of the three selected strains was performed. As shown in Fig. 1d, *H. uvarum* FS35 was unable to produce any BAs, but *S. cerevisiae* HL10 and *S. cerevisiae* HL17 showed the ability to produce tryptamine and 2-phenylethylamine, suggesting that *H. uvarum* FS35 able to degrade BAs do not contribute to BA formation. Thus, *H. uvarum* FS35 was finally selected to evaluate its potential applicability in removing putrescine from culture medium and actual wine fermentation process, proposing a technological improvement to reduce the abundant putrescine content in wine.

### 3.2. Putrescine degradation characterization of *H. Uvarum* FS35

New experiments were conducted to gain a deeper insight into the putrescine-degrading activity exhibited by *H. uvarum* FS35. The YCB medium provides less nitrogen source which may support the use of putrescine as an alternative source of nutrient. As shown in Fig. 2a, the OD600 values of *H. uvarum* FS35 in YCB medium supplemented with putrescine was not significantly different from that of the control group, indicating that the addition of 50 mg/L of putrescine would not have a negative effect on the growth of this strain. Then, the mode of action to degrade putrescine was studied in active and inactive cells. The results showed that autoclaved cells lost its ability to remove putrescine, while active cells were able to eliminate up to 96 % of this amine, and almost no putrescine was detected in the intracellular extract of the active cells (Fig. 2b), suggesting that the putrescine was eliminated through biodegradation rather than simple physical adsorption or bioaccumulation. We therefore started out to investigate the dynamics of putrescine degradation by *H. uvarum* FS35. The results demonstrated that putrescine was degraded gradually in YCB medium within 24 h, in particular,  $>17.5$  mg/L and 44.5 mg/L of this amine were eliminated after 4 h and 12 h of incubation, respectively (Fig. 2c). Similarly, in MSM, putrescine of  $>21.5$  mg/L and 45.5 mg/L were eliminated after 24 h and 48 h of incubation, respectively (Fig. 2d). The degradation time of putrescine in MSM was longer than that in YCB medium, which may be due to the preferential use of other yeast assimilable nitrogen in MSM by *H. uvarum* FS35. The ability to degrade BAs in culture media or food-stuffs is based on the fact that some microorganisms can produce BA-degrading enzymes, such as amine oxidases (AOs) and multi-copper oxidase (MCO) (Alvarez et al., 2014). AOs are a large group of

enzymes catalyzing the degradation of BAs to metabolites that can be used by microorganisms as an energy source for growth. Laccase is the largest protein superfamily of MCO, which catalytically oxidize polyphenols and diamine while reducing molecular oxygen to water (Li & Lu, 2020). Several studies have described these enzymes and oxidation pathways of BAs (Wang, Billett, Borchert, Kuhn, & Ufer, 2013; Kandasamy et al., 2016). Thus, we determined the activities of a class of AOs (MAO, DAO) and laccase to explore the probable mechanism of putrescine degradation by *H. uvarum* FS35. As shown in Fig. 2e, the activities of these enzymes in the strain increased first and then decreased over time, and the strongest activities were exhibited at 4–6 h, which was consistent with the significant decrease of putrescine content in the same period. In addition, the activities of AOs in the medium added with putrescine was significantly higher ( $P < 0.05$ ) than that of laccase, indicating that the presence of putrescine could upregulate the expression level of genes regulating amine-degrading enzymes, and AOs may play an important role in putrescine degradation of *H. uvarum* FS35. Furthermore, the concentration of GABA, the main product of putrescine degradation via AOs, was detected in the medium supernatant of *H. uvarum* FS35. The results showed that GABA content was significantly higher ( $P < 0.05$ ) in the medium added with putrescine than that of the control group, and the highest content was detected at 6–8 h (Fig. 2f), suggesting that GABA may be one of the metabolites of putrescine degradation by AOs in *H. uvarum* FS35, but more degradation products and their detailed pathways need to be further studied and verified at the molecular level.

### 3.3. Influence of enological factors on the growth and putrescine degradation ability of *H. Uvarum* FS35

#### 3.3.1. Temperature and pH

Because yeast and other microorganisms have to face stress conditions such as low pH, ethanol and SO<sub>2</sub> during wine fermentation, understanding better the enological conditions affecting the growth and putrescine-degrading ability of *H. uvarum* FS35 might be practically important for its further application in wine production.

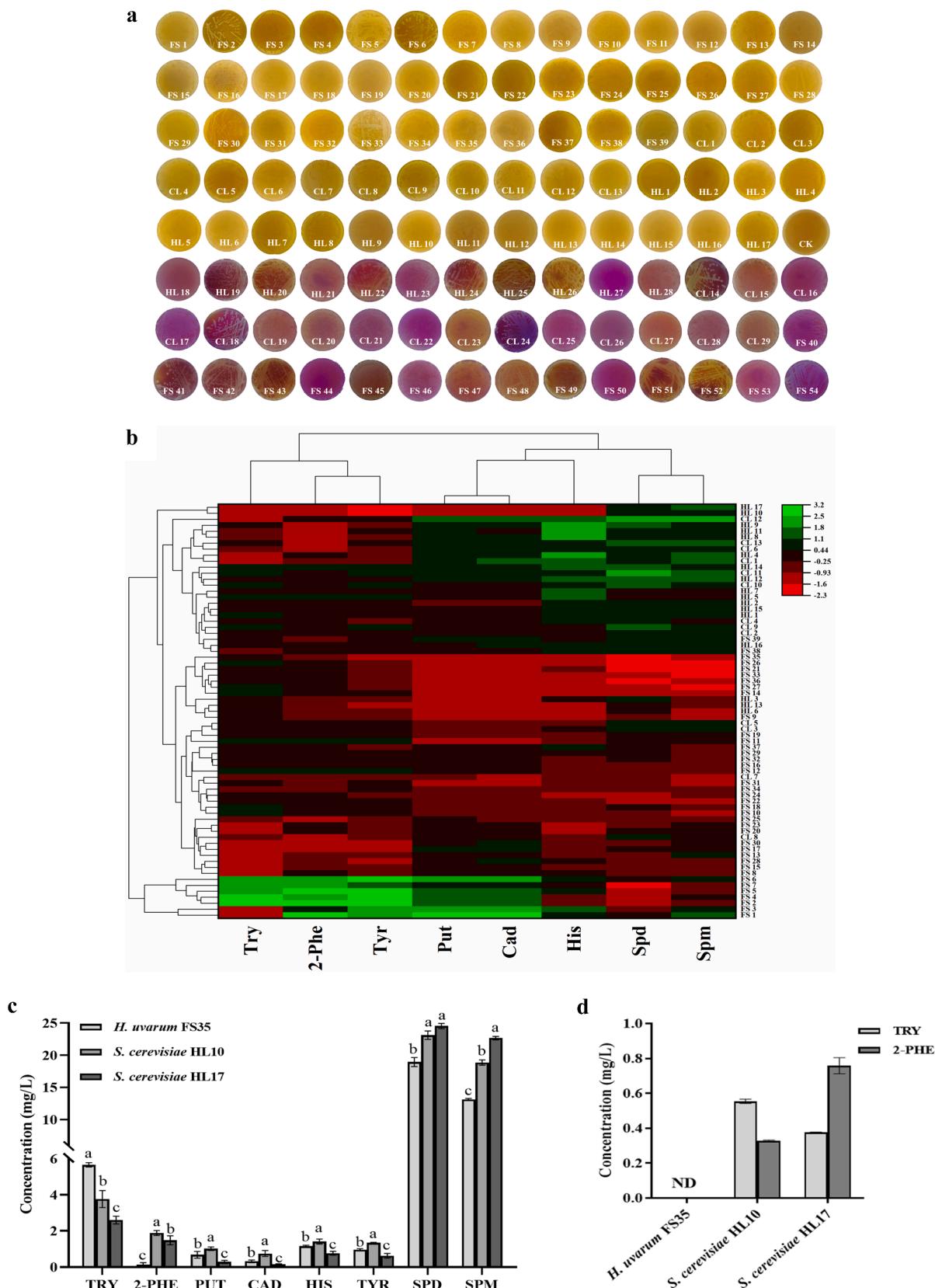
As shown in Fig. 2g, *H. uvarum* FS35 grew well at temperatures ranging from 20 °C to 30 °C, and the highest putrescine degradation activity occurred at 30 °C, at this temperature,  $> 47$  mg/L of the putrescine was degraded. In addition, the fact that the growth of *H. uvarum* FS35 is retarded at 40 and 50 °C might explain why of its putrescine degradation ability is limited at the same temperatures.

All pH values showed good results for the growth and putrescine reduction of *H. uvarum* FS35, and the putrescine-degrading ability at pH 3–4 was slightly lower in comparison to that of the pH 6–7 (Fig. 2h), indicating that either genes encoded amine-degrading enzymes were not totally activated or the activities of the amine-degrading enzymes were not optimal at lower pH values.

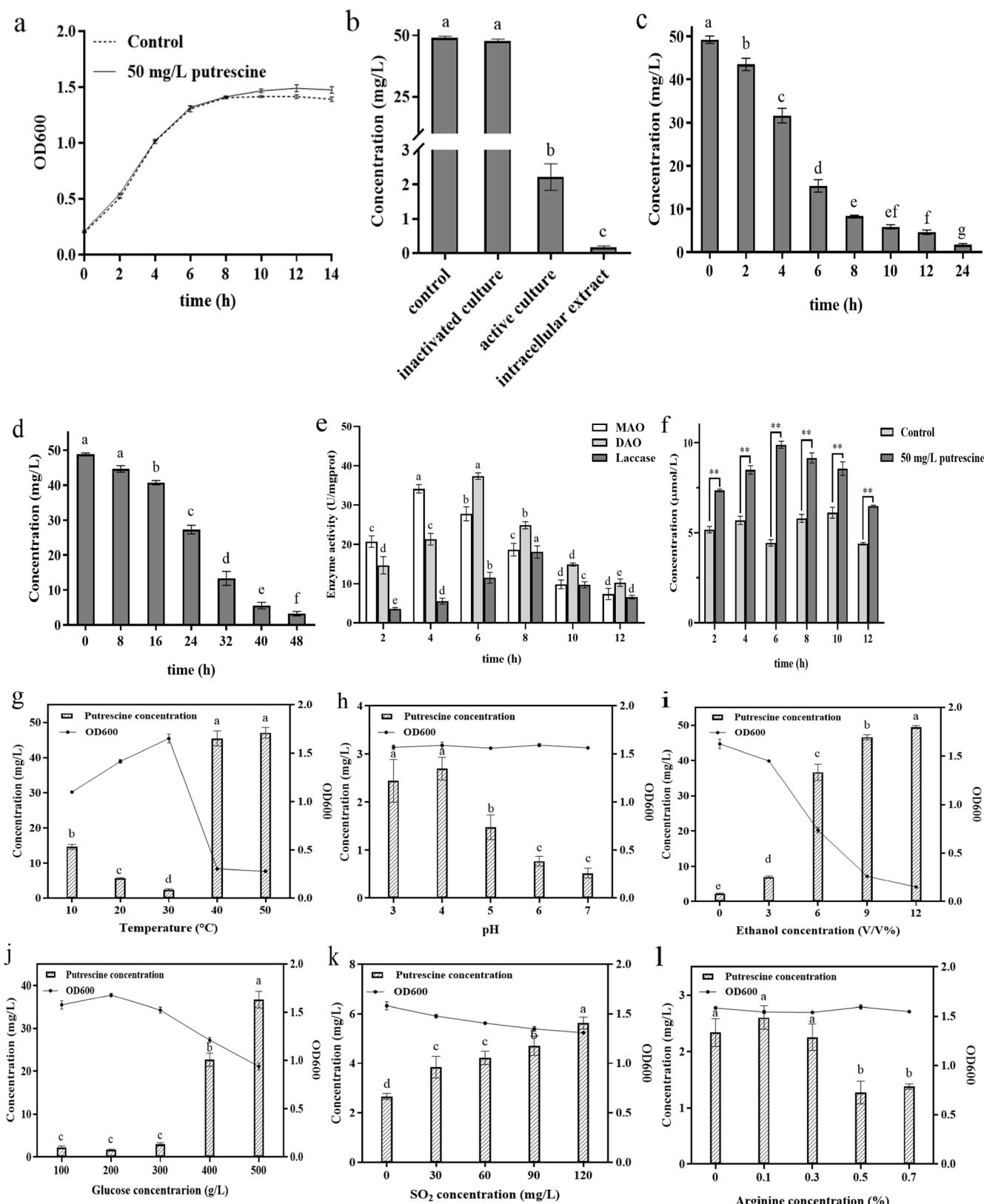
#### 3.3.2. Ethanol, glucose and SO<sub>2</sub>

Results showed that the presence of wine components such as ethanol (Fig. 2i) and glucose (Fig. 2j) strongly inhibited the growth and putrescine-degrading ability of *H. uvarum* FS35. In the presence of 12 % (the normal degree in wine) of ethanol, the growth was almost completely inhibited and only 6.9 % of the putrescine were degraded. Similarly, 26.5 % of putrescine were degraded when supplemented with 500 g/L of glucose. The results showed that the strain and amine-degrading enzymes seemed to be sensitive to the presence of ethanol, but exhibit high tolerance to glucose, which was consistent with the previous study (Zaman et al., 2014), suggesting that this strain may be suitable for degrading putrescine present in grape must or in the initial stage of AF.

The effect of SO<sub>2</sub>, the additive most employed in winemaking because of its antioxidant and selective antimicrobial properties, was tested at normal concentrations (Fig. 2k). The growth and putrescine-degrading ability of *H. uvarum* FS35 reduced slightly with the increase



**Fig. 1.** a. The chromogenic results of BCP staining screening. b. Heatmap of BA content in the presence of yeasts in MSM supplemented with 25 mg/L of each amine. c. The BA content in the presence of BA-degrading yeasts in MSM supplemented with 25 mg/L of each amine. d. The ability of selected strains to produce BAs. TRY: tryptamine; 2-PHE: 2-phenylethylamine; PUT: putrescine; CAD: cadaverine; HIS: histamine; TYR: tyramine; SPD: spermidine; SPM: spermine. Different letters represent significant difference ( $P < 0.05$ ).



**Fig. 2.** a. The growth of *H. uvarum* FS35 in YCB medium. b. The mode of action of putrescine degradation by *H. uvarum* FS35 in PBS (0.1 M, pH 7.4). c. The dynamics of putrescine degradation by *H. uvarum* FS35 in YCB medium added with 50 mg/L of putrescine. d. The dynamics of putrescine degradation by *H. uvarum* FS35 in MSM added with 50 mg/L of putrescine. e. Changes of monoamine oxidase, diamine oxidase and laccase activities of *H. uvarum* FS35 in YCB medium added with 50 mg/L of putrescine. f. Changes of  $\gamma$ -amino-butrylic acid content of *H. uvarum* FS35 in YCB medium. Effects of (g) temperature, (h) pH, (i) ethanol, (j) glucose, (k) SO<sub>2</sub>, (l) arginine, and (m) ornithine on the growth and putrescine-degrading activity of *H. uvarum* FS35 in MSM supplemented with 50 mg/L of putrescine. Different letters represent significant difference ( $P < 0.05$ ). \*\*,  $P < 0.01$  in comparison to the control group.

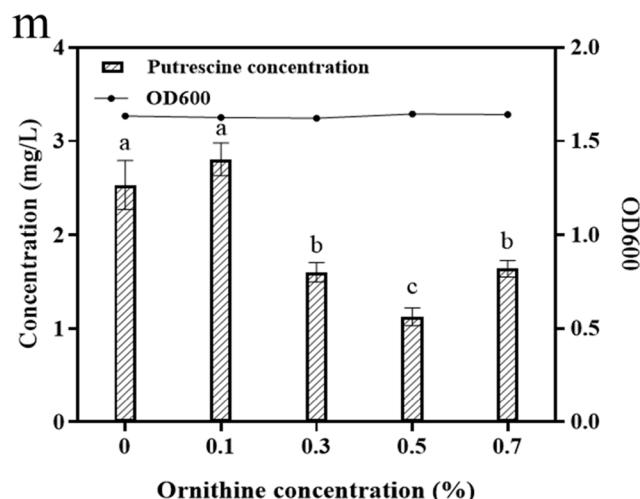


Fig. 2. (continued).

of SO<sub>2</sub> concentration, and exhibited the ability to degrade 88.7 % of putrescine in the presence of 120 mg/L of SO<sub>2</sub>, suggesting that this strain could be used to degrade putrescine at high SO<sub>2</sub> levels.

### 3.3.3. Arginine and ornithine

Arginine and ornithine are important precursor amino acids in the synthesis of putrescine, and their existence may affect the degradation of putrescine as an available nitrogen source. As shown in Fig. 2l-m, *H. uvarum* FS35 grew well in the absence or presence of various levels of arginine and ornithine, and the highest level of putrescine degradation occurred in the presence of 0.5 % arginine and ornithine, respectively, in which up to 98 % of putrescine was degraded, suggesting that the presence of arginine and ornithine would not have a negative effect on the putrescine degradation.

### 3.4. Transcriptome analysis of putrescine degradation mechanism of *H. uvarum* FS35

#### 3.4.1. Differential gene expression profile analysis

The gene expression profile of *H. uvarum* FS35 was different between C\_8 h and T\_8 h (Fig. 3a). The existence of DEGs induced by adding 50 mg/L of putrescine was confirmed, of which 929 DEGs were upregulated, whereas 832 DEGs were downregulated (Fig. 3b). As shown in Fig. 3c, The DEGs encoding binding, cell part and cellular process occupied an important position in the biological process, cellular component and molecular function category of GO annotation, respectively. Notably, many DEGs related to catalytic activity, cell part, cellular and metabolic process displayed ascending expression by putrescine induction.

The expression profile of genes involved in different kinds of metabolism pathways annotated and classified by the KEGG database was shown in Fig. 3d-e. The number of upregulated DEGs related to amino acid, carbohydrate, energy, lipid, cofactor, and vitamin metabolism was higher than that of downregulated DEGs, illustrating that putrescine may promote the growth and metabolism of *H. uvarum* FS35 as an additional nutrient source. Most of the upregulated DEGs in metabolism category were connected with amino acid, illustrating that putrescine degradation may improve the metabolism and biosynthesis of other amino acids, such as glycine, serine and threonine metabolism, lysine biosynthesis, cysteine and methionine metabolism, phenylalanine, tyrosine and tryptophan biosynthesis. The DEGs in arginine and proline metabolic pathways revealed that ALDH was upregulated in the direct oxidation and acetylation pathway of putrescine degradation, while PAO4 was downregulated to limit the oxidation of spermine and spermidine to putrescine, resulting in poor degradation of these two

polyamines. Importantly, the expression of CuAO1 involved in the degradation of phenyl-ethylamine, tyramine, and putrescine to phenyl-acetaldehyde, 4-hydroxy-phenylacetaldehyde, and 4-amino-butanal, respectively, was upregulated, indicating that this gene may play an important role in putrescine degradation, and CuAO1 (EC 1.4.3.22) protein may have a wide range of substrates.

#### 3.4.2. Validation of RNA-seq by qRT-PCR

The transcription results were verified using qRT-PCR analysis of the key genes related to putrescine metabolism. It was found that the Pearson correlation coefficient between the qRT-PCR results and the data of transcriptomic analysis was 0.9747, which suggested that the RNA-seq data were convincing (Fig. 3f-g). Therefore, based on the detected upregulated key genes and the literature survey, the possible putrescine degradation pathway of *H. uvarum* FS35 can be described as follows (Fig. 3h). The degradation started with the transport of putrescine into cells under the action of *Tpo1*, then the upregulated of *CuAO1* indicated that putrescine was oxidized to 4-amino-butanal, which was further oxidized to 4-amino-butanoate through the regulation of *ALDH*. 4-amino-butanoate was a main intermediate of putrescine degradation detected in *H. uvarum* FS35, as shown in Fig. 2f. Finally, succinate was produced with the upregulation of *gabT* and *gabD*, and entered the TCA cycle.

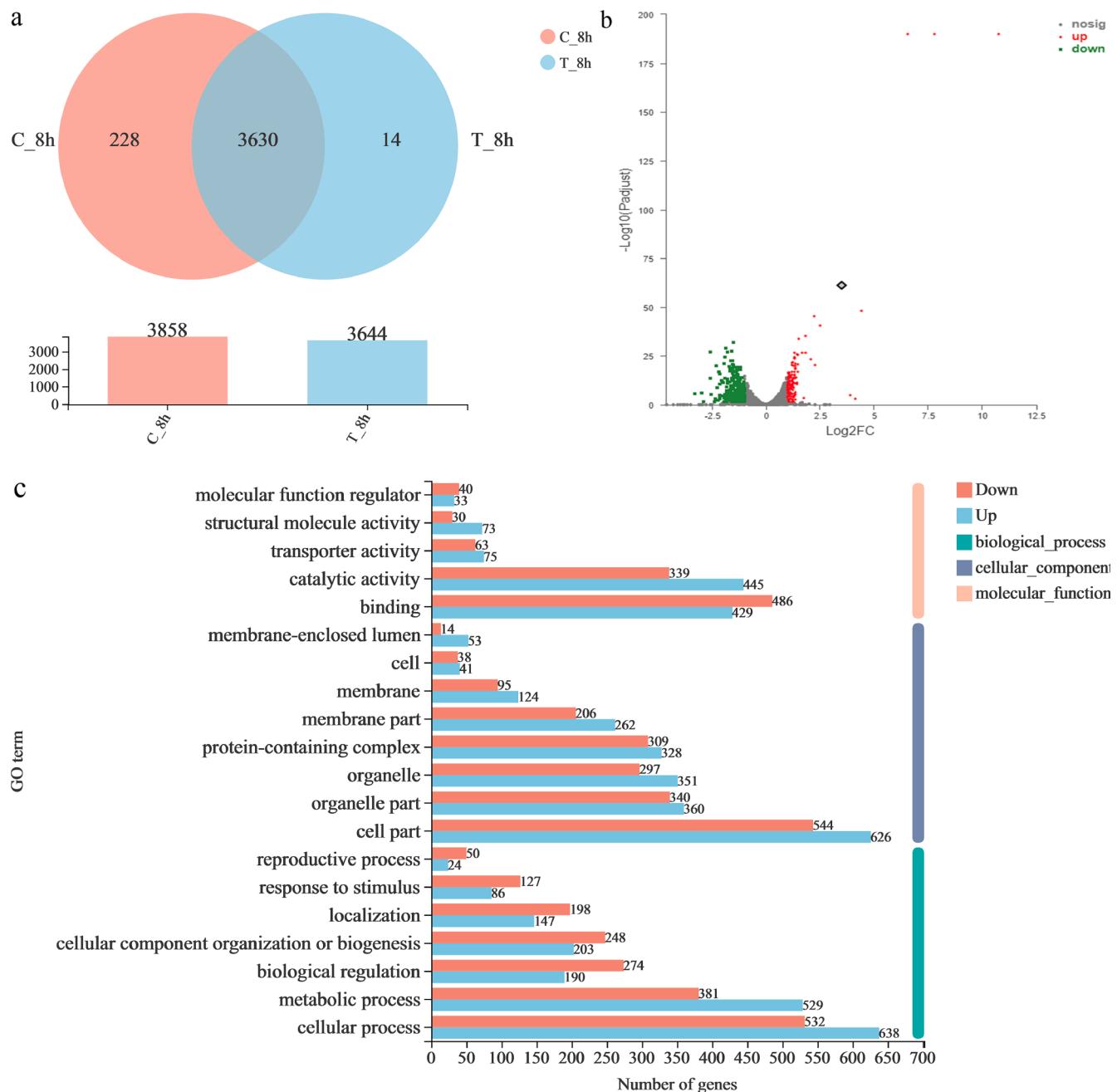
### 3.5. Expression and purification of recombinant CuAO1

To characterize the biochemical properties of CuAO1 from *H. uvarum* FS35, the expression and purification of recombinant CuAO1 protein were performed. For expression, *CuAO1* gene was ligated to pPIC9K expression vector and transformed into *P. pastoris* GS115 (Fig. 4a). A positive recombinant pPIC9K-CuAO1 plasmid was isolated from *P. pastoris* GS115 (Fig. 4b). Western blot was further used to assess whether the expression of recombinant CuAO1 was successful. The band with a molecular weight of approximately 130 kDa was identified (Fig. 4c), indicating that the target protein was successfully expressed with some modification. The purified CuAO1 showed a specific activity of  $0.56 \pm 0.009$  U/mg prot with putrescine as a substrate.

#### 3.6. Biochemical characterization of recombinant CuAO1

##### 3.6.1. Effects of temperature, pH, ethanol and glucose on CuAO1 activity

The optimal temperature of CuAO1 activity was found to be 55 °C, although the enzyme remained active over a rather wide temperature range, from 4 to 75 °C (Fig. 5a). Besides, the heat inactivation experiment revealed that CuAO1 is a relatively thermostable enzyme (Fig. 5b),



**Fig. 3.** Transcriptome analysis of *H. uvarum* FS35 treated with or without 50 mg/L of putrescine. a. Venn assay showed the profiles of gene expression in C\_8h and T\_8h. b. Volcano plot of differentially expressed genes (DEGs) between C\_8h and T\_8h. Red (upregulation) and green (downregulation) dots indicated DEGs ( $|\log_2(\text{Fold Change})| > 1$ ,  $P\text{-adjust} (\text{FDR}) < 0.05$ ). Gray dots were not considered as significantly DEGs. c. Function annotation analysis of the DEGs in C\_8h vs T\_8h. d. The upregulated DEGs of KEGG annotation analysis in C\_8h vs T\_8h. e. The downregulated DEGs of KEGG annotation analysis in C\_8h vs T\_8h. f. Validation of RNA-seq data using qRT-PCR analysis of the selected key genes. g. Comparison of the expression levels of key genes involved in putrescine degradation obtained from RNA-seq with those from qRT-PCR. h. Proposed degradation pathways of putrescine by *H. uvarum* FS35. *Tpo1*: polyamine transporter 1; *CuAO1*: copper amine oxidase 1; *ALDH*: aldehyde dehydrogenase; *gabT*: 4-aminobutyrate aminotransferase; *gabD*: succinate-semialdehyde dehydrogenase. The red arrows indicated the upregulation of gene expression levels.

which retained > 30 % of the activity after a pre-incubation treatment at 65 °C. In contrast, it was found that the putrescine oxidase from *K. varians* suffered rapid inactivation at 65 °C (Callejón et al., 2015). Moreover, *H. uvarum* FS35 CuAO1 was found to be active over a relatively broad range of pH, including values from 6.5 to > 8.5 (Fig. 5c). Low activity was detected below pH 5.5, suggesting that the pH of grape must (3.5–4.5) or wine (3.0–4.0) would inhibit the activity of CuAO1.

CuAO1 activity was evaluated in the presence of different

concentrations of ethanol and glucose. Ethanol strongly inhibited CuAO1, reducing the activity to < 41 % at 15 % (v/v%) concentration (Fig. 5d). Glucose had little effect on the activity of CuAO1, because its activity did not change significantly in the concentration range of 0–300 g/L (Fig. 5e).

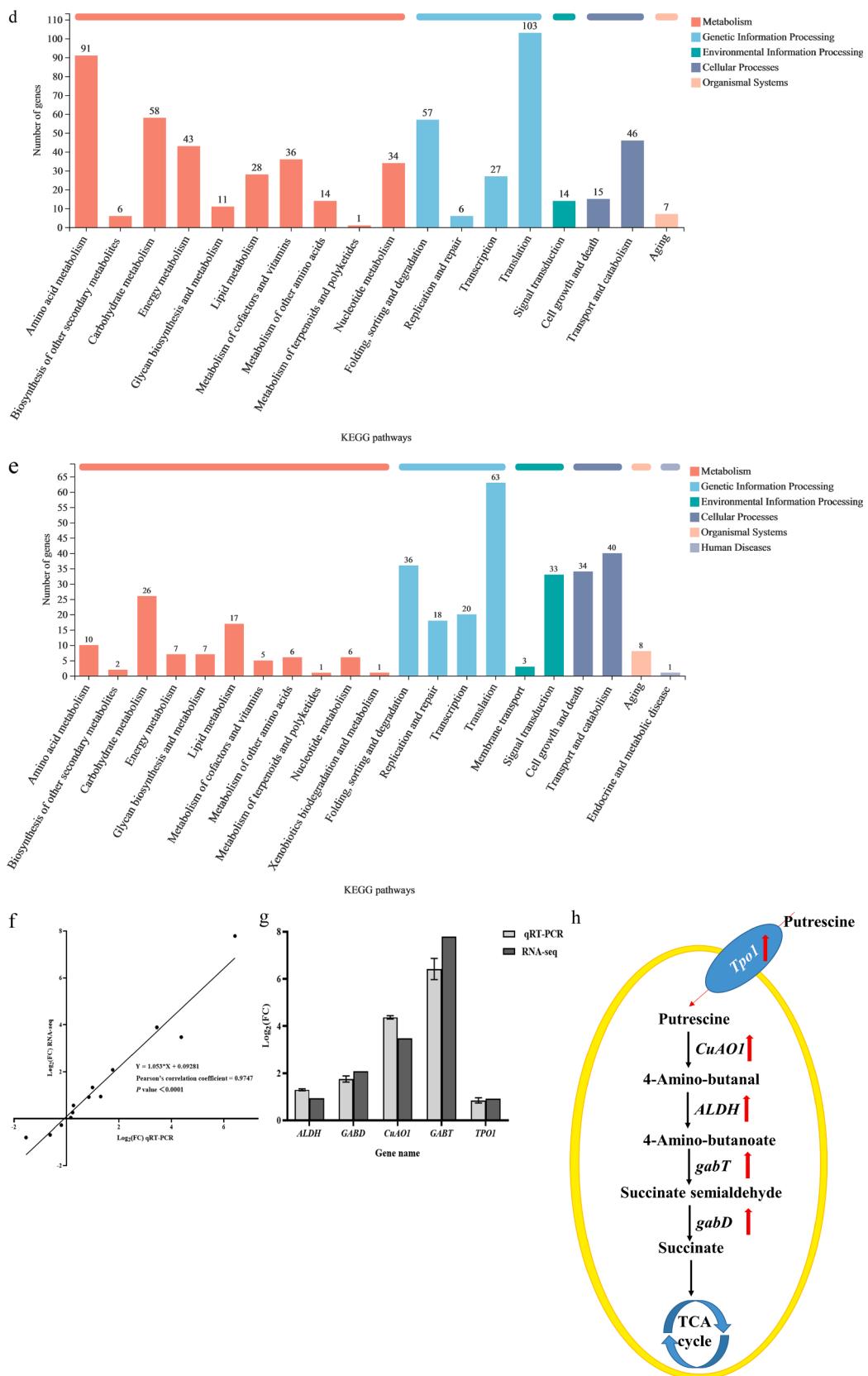
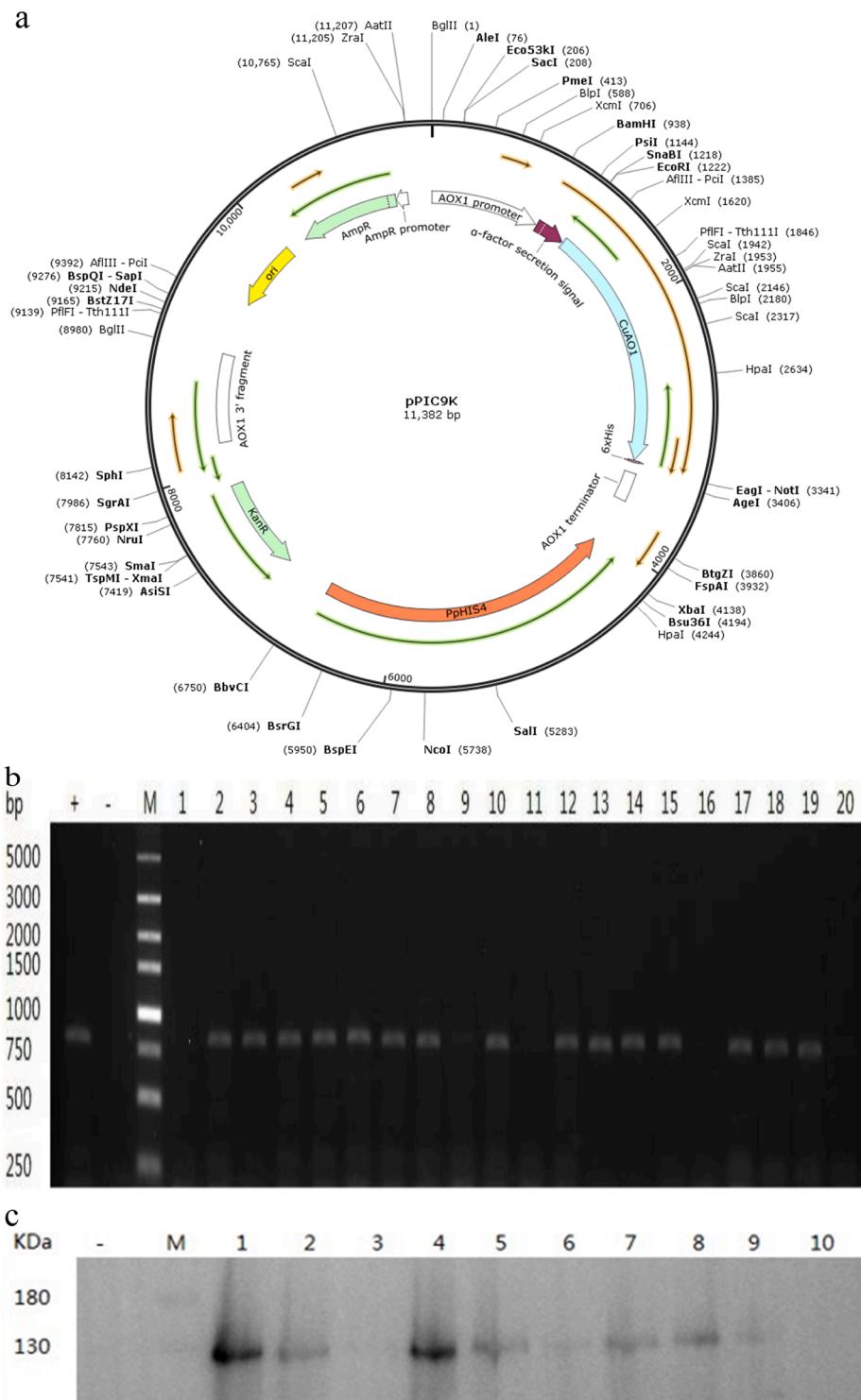


Fig. 3. (continued).

### 3.6.2. Effects of metal ions, enzyme inhibitors, surfactants and proteinases on CuAO1 activity

The effects of different metal ions on CuAO1 activity were shown in

**Fig. 5f.** Na<sup>+</sup>, Fe<sup>3+</sup>, and Al<sup>3+</sup> severely inhibited CuAO1 activity. In contrast, Cu<sup>2+</sup>, Zn<sup>2+</sup>, Ni<sup>2+</sup>, Li<sup>+</sup>, and Co<sup>2+</sup> were able to slightly enhance its activity compared to the control. It has been hypothesized that the



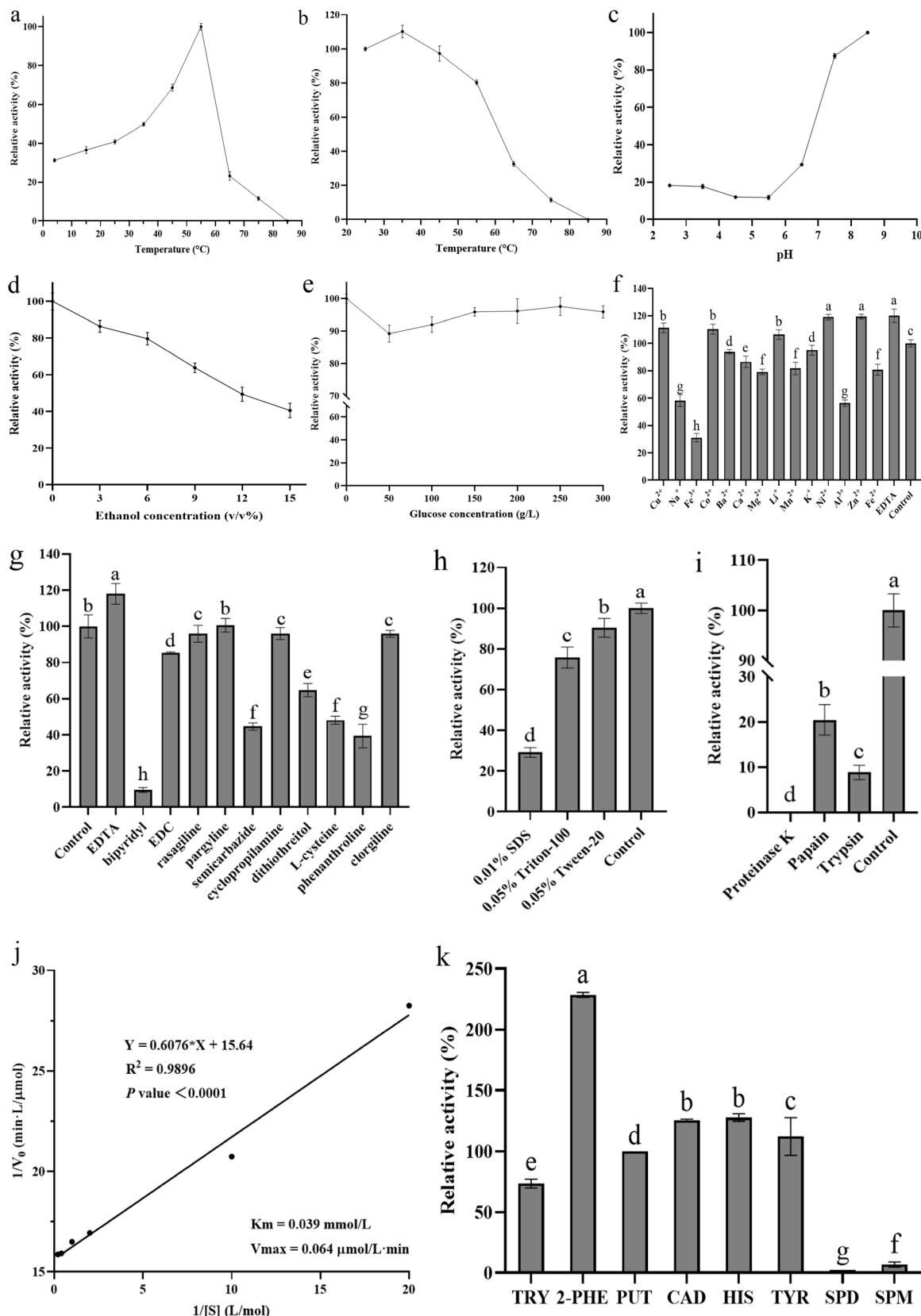
**Fig. 4.** a. The construction of recombinant plasmid pPIC9K-CuAO1. b. PCR identification of yeast transformants. M: 5000 Da Marker; +: positive control; -: negative control; 2-8, 10, 12-15, and 17-19: PCR results of high copy recombinant strains. 1, 9, 11,16, and 20: PCR results of low copy recombinant strains. c. Western blot of induced expression supernatants of recombinant strains. -: Western blot of the supernatant of the uninduced recombinant strain; M: the standard protein marker (180 kDa); 1-2, and 4-9: recombinant strains successfully expressing CuAO1; 3 and 10: recombinant strains that failed to express CuAO1.

positive effect of Cu<sup>2+</sup> is due to its filling in the catalytic active sites of CuAO1. However, the inhibition by some other metal ions was mainly by chelating copper atoms or modifying amino acid residues in the protein to change its structure (Das, Li, Mai, & An, 2018).

The relative activity of CuAO1 after adding different inhibitors for amine oxidases, were shown in Fig. 5g. CuAO1 retained 100 % activity in the presence of four MAO inhibitors (clorgiline, rasagiline, pargyline, or cyclopropylamine), suggesting that no FAD cofactor is involved in catalytic activity. EDC, a carboxyl-modifying agent, produced a 14.8 % decrease in enzyme activity. Similarly, the carbonyl modifier

semicarbazide, which inhibited a specific subgroup of copper-containing amine oxidases, caused an inhibition of nearly 55.4 %. On the other hand, although EDTA did not reduce the catalytic activity, the other two metal chelating agents, bipyridyl and phenanthroline, significantly inhibited the activity of CuAO1 by 90.5 and 60.6 %, respectively, indicating that metallic cofactor was involved in catalytic activity. In addition, as strong reducing agents, L-cysteine and dithiothreitol revealed important inhibitor effects on CuAO1 activity.

CuAO1 activity was slightly inhibited by 0.05 % Tween 20 and 0.05 % Triton-100. However, 0.01 % SDS significantly inhibited the activity



**Fig. 5.** Effects of temperature on (a) activity and (b) stability of recombinant CuAO1. Effects of (c) pH, (d) ethanol concentration, (e) glucose concentration, (f) metal ions, (g) enzyme inhibitors, (h) surfactants, and (i) proteases on activity of recombinant CuAO1. j. The kinetic parameters ( $K_m$  and  $V_{max}$ ) of recombinant CuAO1 for putrescine. k. Substrate specificity analysis of recombinant CuAO1.

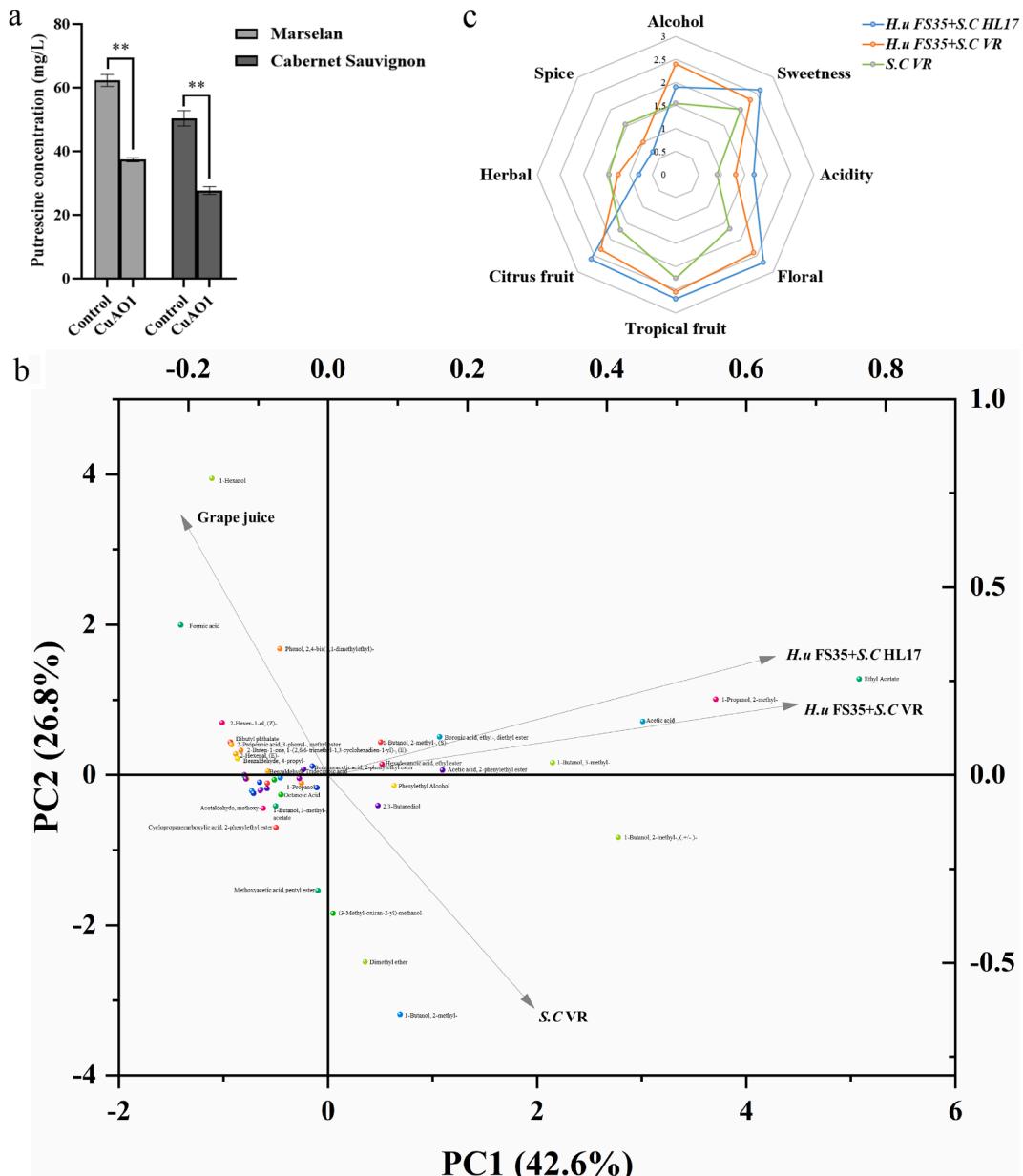
of CuAO1 by 70.8 %, because SDS denatured the protein as a strong anionic surfactant (Fig. 5h). In addition, the CuAO1 activity was completely lost when treated with proteinase K, and was obviously affected after being treated with papain and trypsin (Fig. 5i).

### 3.6.3. The steady state kinetic parameters for putrescine

The double-reciprocal plot of calculated initial velocities versus substrate concentrations was used to derive  $K_m$  and  $V_{max}$ . The resulting kinetic parameters were as follows:  $K_m = 39 \mu\text{M}$  and  $V_{max} = 0.064 \mu\text{mol/L}\cdot\text{min}$  (Fig. 5j). The  $K_m$  of CuAO1 for putrescine was slightly higher than that reported by *Candida guilliermondii* of 20  $\mu\text{M}$  (Gunasekaran & Gunasekaran, 1999), and lower than that reported by Callejón et al. (2015) of 94  $\mu\text{M}$ , indicating that CuAO1 showed a great affinity with putrescine.

### 3.6.4. Substrate specificity analysis

The substrate specificity analysis showed that none of the tested



**Fig. 6.** a. Putrescine content in Marselan and Cabernet Sauvignon wines treated with recombinant CuAO1. b. Loading and score plot of first two principal components for classification of Cabernet Sauvignon wine samples inoculated with different strain combinations according to their volatile compounds. c. Aroma radar map of sensory evaluation.

### 3.7. The performance of recombinant CuAO1 and *H. Uvarum* FS35 in wine production

#### 3.7.1. Putrescine degradation in wine by recombinant CuAO1

As shown in Fig. 6a, the recombinant CuAO1 was able to degrade 25.8 and 21.8 mg/L of putrescine in Marselan and Cabernet Sauvignon wines, respectively. Obviously, wine conditions are not optimal for CuAO1 activity, and the limiting factors may be ethanol and low pH. According to our previous study, the highest putrescine content in Marselan and Cabernet Sauvignon wines were 27.6 and 21.6 mg/L, respectively (Han et al., 2022). Therefore, for the wine samples readily available to Chinese consumers we investigated, although CuAO1 had limited activity in wine, it could effectively reduce putrescine content to a safe level. Therefore, the purified CuAO1 would be a potential measure to eliminate putrescine in wines with very low pH or high ethanol content.

#### 3.7.2. The physicochemical analysis of wines fermented with *H. Uvarum* FS35

The physicochemical parameters, including putrescine, glucose, fructose, glycerol, ethanol, and organic acid content, of wines fermented with different strain combinations were shown in Table 1. The highest level of putrescine was detected in wines from a single culture with commercial yeast *S. cerevisiae* VR, whereas no putrescine was detected in wines from sequential inoculation with *H. uvarum* FS35, indicating that *H. uvarum* FS35 performed prominent putrescine-degrading ability in practical fermentation. High amount of residual fructose was detected in groups with *S. cerevisiae* VR, and all groups were able to use up almost all glucose, which confirmed that *S. cerevisiae* HL17 was capable of utilizing glucose as adequate as *S. cerevisiae* VR, and its affinity to fructose is more satisfying. In addition, the group with *S. cerevisiae* HL17 exhibited higher ethanol content, which is most likely due to the complete consumption of sugars by this strain. Glycerol affects the fullness of the wine when the concentration exceeds its taste threshold of 5.2 g/L (Wei et al., 2020). The groups with *H. uvarum* FS35 could produce more glycerol, indicating that *H. uvarum* FS35 exhibited a property of high-yield glycerol.

Organic acid content is an important indicator relating to the balance of wine taste and aroma. Lower level of citric acid and higher level of lactic acid were detected in groups involving *H. uvarum* FS35, which may have a positive influence on the mouthfeel perception of wine. Malic acid is an acid with a green and astringent taste that can be synthesized by yeast during AF (Han et al., 2021). Higher malic acid level could be found in groups involving *S. cerevisiae* VR, suggesting that *S. cerevisiae* VR may have the ability to produce malic acid. Moreover, succinic acid is an acid with a salty and bitter taste that might have an adverse impact on the quality of wine at high concentration (Restuccia et al., 2017). The succinic acid content of the group involving *S. cerevisiae* HL17 was notably lower, suggesting that *S. cerevisiae* HL17 may have the potential to weaken the adverse effects caused by succinic acid. In addition, non-*Saccharomyces* yeasts are considered to be high

producers of acetic acid (Contreras, Hidalgo, Henschke, Chambers, Curtin, & Varela, 2014). Higher acetic acid content was observed in the fermentation with *H. uvarum* FS35 in our study, but the concentration in all treatments was within the acceptable level of grape wine ( $\leq 1.2$  g/L) (Viana, Gil, Genovés, Vallés, & Manzanares, 2008).

#### 3.7.3. The volatile component analysis of wines fermented with *H. Uvarum* FS35

A total of 86 different aroma compounds were detected. All aroma components other than ethanol were classified into esters, alcohols, acids, and carbonyls, which varied with the yeast species. Compared with grape juice, the variety of aroma compounds detected in all fermentations were significantly higher, and numerically more kinds of aroma compounds were detected in sequential fermentation groups, almost double to that of grape juice.

Although esters and alcohols were originally present in grape juice, they were mainly produced by yeast during AF. In our study, the results revealed that esters were the group with the highest content, which had an important impact on the wine flavor. Among them, ethyl acetate was the most abundant, with concentrations of 14.2–21.2 mg/L (rOAV: 89.9–134.1), and wines involving *H. uvarum* FS35 had significantly higher levels of it, which confirmed the high production of acetate ester and the characteristics of sweet and fruity aromas by *H. uvarum* FS35 (Peinado, Moreno, Bueno, Moreno, & Mauricio, 2004). In addition, *H. uvarum* FS35 + S.C VR group produced the highest level of ethyl palmitate (rOAV: 20.6), and ethyl caprate (rOAV: 61.7) and diethyl succinate (rOAV: 11.7) were also largely synthesized in this group, indicating that *H. uvarum* FS35 + S.C VR group may possess fruity, floral, and sweet features. Importantly, the production of hexanoic acid was negatively correlated with the level of ethyl caproate (-0.530,  $P < 0.05$ ). This result may be explained by the esterification of acids during the synthesis of corresponding ethyl esters. In agreement with a previous study (Song et al., 2015), alcohols were also one of the largest groups of volatiles identified in wine. In this experiment, the concentration of higher alcohols was within the acceptable range ( $\leq 350$  mg/L) (Wei et al., 2020). Variations were noticed among yeasts with respect to their ability to produce phenylethyl alcohol (rOAV: 45.8–138.6), and fermentations involving *H. uvarum* FS35 produced the highest concentration of this compound, suggesting that *H. uvarum* FS35 may contribute rose and honey flavors to wine aroma (Peinado, Moreno, Bueno, Moreno, & Mauricio, 2004).

#### 3.7.4. PCA of main volatile compounds

49 main aroma compounds were selected for PCA, as shown in Fig. 6b. It could be observed that the variance contribution rate of the first principal component (PC1) was 42.6 %, that of the second principal component (PC2) was 26.8 %, and the cumulative variance contribution rate reached 69.4 %, indicating that most of the information about the original aroma could be retained. The PC1 could be interpreted as an indicator to evaluate the richness of the fermentative aroma of the samples and distinguish grape juice from wine. The grape juice contained little fermentative aroma was located in the negative axis of the

**Table 1**

The profile of physicochemical parameters in wines fermented by different strain combinations.

	PUT (mg/L)	Glucose (g/L)	Fructose (g/L)	Glycerol (g/L)	Ethanol (v/ v%)	Citric acid (g/L)	Tartaric acid (g/L)	Malic acid (g/L)	Succinic acid (g/L)	Lactic acid (g/L)	Acetic acid (g/L)
Grape juice	46.10 $\pm$ 1.71 <sup>a</sup>	118.53 $\pm$ 0.49 <sup>a</sup>	129.27 $\pm$ 0.78 <sup>a</sup>	0.64 $\pm$ 0.23 <sup>d</sup>	0.15 $\pm$ 0.03 <sup>d</sup>	1.03 $\pm$ 0.08 <sup>b</sup>	5.05 $\pm$ 0.05 <sup>a</sup>	2.40 $\pm$ 0.03 <sup>d</sup>	0.39 $\pm$ 0.08 <sup>c</sup>	1.00 $\pm$ 0.03 <sup>c</sup>	0.30 $\pm$ 0.01 <sup>c</sup>
S.C VR	17.40 $\pm$ 1.26 <sup>b</sup>	0.63 $\pm$ 0.02 <sup>c</sup>	3.50 $\pm$ 0.07 <sup>b</sup>	6.95 $\pm$ 0.02 <sup>c</sup>	13.60 $\pm$ 0.03 <sup>b</sup>	1.14 $\pm$ 0.04 <sup>a</sup>	4.91 $\pm$ 0.07 <sup>b</sup>	4.06 $\pm$ 0.11 <sup>a</sup>	2.19 $\pm$ 0.03 <sup>a</sup>	0.97 $\pm$ 0.03 <sup>c</sup>	0.46 $\pm$ 0.01 <sup>b</sup>
FS35 + S. C VR	N.D. 0.00 <sup>c</sup>	0.63 $\pm$ 0.03 <sup>c</sup>	1.72 $\pm$ 0.02 <sup>a</sup>	8.10 $\pm$ 0.04 <sup>c</sup>	13.03 $\pm$ 0.01 <sup>b</sup>	1.05 $\pm$ 0.02 <sup>c</sup>	4.24 $\pm$ 0.02 <sup>c</sup>	3.71 $\pm$ 0.06 <sup>b</sup>	2.17 $\pm$ 0.05 <sup>a</sup>	2.86 $\pm$ 0.36 <sup>a</sup>	1.09 $\pm$ 0.01 <sup>a</sup>
FS35 + HL17	N.D. 0.10 <sup>b</sup>	1.40 $\pm$ 0.11 <sup>d</sup>	0.56 $\pm$ 0.01 <sup>b</sup>	7.55 $\pm$ 0.04 <sup>a</sup>	14.23 $\pm$ 0.03 <sup>b</sup>	1.03 $\pm$ 0.03 <sup>b</sup>	2.96 $\pm$ 0.03 <sup>d</sup>	3.24 $\pm$ 0.12 <sup>c</sup>	1.67 $\pm$ 0.05 <sup>b</sup>	1.98 $\pm$ 0.16 <sup>b</sup>	1.12 $\pm$ 0.01 <sup>a</sup>

PUT: Putrescine; N.D.: not detected or below limit of quantitation. Results are expressed as mean  $\pm$  S.D. ( $n = 3$ ). Different superscripts in the same column indicated significant difference in the content of the measured index,  $P < 0.05$ .

PC1, while the wine samples were all located in the positive axis. Among the 3 wine samples, the PC1 scores of groups involving *H. uvarum* FS35 were significantly higher, indicating that the sequential inoculation with *H. uvarum* FS35 could significantly increase the content of esters and other fermented fragrances in wine. The PC2 could be regarded as an index to evaluate the richness of typical aroma. The groups cultured with *H. uvarum* FS35 (except grape juice) were located in the positive axis of the PC2, while the group monoculture with *S. cerevisiae* VR were located in the negative axis, which may due to the obvious floral and fruity characteristics contributed by *H. uvarum* FS35. Therefore, there were significant differences in aroma between wine fermented by indigenous yeast and wine produced by commercial *S. cerevisiae* VR, demonstrating the great potential of native yeast in wine fermentation.

### 3.7.5. Sensory analysis

The sensory evaluation results of 3 samples were shown in Fig. 6c. It can be noticed that *H. uvarum* FS35 has great influence on aroma characteristics of wine. Among them, both *H.u* FS35 + *S.C* VR group and *H.u* FS35 + *S.C* HL17 group showed high intensity of sweetness, floral, tropical fruit and citrus fruit aroma. *S.C* VR group showed obvious characteristics in herbal and spice, but relatively weak in overall aroma intensity. Although *H.u* FS35 + *S.C* VR and *H.u* FS35 + *S.C* HL17 group exhibited obvious characteristics in alcohol and acidity, respectively, their floral and fruity aroma intensity was outstanding, and all sensory evaluators found significant differences in aroma between the two groups and *S.C* VR group, this may due to the presence of relatively high concentration of phenylethyl alcohol, ethyl acetate and  $\beta$ -phenylethyl acetate in both samples, resulting in a certain amount of floral and fruity aroma.

## 4. Conclusions

Various fermented, seasoned or conserved foodstuffs (wine, beer, cheese, fish, chocolate and soybean products) were reported to contain high content of BAs, resulting in harmful effects on human body. In this study, *H. uvarum* FS35, a strain with great ability to degrade BAs, especially putrescine was finally selected from 111 strains of yeast through screening, stress tolerance and winemaking performance evaluation. Then, CuAO1 of *H. uvarum* FS35, an amine oxidase leading to a direct oxidative deamination of putrescine to 4-aminobutyrate and, ultimately, to succinate, was characterized through the analysis of transcriptome and enzymatic activity. It is expected to further study the fermentation characteristics in pilot production and other putrescine degradation pathways of this strain, which is of great significance for brewing safe and high-quality wine in practical production.

## CRediT authorship contribution statement

**Bing Han:** Data curation, Methodology, Formal analysis, Software, Validation, Visualization, Writing – original draft. **Jie Gao:** Investigation, Methodology, Visualization. **Xiaoyu Han:** Formal analysis, Validation, Visualization. **Huan Deng:** Investigation, Methodology, Visualization. **Tianyang Wu:** Formal analysis, Investigation, Visualization. **Chenyu Li:** Investigation, Methodology, Validation. **Jicheng Zhan:** Validation, Writing – review & editing. **Weidong Huang:** Conceptualization, Funding acquisition, Project administration, Writing – review & editing. **Yilin You:** Writing – review & editing, Conceptualization, Funding acquisition, Project administration, Resources, Supervision.

## 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 material

Supplementary data to this article can be found online at <https://doi.org/10.1016/j.foodres.2022.111923>.

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