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Article

Development of Functional Msalais Wines Rich in Amadori Compounds by Yeast Fermentation

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Abstract

Msalais is a wine made by a series of processes such as boiling and fermentation after hotan red grape juice. Maillard reaction occurs during boiling. Amadori compound is a product of the early stage of Maillard reaction, which has physiological activities such as anti-oxidation, anti-hypertension and anti-hyperglycemia. The purpose of this study was to develop a kind of Msalais rich in Amadori compounds by using the fermentation performance of different yeasts. The optimal fermentation process was obtained by response surface optimization (*Saccharomyces cerevisiae* Y4 and *Wickerhamomyces anomalus* Y2, fermentation temperature 28°C, fermentation time 14 days, yeast inoculation amount 2%, V/V, ratio of *Saccharomyces cerevisiae* and non-*Saccharomyces cerevisiae* 2:1). At the same time, HPLC-ELSD was used to detect Amadori compounds. The contents of Fru-Pro and Fru-Asp in the optimal fermentation process were 0.2867 ± 0.0115 g/L and 0.0203 ± 0.0014 g/L, respectively, which were 0.0702 g/L and 0.026g/L higher than those of 0.2165 ± 0.0022 g/L and 0.0177 ± 0.0008 g/L of commercially available cotton Msalais. With the increase of Amadori compounds, the antioxidant activity was significantly improved. The DPPH free radical scavenging ability was 116.37 ± 1.79 μ mol Trolox/sample, which was 53.01 μ mol Trolox/L sample higher than that of commercial Msalais. The ABTS free radical scavenging ability was 142.51 ± 1.98 μ mol Trolox/L sample, which was 68.23 μ mol Trolox/L sample higher than that of commercial Msalais. The total oxygen free radical absorption capacity was 132.74 ± 6.36 μ mol Trolox/L sample, which was 60.12 μ mol Trolox/L higher than that of the commercial Msalais.

Keywords: Amadori compounds; high-performance liquid chromatography; response surface optimization; antioxidant

1. Introduction

Msalais is a specialty of Xinjiang. It is a delicious and mellow wine drink after boiling and natural fermentation. It has high medicinal value and is rich in amino acids, vitamins, glucose, iron and other nutrients and trace elements needed by the human body. In recent years, as people's attention to healthy eating continues to increase, Msalais has been favored for its unique taste and potential health benefits. Its unique flavor and color are derived from complex biochemical conversion processes[1]. In the traditional process, grape juice is boiled and fermented for a long time to form dark brown and rich aroma. This process involves the deep participation of Maillard reaction. As an early stable intermediate product of Maillard reaction, Amadori compound is not only the precursor of

subsequent flavor substances, but also has physiological activities[2] such as antioxidant activity, which may have a key impact on the storage stability and health attributes of Msalais. At present, there are studies on the end-stage products of the Maillard reaction in Msalais, such as acrylamide and 5-hydroxymethylfurfural[3], but the study of Amadori compounds in Msalais has not been reported. Amadori compound is a carbonyl amine condensation reaction between reducing sugar substances such as glucose and fructose in fruit and vegetable raw materials as carbonyl donors and free amino groups contained in amino acids, peptides and proteins. After Amadori rearrangement, the product-1-amino-1-deoxy-2-keto sugar is formed, that is, Amadori compound. The specific process is shown in Figure 1. Amadori compounds are generally solid, yellow or white, easily soluble in water, methanol and ethanol, have no odor, but are important non-volatile aroma precursors. [4]. In Hotan red grape juice, before boiling, the content of proline was 284.68 mg / mL, and the content of aspartic acid was 8.86 mg / mL[5], which could provide sufficient amino acid substrates for the formation of Amadori compounds, and the two Amadori compounds were proved to have more functions, such as antibacterial, anticancer, antioxidant, etc. [6–8]. In order to improve the function of Msalais, Fru-Pro and Fru-Asp were selected as two Amadori compounds for subsequent research.

Amadori compounds, whose substrates reducing sugars and amino acids are the basic nutrients of food, have high reactivity. Therefore, Amadori compounds are widely found in tomato powder, chili powder, black garlic and dried fruits and vegetables[9]. In the process of food processing, transportation, storage and even the ripening of sugary fruits and vegetables, Maillard reaction occurs to varying degrees and accumulates various Maillard products in food. Therefore, Amadori compounds are also widely found in various processed foods, but the content varies greatly according to different food raw materials and processing methods. Because of the rich in free amino acids and reducing sugars, the content and types of Amadori compounds in fruit and vegetable products are abundant. Amadori compounds have long been considered to have a negative impact on food quality and nutrition and human health[10]. For example, the loss of active ingredients such as amino acids and sugars in food; the bioavailability of Amadori compounds is low. After being partially absorbed, they are excreted out of the body and cannot be used by organisms, which reduces the nutritional value of food[11]; amadori compounds may also be further generated by Maillard reaction to some harmful compounds, such as acrylamide[12]. However, with the deepening of research, some Amadori compounds have been proved to have beneficial physiological effects on the human body. Yu[13] studies have shown that Amadori compounds can inhibit cardiovascular and cerebrovascular diseases, because they can effectively inhibit the activity of angiotensin converting enzyme; ha[14] et al found that Amadori compounds with arginine residues can inhibit the activity of pancreatic amylase and glucosidase, thereby reducing the digestion and absorption of carbohydrates in the gastrointestinal tract, reducing the increase of postprandial blood glucose, and thus playing a role in lowering blood glucose. Mossine[15] found that Fru-His can synergistically inhibit the proliferation of prostate cancer cells in vitro and in vivo, and the experimental diet supplemented with tomato paste and Fru-His can reduce the carcinogenic effect of carcinogenic rat prostate by 6 times. Therefore, more and more studies have focused on how to improve Amadori compounds by optimizing food processing technology.

At present, there have been reports on various detection methods of Amadori compounds. A classic amino acid analysis method, post-column ninhydrin derivatization assay, has been used for the analysis of Amadori compounds[16]. However, this method has many shortcomings, such as insufficient separation, poor sensitivity, and time-consuming. The method of using trimethylsilane to partially derivatize sugars in the gas phase[17] has also been used for the analysis of Amadori compounds, but this method requires a long time of derivatization, and the separation process is extremely complicated due to the formation of tautomers. Yu et al.[18] used ligand exchange and scanning capillary electrophoresis to directly detect Amadori compounds by ultraviolet detection (UV). At present, the most commonly used methods for the analysis and determination of Amadori compounds are high performance liquid chromatography, ion chromatography, etc. High performance liquid chromatography mainly uses different types of chromatographic columns to

separate Amadori compounds from other substances and then detect them with ultraviolet detector, fluorescence detector or evaporative light scattering detector. Most Amadori compounds have low ultraviolet absorption or no fluorescence characteristics, so it is usually necessary to introduce derivatization steps to improve the detection sensitivity [18], while the evaporative light detector responds to all substances and can detect Amadori compounds without complex pre-treatment. Li[19] synthesized and purified four Amadori compounds under aqueous conditions and used high performance liquid chromatography-evaporative light detector to detect their purity up to 98% High performance anion chromatography tandem pulsed amperometric detector (HPAEC-PAD)[20] and high performance anion chromatography tandem mass spectrometry (HPAEC-MS) have also been widely used in the qualitative analysis of Amadori compounds. In addition, high performance liquid chromatography-tandem mass spectrometry has also been widely used.

Based on the above background, this study aims to develop a Msalais rich in Amadori compounds by using the fermentation performance of different yeasts. The Amadori compounds in Msalais were detected by high performance liquid chromatography-evaporative light detector, and different yeasts were screened and their fermentation performance was evaluated. Through single factor experiment and response surface optimization, the strain and optimum fermentation conditions of mixed fermentation were determined. The antioxidant capacity of the developed Msalais was studied, and the effect of the increase of Amadori compound content on the antioxidant capacity of Msalais was evaluated. These results may provide a new way for the study of microorganisms in Maillard reaction and open up the possibility for the wide application of Amadori compounds in food industry and other industries.

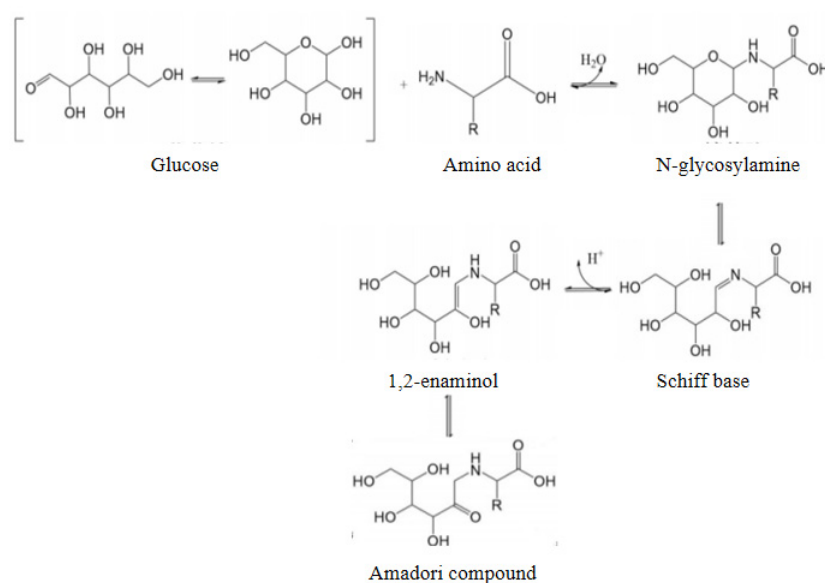


Figure 1. The formation of Amadori compounds.

2. Materials and Methods

2.1. Chemicals

Hetian red grapes sourced from Awati County (Xinjiang, China); Fru-Asp (1-deoxy-1-L-aspartate-D-fructose), Fru-Pro (1-deoxy-1-L-proline-D-fructose), purity > 95%, from TRC Company (Canada); Acetonitrile (chromatographically pure); 2,3,5-triphenyltetrazolium chloride, from Sinopharm Chemical Reagent Co., Ltd. (Shanghai, China); Dowex 50WX4 Hydrogen Ion Exchange Resin (200~400 mesh) from J&K Scientific (Beijing, China); Ammonium formate (chromatographically

pure) from Weiqi Boxing Biotechnology Co., Ltd. (Wuhan, China); Yeast genomic DNA rapid extraction kit from Solarbio Technology Co., Ltd. (Beijing, China); WL nutrient agar from CoolLebo Technology Co., Ltd. (Beijing, China); Phosphate buffer solution from Kaiji Biotechnology Co., Ltd. (Jiangsu, China); DPPH (1,1-diphenyl-2-picrylhydrazyl), ABTS(2,2'-azino-bis-3-ethylbenzothiazoline-6-sulfonic acid), Tolorx (6-hydroxy-2,5,7,8-tetramethylchroman-2-carboxylic acid) from Yuanye Technology Co., Ltd. (Shanghai,China);Fluorescein sodium, AAPH:(2,2-azobis (2-methylpropylimidazole) dihydrochloride) from Aladdin Biotechnology Co., Ltd. (Shanghai, China); Angel Aroma Active Dry Yeast Powder (AQSX) : Angel Yeast Co., Ltd. (Hubei, China); Other reagents in the test were of analytical purity.

2.2. Instruments

High Performance Liquid Chromatograph-Evaporative Light Scattering Detector from Shimadzu Corporation (Japan); XBridge BEH Amide (5 μ m, 4.6 mm $\times$ 250 mm) Chromatographic column from Waters Company (USA); Open glass sand core chromatography column (20 mm $\times$ 60 cm) from Rhoda Henghui Company (Beijing, China); Rotary evaporator from Haozhuang Instrument Co., Ltd. (Shanghai, China); LDZX-50L Vertical High Pressure Steam Sterilizer from Shen 'an Medical Device Factory (Shanghai, China); GZX-9140MBE Electric Blower Dryer from Boxun Instrument Co., Ltd. (Shanghai, China); Biological microscope from Jiangnan Yongxin Optical Co., Ltd. (Nanjing, China); Constant temperature incubator from Yiheng Technology Co., Ltd. (Shanghai, China); Biological microplate reader from Thermo Fisher Scientific (USA)

2.3. Establishment of HPLC-ELSD Method for the Detection of Amadori Compounds

2.3.1. Chromatographic Conditions

According to the method of Li[19], some modifications are made: The chromatographic column was XBridge BEH Amide (5 μ m, 4.6 mm $\times$ 250 mm). The mobile phase was acetonitrile : ammonium formate=80:20, the flow rate was 1.0 mL / min ; the column temperature was 35°C. The injection volume was 10 μ L; evaporative light scattering detector drift tube temperature 40°C, gain 6, nitrogen as carrier gas. The mobile phase was acetonitrile (phase A) and 10 mmol/L ammonium formate aqueous solution (phase B), respectively. The gradient elution method was used for the determination. The gradient change of mobile phase A was as follows : from 0 min to 5 min, the volume fraction of phase A changed from 80% to 70% ; from 5 min to 10 min, the volume fraction of A phase changed from 70% to 65%. From 10 min to 11 min, the volume fraction of A phase changed from 65% to 80%. From 11 min to 15 min, the volume fraction of A phase changed from 65% to 80%, and the total time was 20 min. The specific changes are shown in Table 1.

Table 1. Table of volume fraction of organic phase in gradient elution.

Time (min)	Volume fraction of B phase (%)
0	80
5	70
10	65
15	80
20	80

2.3.2. Sample Pretreatment

The 500mL sample solution was diluted with equal volume of ultrapure water, added with an appropriate amount of activated carbon powder to adsorb impurities such as pigments, and centrifuged at 6000rpm for 10min. The supernatant was filtered by medium-speed qualitative filter paper and allowed to stand for later use. The pretreated Dowex 50WX4 hydrogen ion exchange resin

(200~400 mesh) was loaded into the chromatographic column, and the excess water was discharged by settling with ultrapure water to half of the column height. A 1L diluted sample solution was injected into the chromatographic column, and after it flowed out naturally, it was washed with a large amount of ultrapure water to remove impurities such as glucose. Elution was performed with 0.2 mol/L ammonia water, and the TTC test positive eluent (each 10mL as a group) was collected in sections until the TTC test was negative[21]. Then the resin was washed with ultrapure water to neutral, soaked in 0.1mol/L dilute hydrochloric acid for 30min, washed with ultrapure water again to remove the acid solution, and the resin was recovered for backup. The collected eluent was vacuum-distilled at 55°C for 30min and concentrated for analysis.

2.3.3. Methodological Investigation

1. Standard stock solution preparation and system adaptability test

The 10 mg Fru-Asp and Fru-Pro standards were accurately weighed, dissolved in ultrapure water and diluted to 10 mL volumetric flasks to prepare a 1000µg/mL standard stock solution, which was stored in dark at 4 °C. In the actual detection, the Fru-Pro standard was dissolved in ultrapure water and diluted to 500, 400, 300, 200, 100 µg/mL, and the Fru-Asp was dissolved in ultrapure water and diluted to 5, 10, 100, 200, 300µg/mL. The HPLC-ELSD detection was performed according to the chromatographic conditions of 2.2.1, and the chromatogram was recorded.

2. Linear relationship investigation

The Fru-Pro standard was dissolved in ultrapure water and diluted to 500, 400, 300, 200, 100µg/mL, and the Fru-Asp was dissolved in ultrapure water and diluted to 5, 10, 100, 200, 300µg/mL. HPLC-ELSD detection was performed according to the chromatographic conditions of 2.2.1. The standard curve was drawn with the peak area as the y-axis and the standard concentration of Fru-Pro and Fru-Asp as the x-axis, and the correlation coefficient R^2 was calculated.

3. Accuracy test

The Fru-Pro standard was dissolved in ultrapure water and diluted to 250µg/mL, and a total of 3 Fru-Pro aqueous solutions were prepared. The Fru-Asp was dissolved in ultrapure water and diluted to 100µg/mL. A total of 3 parts of Fru-Asp aqueous solution were prepared. Six standard solutions were detected by HPLC-ELSD according to the chromatographic conditions of 2.2.1. The peak area was recorded and the relative standard deviation RSD was calculated.

4. Precision test

The Fru-Pro standard was dissolved in ultrapure water and diluted to 250 µg/mL. Fru-Asp was dissolved in ultrapure water and diluted to 100 µg/mL. HPLC-ELSD detection was performed according to the chromatographic conditions of 2.2.1. Each standard solution was injected repeatedly for 6 times, and the peak area was recorded and the relative standard deviation RSD was calculated.

5. Repeatability test

Msalaisi from Miandu Distillery was used as the sample solution. The sample solution was pretreated according to 2.2.2 to obtain the sample solution to be tested. 1mL of the sample solution to be tested was taken and detected by HPLC-ELSD according to the chromatographic conditions of 2.2.1. The sample was injected repeatedly for 6 times, the peak area was recorded, and the content of Amdori compound and relative standard deviation RSD were calculated according to the standard curve.

6. Stability test

The sample solution was taken and pretreated according to the method of 2.2.2 to obtain the sample solution to be tested. The sample solution to be tested was placed at 20°C for 0, 6, 12, 18, 24, 30, 36h, respectively. HPLC-ELSD detection was performed according to the chromatographic conditions of 2.2.1. The peak area at 0 h was used as a comparison to calculate the content changes and RSD values of Amadori compounds at different time points.

7. Determination of sample content

The sample solution was taken, and the sample solution was pretreated according to the method of 2.2.2 to obtain the sample solution to be tested. The 1 mL sample solution to be tested was accurately sucked and repeated three times to obtain three sample solutions to be tested. HPLC-ELSD detection was performed according to the chromatographic conditions of 2.2.1, and the concentrations of the two Amadori compounds were calculated according to the standard curve.

8. Standard addition recovery test

The sample solution was pretreated according to the method of 2.2.2 to obtain the sample solution to be tested. The sample solution to be tested was divided into two groups, one group was added with 0.5 mL 250µg/mL Fru-Pro standard solution, and the other group was added with 0.5 mL 100µg/mL Fru-Asp standard solution, 6 parts in each group. HPLC-ELSD detection was carried out according to the chromatographic conditions of 2.2.1, and the average recovery rate and RSD value were calculated.

2.4. Preparation of Grape Juice Rich in Amadori Compounds

The Hotan red grape with more than 80% maturity, full particles and no mechanical damage was selected and washed. After removing the stem and crushing, the grape juice was boiled and concentrated to a sugar content of 21°BX after pressing and extracting the juice. The grape skin and water were boiled and concentrated to a sugar content of 21°BX at a ratio of 2:1(kg:L), and the oil bath was heated using a laminated pan, The specific process is shown in Figure 2. The filtered grape skin juice and grape juice were mixed and poured into a laminated pan to be boiled ; the heating concentration temperature was set at 140°C, and the final sugar content was 27°BX. After cooling to room temperature (20-23°C), it was frozen at -18°C.

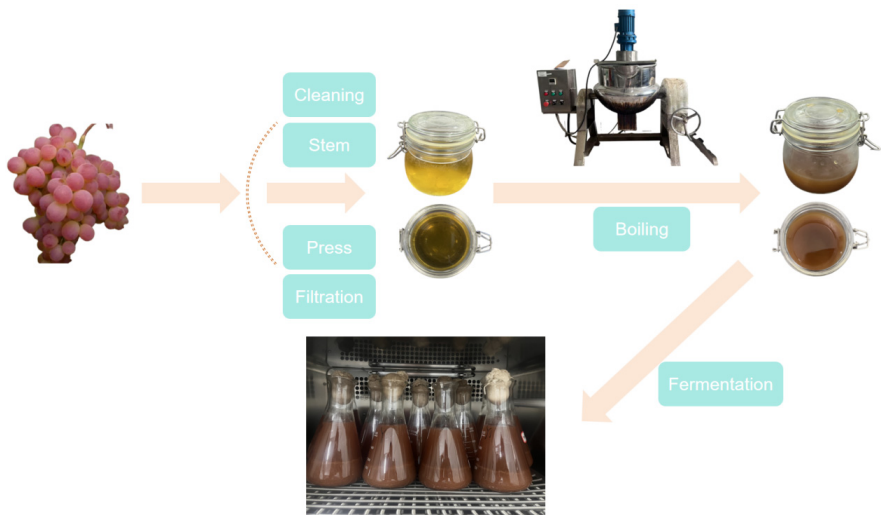


Figure 2. The process of making grapes into Msalais.

2.5. Screening of Yeast

2.5.1. Isolation and Purification of Yeast

Preparation of YPD medium: glucose 2%, peptone 2%, yeast dip 1%, chloramphenicol 0.001%, and solid medium with 2% agar powder, the above material ratios are volume ratios[22].

Accurate weighing of soil and grapevine samples was conducted, followed by their placement into conical flasks containing 100 milliliters of sterile water. The samples were then subjected to rigorous shaking to ensure homogeneity. The 10⁻¹ to 10⁻⁷ gradient dilutions were prepared

sequentially, and 100 microliters of each sample was inoculated into WL medium by the dilution spread plate method. An inverted incubation at 28°C was performed for a period of 48 hours. Following this incubation, characteristic single colonies were selected and inoculated into fresh YPD medium using the streak plate method. This process was repeated until pure culture strains exhibiting consistent morphology were obtained. The appropriate amount of 30% glycerol aqueous solution was prepared and placed in a high-pressure steam sterilization pot for sterilization. After sterilization, it was taken out and cooled to room temperature (20-23°C) and transferred to a 2mL cryopreservation tube. The isolated strain was placed in a cryopreservation tube, shaken well, and the cryopreservation tube was stored in a refrigerator at -80°C. The morphology of the colonies (e.g., colony size, color, surface features, etc.) was observed by light microscope, and their characteristics were recorded[22].

2.5.2. Molecular Biological Identification of Yeast

The isolated and purified suspected yeast strains were inoculated into YPD liquid medium and activated at 28°C for 48h. The bacteria were collected by centrifugation, and the genomic DNA was extracted by yeast genomic DNA rapid extraction kit. PCR amplification was performed using ITS1 (5'-TCCGTAGGTGAACCTGCGG-3' NR_171887.1) and ITS4 (5'-TCCTCCGCTTATTGATATATGC-3' NR_168827.1) as primers. The reaction system (25μL) : ddH₂O 10.5μL, Taq mix 12.5μL, forward/reverse primers 0.5 μL, template DNA 1μL. PCR conditions : 94°C for 5min; 94°C denaturation 30s, 58°C annealing 30s, 72°C extension 35s, 30cycles; the final extension at 72°C for 10min[23]. The amplified products were detected by gel electrophoresis and sent to Xi 'an Qingke Biotechnology Co., Ltd. for sequencing. The sequencing results were compared by NCBI database BLAST, and the phylogenetic tree was constructed by MEGA11 Neighbor-Joining method.

2.5.3. Rescreening of Yeast

1. Preparation of yeast seed solution

The preserved yeast strains were inoculated in YPD liquid medium and cultured at 28°C and 150rpm for 48h. The cultured yeast was transferred to a sterile centrifuge tube and centrifuged at 8000rpm for 5min. The precipitate was taken and dissolved in sterile water. The OD₆₀₀ was measured by a microplate reader until the OD₆₀₀ was 0.6-0.8. At this time, the yeast concentration was about 6-8×10⁷ cells/mL. The prepared yeast seed solution was stored at 4°C for later use.

2. Determination of gas production performance

The yeast seed solution was inoculated into the Duchenne fermentation tube (containing YPD liquid medium) at 2 % (v/v), and cultured at 28°C for 48h. The gas production in the fermentation tube (such as the number of bubbles, volume change) and the odor characteristics of the fermentation broth were observed and recorded regularly to evaluate the gas production capacity of the strain[22].

3. Determination of alcohol tolerance

The yeast seed solution was inoculated into YPD medium containing different concentrations of anhydrous ethanol (final concentrations were 6%, 9%, 12%, 15%, 18% v/v) at 2% (v/v) inoculation amount, and YPD medium without ethanol was used as blank control. After 48h of culture at 28°C, the OD value at 600 nm wavelength was measured by microplate reader to evaluate the growth of the strain at different alcohol concentrations[22].

4. Determination of acid resistance

The yeast seed solution was inoculated into YPD medium with pH of 2.0, 2.5, 3.0, 3.5 and 4.0 (adjusted with citric acid) at 2% (v/v) inoculation amount, and YPD medium without pH adjustment was used as blank control. After incubation at 28°C for 48h, the OD value at 600 nm wavelength was measured to analyze the growth adaptability of the strain under different acidic conditions[22].

5. Determination of sugar resistance

The yeast seed solution was inoculated into YPD medium with glucose concentrations of 300, 350, 400, 450 and 500g/L by 2% (v/v) inoculation amount, and YPD medium without additional glucose was used as blank control. After incubation at 28°C for 48h, the OD value at 600nm wavelength was measured to investigate the growth characteristics of the strain in high glucose environment[22].

2.6. *Msalais Fermentation Single Factor Test and Response Surface Optimization Test*

2.6.1. Effect of Yeast Addition on the Content of Amadori Compounds in Msalais

Five fermentation tanks were taken, and 1 L of grape juice rich in Amadori compounds (see 2.4 for preparation method) was taken in the fermentation tank. In each fermentation tank, 1%, 2%, 3%, 4%, 5% yeast seed liquid (v/v) was added in turn. The ratio of *Saccharomyces cerevisiae* to non-*Saccharomyces cerevisiae* was 2:1 (the total volume of *Saccharomyces cerevisiae* and non-*Saccharomyces cerevisiae* was the volume of yeast seed solution), and the fermentation was carried out at 28°C for 14days (336 h). The above steps were repeated three times. After the fermentation, the content of Amadori compounds in each fermentation tank was detected. Determine the optimal yeast addition amount.

2.6.2. Effect of Fermentation Temperature on the Content of Amadori Compounds in Msalais

Five fermentation tanks were taken, and 1L of grape juice rich in Amadori compounds (see 2.4 for preparation method) was taken in the fermentation tank, and 2% yeast seed solution (v/v) was added to each fermentation tank. The ratio of *Saccharomyces cerevisiae* to non-*Saccharomyces cerevisiae* was 2:1 (the total volume of *Saccharomyces cerevisiae* and non-*Saccharomyces cerevisiae* was the volume of seed liquid). The five fermentation tanks were placed in an incubator at fermentation temperatures of 22°C, 25°C, 28°C, 31°C, and 34°C, respectively, and fermented for 14 days (336 h), repeating the above steps three times. After the fermentation, the content of Amadori compounds in Msalais was detected in each fermentor to determine the optimal fermentation temperature[24].

2.6.3. Effect of Fermentation Time on the Content of Amadori Compounds in Msalais

Five fermentation tanks were taken, and 1 L of grape juice rich in Amadori compounds (see 2.4 for preparation method) was taken in the fermentation tank, and 2% yeast seed solution (v/v) was added to each fermentation tank. The ratio of *Saccharomyces cerevisiae* to non-*Saccharomyces cerevisiae* was 2:1 (the total volume of *Saccharomyces cerevisiae* and non-*Saccharomyces cerevisiae* was the volume of seed liquid), and the five fermentation tanks were placed at a fermentation temperature of 28°C for 7d (168h), 14 d (336h), 21d (504h), 25d (600h), 30d (720h) fermentation, and the above steps were repeated three times. After the fermentation, the content of Amadori compounds in Msalais was detected in each fermentor to determine the optimal fermentation time.

2.6.4. The Effect of the Ratio of *Saccharomyces Cerevisiae* to Non-*Saccharomyces Cerevisiae* on the Content of Amadori Compounds in Msalais

Five fermenters were selected, and 1L grape juice rich in Amadori compounds (preparation method is shown in 2.4) was taken in the fermenter. In each fermenter, 2 % yeast seed solution (v/v) was added according to the ratio of *Saccharomyces cerevisiae* to non-*Saccharomyces cerevisiae*: 1:1, 1:2, 2:1, 1:3, 3:1 (the total volume of *Saccharomyces cerevisiae* and non-*Saccharomyces cerevisiae* was the volume of yeast seed solution). The fermentation temperature was 28°C, and the fermentation was carried out for 14 days (336h). The above steps were repeated three times. After the fermentation, the content of Amadori compounds in Msalais was detected in each fermenter. Determine the optimal ratio of *Saccharomyces cerevisiae* to non-*Saccharomyces cerevisiae*.

2.6.5. Msalais Fermentation Response Surface Test

According to the optimal condition range determined by single factor test, the content of Fru-Pro and Fru-Asp were used as evaluation indexes. The Box-Behnken experimental design method was used to select the amount of yeast, fermentation temperature, fermentation time, and the ratio of *Saccharomyces cerevisiae* to non-*Saccharomyces cerevisiae*. The four-factor three-level response surface design is shown in Table 2, and the optimal fermentation conditions are obtained according to the response surface optimization test.

Table 2. Response surface test factors and levels

Level	Factor			
	A The ratio of <i>Saccharomyces cerevisiae</i> to non- <i>Saccharomyces cerevisiae</i>	B yeast addition amount (%)	C fermentation temperature (°C)	D Fermentation time (All day)
-1	1:1	1	25	7
0	2:1	2	28	14
1	3:1	3	31	21

2.7. Antioxidant Experiment

2.7.1. Determination of DPPH Free Radical Scavenging Ability

The 25μL sample was mixed with 200μL freshly prepared 0.35mmol/L DPPH/methanol solution and placed in dark at room temperature(20-23°C) for 2h. Methanol was used as a blank to determine the absorbance at 517nm. The absorbance of the sample solution was recorded as Aa, and the absorbance of the blank was recorded as Ab. Trolox was dissolved in methanol with a concentration gradient of 0, 10, 20, 30, 40 μg/mL[25]. The antioxidant activity of Msalais was expressed as μmol Trolox/L sample (Trolox equivalent).

$$\text{Scavenging rate of DPPH radical (\%)} = \frac{Aa - Ab}{Aa} \times 100\%$$

(1)

2.7.2. Determination of ABTS Free Radical Scavenging Ability

The configuration of ABTS working solution: The ABTS mother liquor with a concentration of 7mmol/L and the potassium persulfate solution with a concentration of 2.45mmol/L were mixed in an equal volume ratio, and placed in the dark for 12-16h at room temperature (20-23°C). The configured ABTS working solution was diluted to an absorbance of 0.700±0.020 for use. The 0.1mL sample was mixed with 3.9mL reaction solution, placed in the dark at room temperature (20-23°C) for 30min, with methanol as a blank, 200μL of the mixed solution was added to the 96-well microtiter plate, and then the absorbance was measured at the wavelength of 735nm. The absorbance of the sample solution was recorded as Aa, and the absorbance of the blank was recorded as Ab. The concentration gradient of water-soluble vitamin E (Trolox) was 0, 10, 20, 30, 40 μg / mL[26].

$$\text{Scavenging rate of ABTS radical (\%)} = \frac{Aa - Ab}{Aa} \times 100\%$$

(2)

2.7.3. Determination of Total Oxygen Radical Absorbance Capacity (ORAC)

The sample solution and Trolox standard solution 20μL and 200μL fluorescein sodium solution (96nmol/L) were added to the 96-well plate, and 20μL phosphate buffer (100mmol/L) was added to the blank control. After mixing, it was preheated at 37°C for 20min, and 20μL AAPH (153mmol/L) solution was quickly added and mixed well. The 96-well plate was placed in a microplate reader for

fluorescence intensity measurement. Determination conditions:excitation wavelength $\lambda_{excitation}=485nm$, absorption wavelength $\lambda_{emission}=535nm$, fluorescence value was measured every 2.5min, a total of 35cycles, fluorescence intensity were recorded as $f_1, f_2, \dots$. The f_{35} was subjected to Trolox calibration in each test, and the ORAC value of the sample was expressed as Trolox equivalent[27].

$$AUC=(0.5 \times f_1 / f_1 + f_2 / f_1 + f_3 / f_1 + \dots + f_i / f_1 + \dots f_{34} / f_1 + 0.5 \times f_{35} / f_1) \times 2.5$$
$$\Delta AUC=AUC_{sample} - AUC_{blank}$$
(3)

3. Results and Discussion

3.1. Validation of HPLC-ELSD Method for the Detection of Two Amadori Compounds

3.1.1. System Adaptability Results

The two Amadori compound standard solutions were injected for analysis, and the high performance liquid chromatograms of Fru-Asp and Fru-Pro standard solutions were obtained. The results are shown in Figure 2. It can be seen from Figure 2 that the peak time of Fru-Pro and Fru-Asp were 9.16min and 11.78min, respectively. The two Amadori compounds could be detected within 20 min.

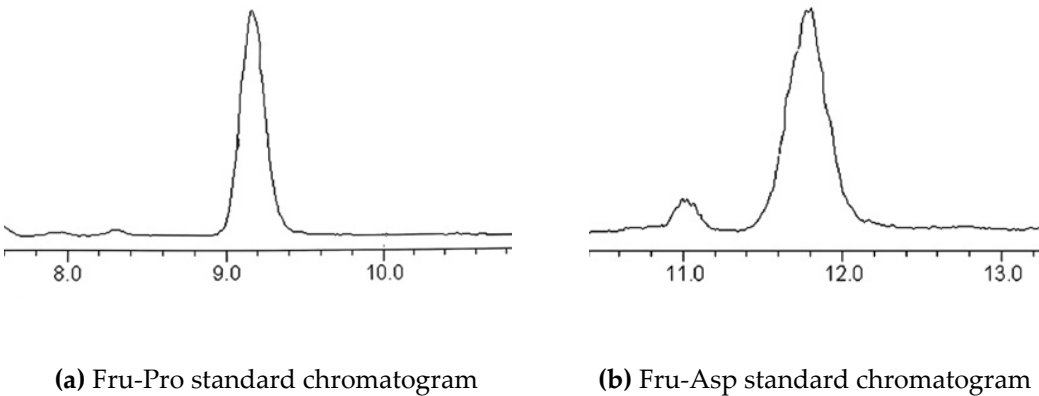


Figure 3. Standard chromatograms of two Amadori compounds.

3.1.2. Results of Linear Relationship Investigation

The standard curve regression equation and correlation coefficient results of two Amadori compounds are shown in Figure 4.

From Figure 4, The standard curve regression equation of Fru-Pro was $y=1962.2x-83672$, and the standard curve regression equation of Fru-Asp was $y=694.17x+2227$. The standard curve regression equation of Fru-Pro was $y=1962.2x-83672$, and the standard curve regression equation of Fru-Asp was $y=694.17x+2227$. Fru-Pro showed a good linear relationship in the range of 100-500 $\mu g/mL$, Fru-Asp showed a good linear relationship in the range of 5-300 $\mu g/mL$, and the correlation coefficient R^2 was ≥ 0.9995 . It shows that Fru-Pro and Fru-Asp have a good linear relationship with the chromatographic peak area in the corresponding mass concentration range, and the detection sensitivity of this method is high.

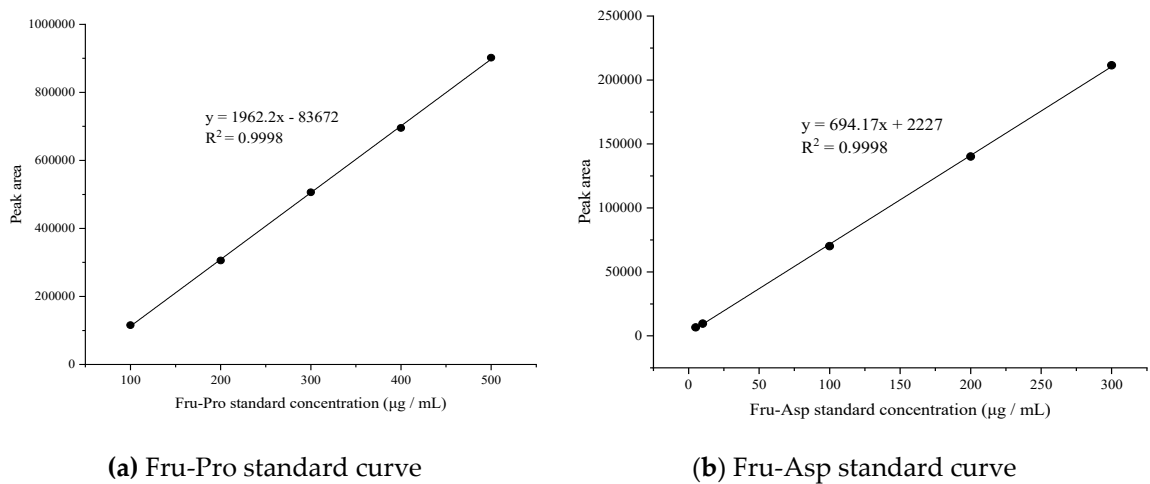


Figure 4. Standard curves of two Amadori compounds.

3.1.3. Accuracy Test Results

The accuracy test results of the two Amadori compounds are shown in Table 3. It can be seen from Table 3 that the relative standard deviations of the accuracy test results of Fru-Pro and Fru-Asp were 2.83% and 9.49%, respectively, and the relative standard deviations were less than 10%, indicating that the accuracy of the method was good.

Table 3. Accuracy test results of Fru-Pro and Fru-Asp.

Name	Known concentrationPeak area number	Measured value (g/L)	Average measured value (g/L)	Correlation coefficient (R²)	Standard deviation (g/L)	Relative standard deviation (%)
Fru-Pro (0.25g/L)	1	405168	0.2492	0.9998	0.0070	2.83
	2	384965	0.2389			
	3	411152	0.2522			
Fru-Asp (0.01g/L)	1	69154	0.0099	0.9998	0.0011	9.49
	2	81432	0.0114			
	3	85319	0.0120			

3.1.4. Precision Test Results

The precision test results of the two Amadori compounds are shown in Table 4. It can be seen from Table 4 that the relative standard deviations of the precision test results of Fru-Pro and Fru-Asp were 4.59% and 8.72%, respectively, and the relative standard deviations were less than 10%, indicating that the instrument performance was relatively stable and the precision of the method was good.

Table 4. Precision test results of Fru-Pro and Fru-Asp.

Name	Known concentrationPeak area number	Measured value (g/L)	Average measured value (g/L)	Correlation coefficient (R²)	Standard deviation (g/L)	Relative standard deviation (%)
Fru-Pro (0.25g/L)	1	405168	0.2492	0.9998	0.0110	4.59
	2	355947	0.2241			
	3	366948	0.2297			
	4	385264	0.239			
	5	410516	0.2519			
	6	395678	0.2443			
Fru-Asp (0.01g/L)	1	71125	0.0099	0.9998	0.0009	8.72
	2	65415	0.0091			

3	70185	0.0098
4	79263	0.0111
5	68894	0.0096
6	81021	0.0114

3.1.5. Repeatability Test Results

The repeatability test results of two Amadori compounds are shown in Table 5. From Table 5, the relative standard deviations of the repeatability test results of Fru-Pro and Fru-Asp were 2.67% and 8.06%, respectively, and the relative standard deviations were all<10 %, indicating that the method had good repeatability.

Table 5. Repeatability test results of Fru-Pro and Fru-Asp.

Name	Repeated injection of the same sample solution (times)	Peak area	Measured value (g/L)	Average measured value (g/L)	Standard deviation (g/L)	Relative standard deviation (%)
Fru-Pro	1	3899421	0.2030	0.2057	0.0055	2.67
	2	4121025	0.2143			
	3	3772005	0.1965			
	4	3921452	0.2041			
	5	3985241	0.2074			
	6	4012585	0.2088			
Fru-Asp	1	135777	0.0192	0.0186	0.0015	8.06
	2	145202	0.0206			
	3	121478	0.0172			
	4	115489	0.0163			
	5	128563	0.0182			
	6	140254	0.0199			

3.1.6. Result of Stability Test

The stability test results of two Amadori compounds are shown in Table 6. It can be seen from Table 6 that the relative standard deviations of Fru-Pro and Fru-Asp were 3.25% and 8.71%, respectively, and the relative standard deviations were less than 10%, indicating that the sample solution was stable within 36h.

Table 6. Stability test results of Fru-Pro and Fru-Asp.

Name	Placement time (h)	Peak area	Measured value (g/L)	Average measured value (g/L)	Standard deviation (g/L)	Relative standard deviation (%)
Fru-Pro	0	4213546	0.2190	0.2161	0.0070	3.25
	6	4225135	0.2196			
	12	3985621	0.2074			
	18	4002145	0.2082			
	24	4115836	0.2140			
	30	4337996	0.2253			
	36	4146713	0.2156			
Fru-Asp	0	130648	0.0185	0.0171	0.0015	8.71
	6	121582	0.0172			
	12	137254	0.0195			
	18	110565	0.0156			
	24	109521	0.0155			
	30	115754	0.0164			
	36	119659	0.0169			

3.1.7. Sample Content Determination Results

HPLC-ELSD was used to detect two Amadori compounds in Msalais-like varieties. The detection chromatograms of Fru-Pro and Fru-Asp were shown in Figure 5. The retention time of Fru-Pro was 9.16 min , and the retention time of Fru-Asp was 11.77 min., and the contents were 0.2165±0.0022g/L and 0.0185±0.0008g/L, respectively. The separation of the two substances is clear.

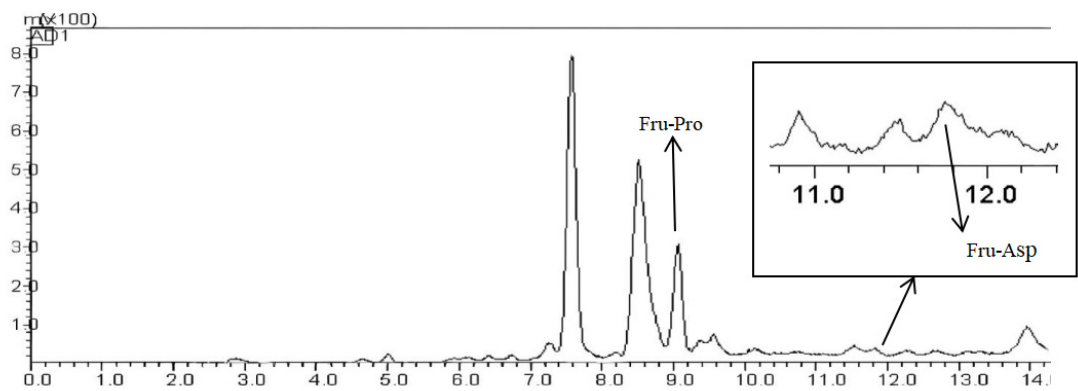


Figure 5. Chromatogram of two Amadori compounds.

3.1.8. Test Results of Recovery Rate

The recovery test results of two Amadori compounds are shown in Table 7. It can be seen from Table 7 that the average spiked recoveries of Fru-Pro and Fru-Asp were 101.21% and 100.57%, respectively. The relative standard deviations of the average recovery test results of each component were 4.97% and 5.87%, respectively. The relative standard deviations were less than 10%, indicating that the method is stable and reliable, and can accurately test the Amadori compounds in the sample.

Table 7. The recovery test results of Fru-Pro and Fru-Asp.

Name	Quality in the sample (mg)	The amount of added standard (mg)	The quality detected(mg)	Recovery rate (%)	average value (%)	Standard deviation (g/L)	Relative standard deviation (%)
Fru-Pro	0.2030	0.1250	0.3307	102.16	101.21	5.03	4.97
	0.2112	0.1250	0.3314	96.16			
	0.2157	0.1250	0.3389	98.56			
	0.2236	0.1250	0.3592	108.48			
	0.2045	0.1250	0.3251	96.48			
	0.2187	0.1250	0.3505	105.44			
Fru-Asp	0.0192	0.0500	0.0710	103.60	100.57	5.91	5.87
	0.0180	0.0500	0.0654	94.80			
	0.0197	0.0500	0.0735	107.60			
	0.0174	0.0500	0.0703	105.80			
	0.0165	0.0500	0.0655	98.00			
	0.0170	0.0500	0.0638	93.60			

3.2. Preliminary Screening of Yeast

3.2.1. Separation of Yeasts in Samples by Dilution Coating Plate Method

Three batches of samples were sampled in Msalais winery. Yeasts were isolated from the mechanical surface, grape epidermis and soil, and the dilution gradients were 10⁻³,10⁻³ and 10⁻², respectively.

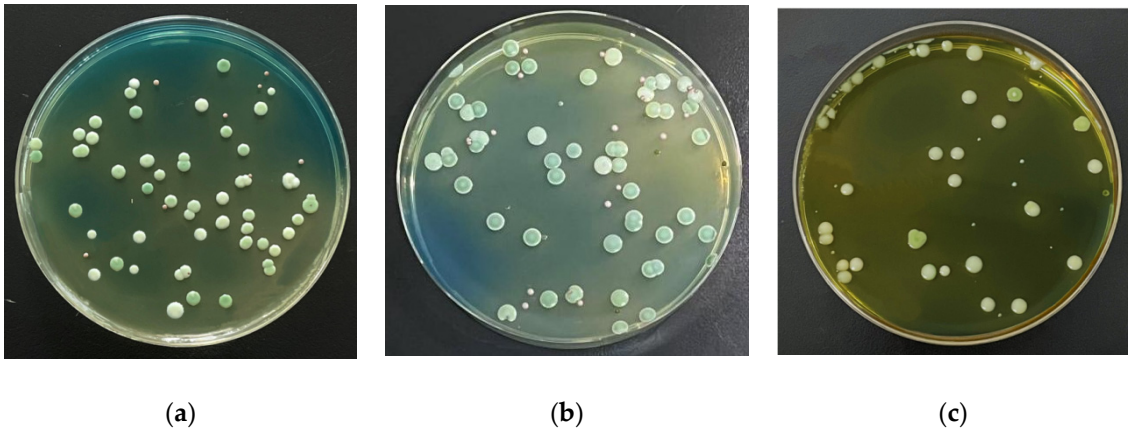
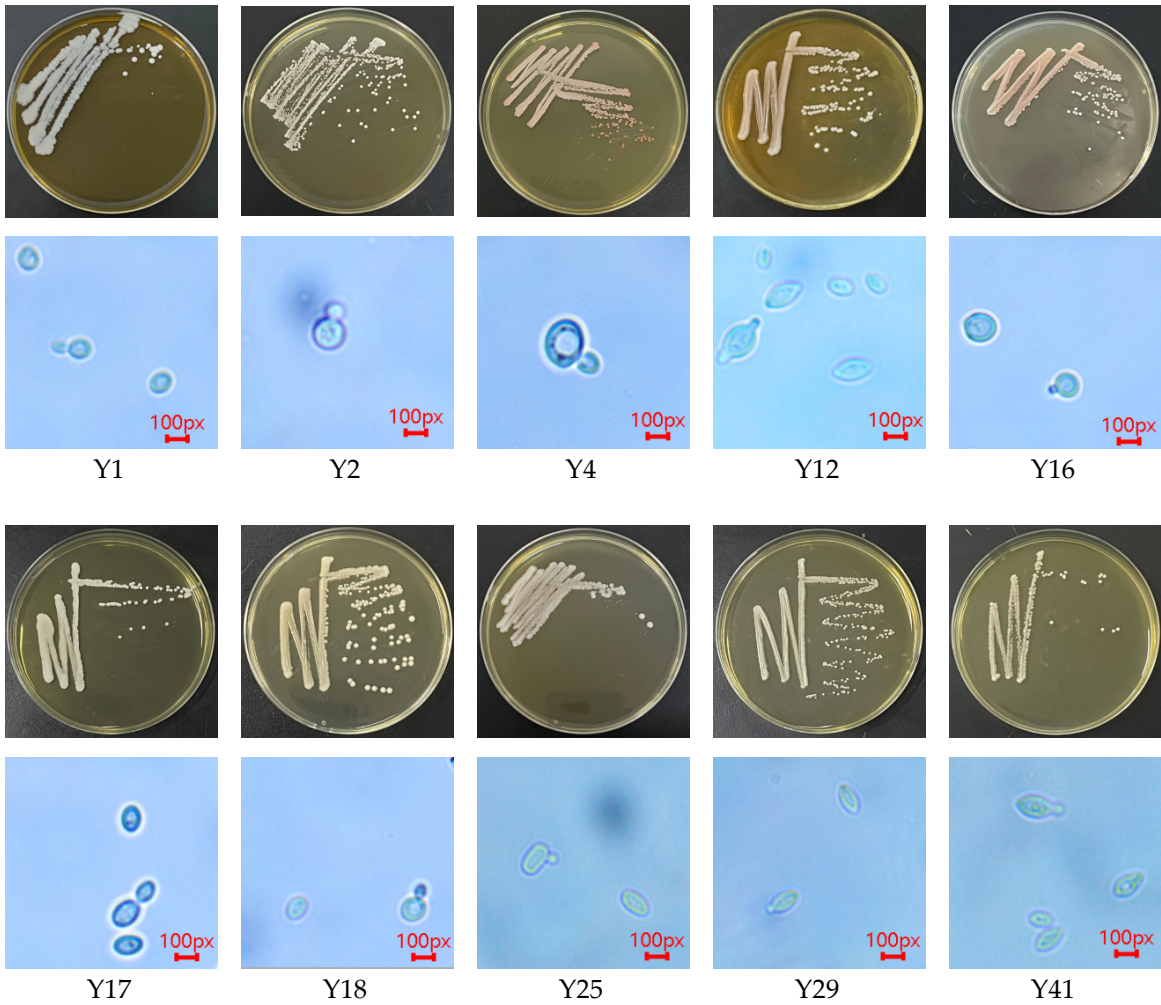


Figure 6. Screening of yeasts by dilution coating plate method.

3.2.2. Morphological Identification of Yeast

Single colonies were picked from the diluted coating plate and inoculated into the new YPD medium using the plate streaking method until a single morphological strain was purified. The colony morphology was observed under a biological microscope and the characteristics were recorded. A total of 15 suspected yeast strains were isolated from the fruit of Hotan red grape, and numbered as Y1, Y2, Y4, Y12, Y16, Y17, Y18, Y25, Y29, Y41, Y61, Y67, Y72, Y91, Y107. The colony morphology and microscopic morphology of the isolated suspected yeast strains were observed[22], as shown in Figure 7.



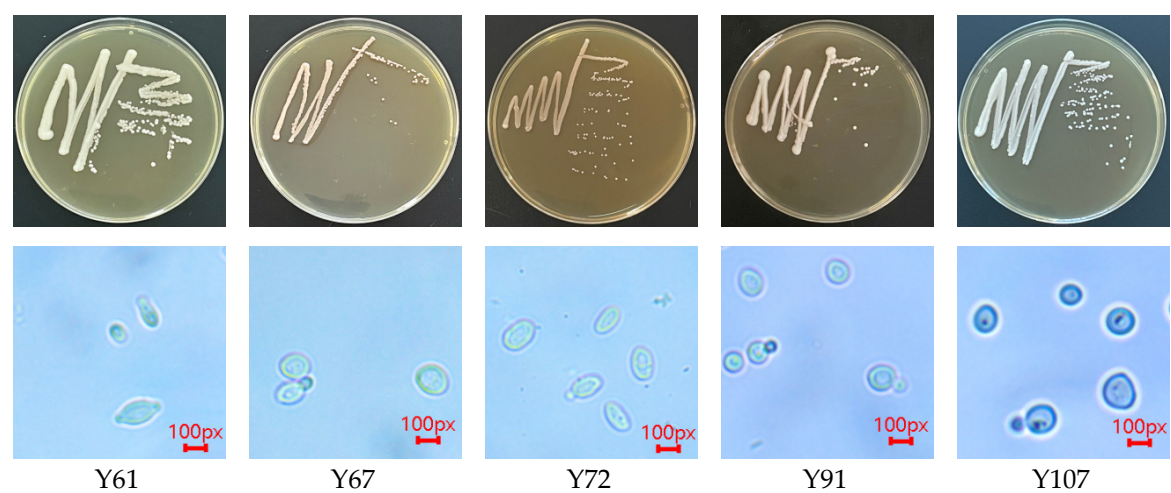


Figure 7. Colony morphology and microscopic morphology of 15 yeast strains.

From Figure 7, it can be seen that the edges of the 15 colonies are neat, the surface is mainly smooth and easy to evoke. The main morphological description is shown in Table 8. The cell morphology of strains Y12, Y16, Y18, Y25, Y67, Y91 and Y107 was spherical, Y17 and Y72 were oval, Y2, Y4, Y29, Y41 and Y61 were lemon-shaped. Among them, Y1, Y12, Y18 and Y107 were single-ended budding, and the remaining 10 strains were two-ended or multi-ended budding. Some of them formed a string of cells, which were like filaments[28]. The microscopic morphology is described in Table 9.

Table 8. Description of colony morphology.

Number of strains	Colony color and morphology
Y1, Y2	Milk white greenish, spherical protrusions, smooth surface, opaque
Y4, Y16, Y18, Y67, Y72, Y91	Rice white purple, spherical protrusions, the surface is rough, opaque
Y12, Y61	Rice white yellowish, spherical protrusions, smooth surface, translucent
Y17	Milk white greenish, spherical protrusions, rough surface, opaque
Y25	Milk white greenish, flat, smooth surface, translucent
Y29	Rice white greenish, spherical protrusions, smooth surface, translucent
Y41, Y107	Milk white, spherical protrusions, smooth surface, opaque

Table 9. Microscopic morphology description table.

Number of strains	micro-morphology
Y1	Spindle, single-ended budding reproduction
Y2, Y4, Y29, Y41, Y61	Lemon-shaped, both ends of the bud reproduction
Y12, Y16, Y18, Y25, Y67, Y91, Y107	Spherical, single-ended budding reproduction
Y17, Y72	Oval, single-ended budding reproduction

3.2.3. Molecular Biological Identification of Yeast

The DNA of 15 yeast strains were extracted and amplified by PCR using primers ITS1 and ITS4. The sequences of the strains were compared by BLAST in NCBI, and the phylogenetic tree[29] was constructed as shown in Figure 8.

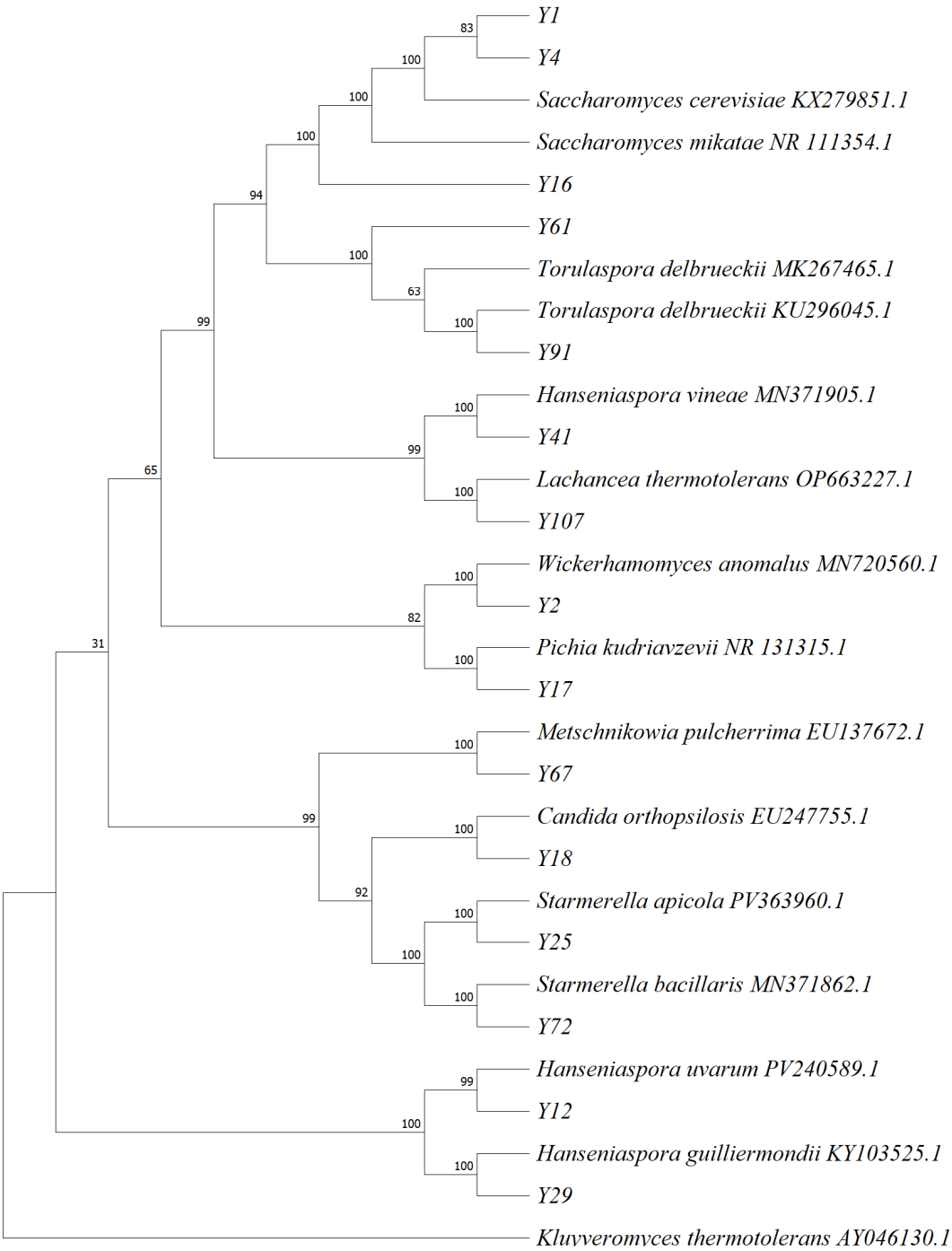


Figure 8. Phylogenetic tree of 15 yeast strains.

From Figure 8, it can be seen that the strains Y1, Y2, Y4, Y12, Y17, Y18, Y25, Y29, Y41, Y61, Y67, Y72, Y91 and Y107 are related to some other reference yeast strains downloaded from NCBI. Among them, strains Y1, Y4 and Y16 had high similarity with *Sacchsromyces cerevisiae*, and the similarity was 100%. Strain Y2 had high similarity with *Wickerhamomyces anomalus*, and the similarity was 100%. Strain Y12 had high similarity with *Hanseniaspora uvarum*, and the similarity was 99%. Strain Y17 had high similarity with *Pichia kudriavzevii*, and the similarity was 100%. Strain Y18 had high similarity with *Candida orthopsilosis*, and the similarity was 100%. Strain Y25 had a high similarity with *Starmerella apicola*, with a similarity of 100%. The strain Y29 had a high similarity with *Hanseniaspora guilliermondii*, and the similarity was 100%. Strain Y41 had high similarity with *Hanseniaspora vineae*, and the similarity was 100%. Strain Y61 had high similarity with *Torulaspora delbrueckii*, and the

similarity was 100%. The strain Y67 had a high similarity with *Metschnikowia pulcherrima*, and the similarity was 100%. Strain Y72 had a high similarity with *Starmerella bacillaris*, with a similarity of 100%. Strain Y91 had high similarity with *Torulaspora delbrueckii*, and the similarity was 100%. Strain Y107 had a high similarity with *Lachancea thermotolerans*, with a similarity of 100%. The similarity was higher than 99%, and the 15 strains could be identified as belonging to the above strains.

3.3. Rescreening of Yeast

3.3.1. Experimental Results of Gas Production Performance of Yeast Strains

The largest gas production is *Saccharomyces cerevisiae* Y4, which has good precipitation and strong wine aroma. Y1, Y16 and AQSX have more gas production and wine aroma. The better the gas production of yeast, the stronger the ability to decompose sugar to produce carbon dioxide and alcohol. The main fermentation product of *Saccharomyces cerevisiae* is alcohol, so the stronger the wine aroma of its fermentation broth proves that it is more suitable for alcohol fermentation. The role of non-*Saccharomyces cerevisiae* is to produce some aroma substances such as alcohol and esters. The stronger the aroma, the more esters it produces, which can improve the flavor of Msalais. Non-*Saccharomyces cerevisiae* Y2, Y12, Y17, Y41, Y61, Y72, Y91 had more gas production and good precipitation. The aroma of Y2 was stronger, and the fermentation broth of Y17 had no obvious aroma and less precipitation.

Table 10. Experimental results of gas production performance of yeast strains.

Number of strains	Gas production situation	Coagulation of strains	Fermentation broth aroma
Y1	+++	The precipitation is in good condition	Wine flavor
Y2	+++	The precipitation is in good condition	Stronger aroma
Y4	++++	The precipitation is in good condition	Stronger wine aroma
Y12	+++	The precipitation is in good condition	Wine flavor
Y16	+++	The precipitation is in good condition	Wine flavor
Y17	+++	Precipitation is poor	No aroma
Y18	++	The precipitation is in good condition	No aroma
Y25	++	The precipitation is in good condition	Wine flavor
Y29	+	The precipitation is in good condition	Wine flavor
Y41	+++	The precipitation is in good condition	Lighter aroma
Y61	+++	The precipitation is in good condition	Wine flavor
Y67	++	The precipitation is in good condition	Wine flavor
Y72	+++	The precipitation is in good condition	Lighter aroma
Y91	+++	The precipitation is in good condition	Wine flavor
Y107	++	The precipitation is in good condition	No aroma
AQSX	+++	The precipitation is in good condition	Wine flavor

Note : “+” indicates gas production, and the more “+” indicates the greater gas production.

3.3.2. Experimental Results of Alcohol Resistance of Yeast Strains

It can be seen from Table 11 that Y1, Y2 and Y4 can grow in large quantities when the alcohol volume fraction is 6% and can grow significantly when the alcohol volume fraction is 9%. Y16, Y107 and AQSX can grow in large quantities when the alcohol volume fraction is 6%. Y12, Y17, Y25, Y29, Y41 and Y72 can grow significantly when the alcohol volume fraction is 6%. Studies have shown that *Saccharomyces cerevisiae* has better alcohol resistance than non-*Saccharomyces cerevisiae* [22]. The growth and fermentation activities of most non-*Saccharomyces* yeasts are significantly inhibited or stopped after the increase of alcohol concentration (The biological mechanism is discussed according to the reviewer 's suggestion). Y1, Y2, Y4, Y16, Y107, AQSX, Y12, Y17, Y25, Y29, Y41, Y72 have a certain alcohol tolerance, so they can be used for subsequent Msalais fermentation.

Table 11. The experimental results of alcohol resistance of yeast strains.

Number of strains	Medium alcohol volume fraction (%)				
	6	9	12	15	18
Y1	+++	++	*	*	*
Y2	+++	++	*	*	*
Y4	+++	++	*	*	*
Y12	++	*	*	*	*
Y16	+++	+	*	*	*
Y17	++	+	*	*	*
Y18	+	*	*	*	*
Y25	++	+	*	*	*
Y29	++	*	*	*	*
Y41	++	+	*	*	/
Y61	+	*	*	*	/
Y67	*	*	*	*	*
Y72	++	*	*	*	*
Y91	+	*	*	*	*
Y107	+++	+	*	*	*
AQ SX	+++	+	*	*	*

Note : “/”: no growth, $OD_{600} \leq 0.1$; “*”: micro-growth, $0.1 < OD_{600} < 0.3$; “+”: growth, $0.3 < OD_{600} < 0.5$; “++” : obvious growth, $0.5 < OD_{600} < 1.0$; “+++”: a large number of growth, $OD_{600} > 1.0$ (Table12, Table 13 are the same).

3.3.3. Experimental Results of Acid Resistance of Yeast Strains

Yeasts Y1, Y2, Y4, Y61 and Y91 can grow in large quantities at pH 3, but Y61 and Y91 can only grow slightly at pH 2.5. Therefore, yeasts Y1, Y2 and Y4 grow best under different acidic conditions. Y12, Y16, Y17, Y18, Y25 and Y29 can grow significantly at pH 3 and pH 2.5, and their tolerance to acidic conditions is strong. Y16, Y41, Y72, Y107 and AQ SX grow significantly at pH 3 and grow at pH 2.5, indicating that they have certain tolerance to acidic conditions. The pH of Msalais is usually between 3.4 and 3.8, and the main source of its acidity is tartaric acid[30]. In traditional processes, grape juice is mixed with grape skin and grape seeds before fermentation. The grape seeds will release potassium ion K + and combine with tartaric acid to form potassium hydrogen tartrate precipitate, which will lead to an increase in pH value. However, Msalais is still generally acidic, so the yeast with strong acid resistance is more suitable for the fermentation of Msalais.

Table 12. The experimental results of acid resistance of yeast strains.

Number of strains	pH				
	2	2.5	3	3.5	4
Y1	*	+	+++	+++	+++
Y2	*	+	+++	+++	+++
Y4	*	+	+++	+++	+++
Y12	*	++	++	+++	+++
Y16	*	+	++	++	+++
Y17	*	++	++	++	++
Y18	*	++	++	+++	+++
Y25	*	++	++	+++	+++
Y29	*	++	++	++	+++
Y41	*	+	++	++	++
Y61	*	*	+++	+++	+++
Y67	*	+	+	++	++
Y72	*	+	++	++	+++

Y91	*	*	+++	+++	+++
Y107	*	+	++	+++	+++
AQ SX	*	+	++	+++	+++

3.3.4. Experimental Results of Sugar Tolerance of Yeast Strains

The yeasts Y1, Y2, Y4, Y12, Y16, Y41, Y61, Y72, Y91, Y107, AQ SX could grow on the medium with different concentrations of glucose, indicating that they had good sugar tolerance. The raw materials used in this study were boiled and concentrated grape juice, and the sugar content could reach 27-30 °BX. The higher the tolerance of yeasts to sugar, the better the growth status of yeasts in the early stage of fermentation. The selected 11 yeasts can be used for concentrated grape juice fermentation Msalais.

Table 13. The experimental results of acid resistance of yeast strains.

Number of strains	Medium sugar concentration (g/L)				
	300	350	400	450	450
Y1	+++	+++	+++	+++	+++
Y2	+++	+++	+++	+++	+++
Y4	+++	+++	+++	+++	+++
Y12	++	++	++	++	++
Y16	+++	+++	+++	+++	+++
Y17	+++	+++	++	++	++
Y18	+++	+++	++	++	++
Y25	+++	+++	++	++	++
Y29	++	++	++	++	++
Y41	+++	+++	++	++	++
Y61	+++	+++	+++	+++	+++
Y67	++	++	+	+	+
Y72	+++	+++	++	++	++
Y91	+++	+++	+++	+++	+++
Y107	+++	+++	+++	+++	+++
AQ SX	+++	+++	+++	+++	+++

3.3.5. Single Yeast Fermentation Experiment

It can be seen from Figure 9 that the content of Fru-Pro in Msalais fermented by non-Saccharomyces cerevisiae Y2, Y12, Y17, Y29, Y41, Y72 and Y91 was the highest. The contents of Fru-Asp in Msalais fermented by Y2, Y12, Y41 and Y72 were the highest. During the fermentation process, some non-Saccharomyces may use Amadori compounds as nutrients, resulting in a decrease in the content of Amadori compounds. Choosing non-Saccharomyces that do not use Amadori compounds as nutrients can effectively increase the content of Amadori compounds in Msalais. The contents of Fru-Pro and Fru-Asp in Msalais fermented by Saccharomyces cerevisiae Y4 were higher than those of Y1, Y16 and AQ SX. Therefore, according to the results of tolerance experiment and single-strain fermentation experiment, Saccharomyces cerevisiae Y4 and non-Saccharomyces cerevisiae Y2, Y12, Y41 and Y72 were selected as the original strains of mixed fermentation to ferment Msalais.

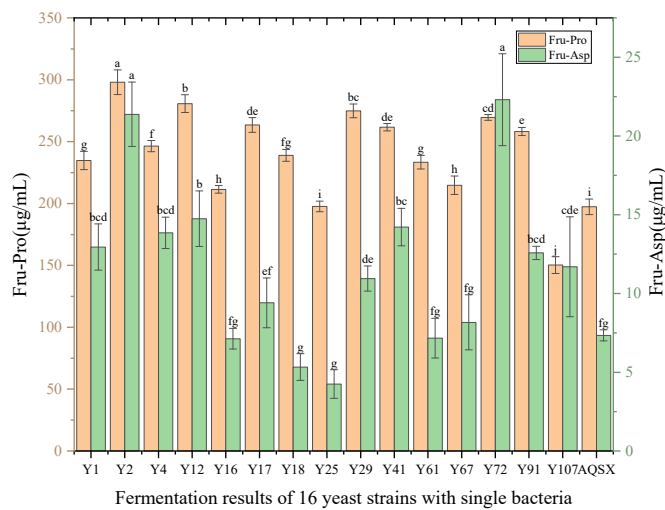
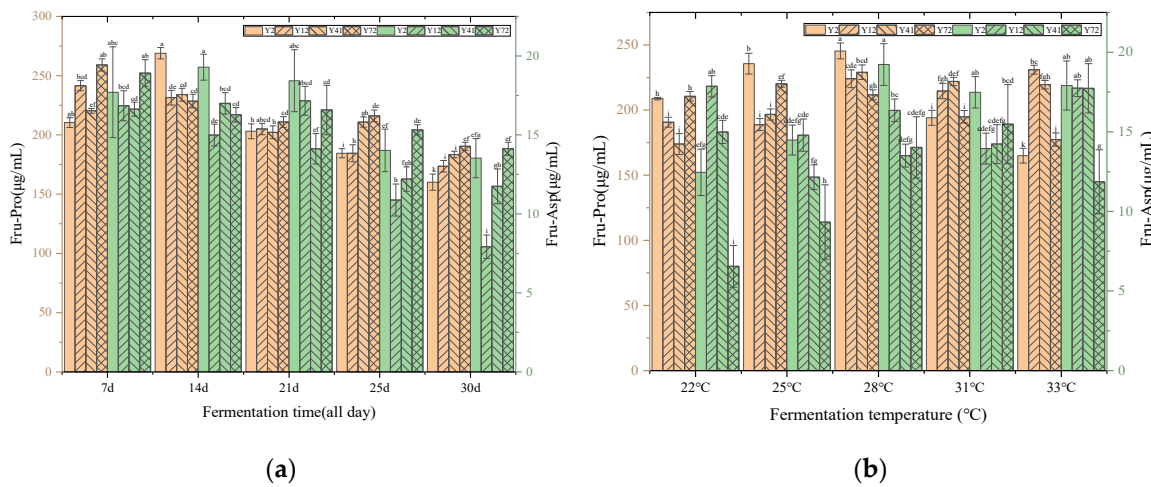


Figure 9. The content of Fru-Pro and Fru-Asp in Msalais fermented by 16 yeasts.

3.4. Single Factor Experiment Results of Fermentation Process

3.4.1. Effect of Yeast Addition on the Content of Fru-Pro and Fru-Asp

During the brewing process, the addition of yeast directly affects the fermentation kinetics and the production of metabolites. It can be seen from Figure 10 (a) that the content of Amadori compounds reached the peak when the yeast addition amount was 2%, and then the content of Amadori compounds decreased with the increase of yeast addition amount. The possible reason is that when the amount of yeast added is 2%, the glucose metabolism rate is balanced with the Amadori compound formation rate ; when the amount of yeast was more than 2%, the content of Amadori compounds decreased due to substrate depletion, ethanol inhibition and metabolic pathway competition[31]. This rule suggests that the amount of yeast added should be accurately controlled in the fermentation process to maximize the production of Amadori compounds and optimize the flavor of wine. The content of Amadori compounds in the mixed fermentation of Y12, Y41, Y72 and Y4 reached the peak when the amount of yeast added was 2% or 3%, but the content was lower than that of the mixed fermentation of Y2 and Y4 when the amount of yeast added was 2%. Therefore, Y2 and Y4 were selected as mixed fermentation strains, and the amount of yeast added 2% was the best level.



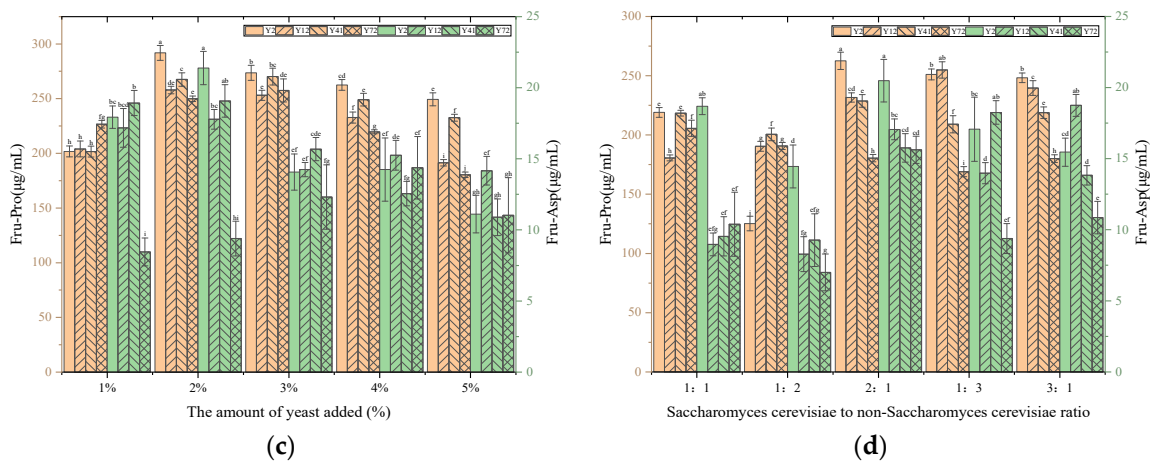


Figure 10. The content of Fru-Pro and Fru-Asp in Msalais fermented by 16 yeasts; (a) Effect of yeast addition on the content of Fru-Pro and Fru-Asp; (b) The content of two Amadori compounds fermented by four yeast strains at different fermentation temperatures; (c) The content of two Amadori compounds fermented by 4 yeast strains at different fermentation time; (d) The content of two Amadori compounds in Msalais fermented by four yeast strains at different ratios of.

3.4.2. Effect of Fermentation Temperature on the Content of Fru-Pro and Fru-Asp

It can be seen from Figure 10 (b) that the content of Amadori compounds increased slowly when the fermentation temperature of 4 strains of yeast and Y4 was 22°C~28°C. When the fermentation temperature was higher than 28°C, the content decreased. The possible reason is that the increase of temperature can accelerate the metabolic rate of yeast, produce too much alcohol and acid[32], and inhibit the formation of Amadori compounds by yeast. At the same time, yeast turns to decompose Amadori compounds after using glucose. Comparing the content of Amadori compounds in four yeast strains at different temperatures, the content of two Amadori compounds in Msalais fermented by Y2 and Y4 at 28°C was the highest. Therefore, Y2 and Y4 were selected as the strains for mixed fermentation, and the fermentation temperature was 28°C.

3.4.3. Effect of Fermentation Time on the Content of Fru-Pro and Fru-Asp

It can be seen from Figure 10 (c) that the content of Amadori compounds increased first and then decreased with the fermentation time. The reason may be that in the early stage of fermentation (0-14 days), the concentration of reducing sugars and amino acids in the raw materials was high, which provided sufficient substrates for the formation of Amadori compounds. At this time, the saccharification and alcoholization of yeasts have not yet been completely dominant. At this stage, yeasts form a large number of Amadori compounds, which accumulate Amadori compounds to the peak. With the prolongation of fermentation time (>14 days), reducing sugars were consumed by yeast, and amino acids were also metabolized by microorganisms or involved in other reactions (such as protein decomposition), resulting in the reduction of raw materials for the synthesis of Amadori compounds[33]. At the same time, the increase of alcohol concentration in the fermentation broth inhibited the formation of Amadori compounds by yeast, which eventually led to a decrease in the content of Amadori compounds. Comparing the content of Amadori compounds of the four yeasts at different fermentation times, Y2 and Y4 had the highest content of two Amadori compounds in the 14d mixed fermentation of Msalais. Therefore, Y2 and Y4 were selected as mixed fermentation strains, and the fermentation time of 14d was the best level.

3.4.4. Effect of the Ratio of *Saccharomyces Cerevisiae* Y4 to Non-*Saccharomyces Cerevisiae* Y2 on the Content of Fru-Pro and Fru-Asp

It can be seen from Figure 10 (d) that the content of Amadori compounds was the highest when the ratio of *Saccharomyces cerevisiae* to non-*Saccharomyces cerevisiae* was 2:1, and the content was

the lowest when the ratio was 1:2. The reason may be that *Saccharomyces cerevisiae* uses reducing sugars (such as glucose, fructose) as the main carbon source, has a fast metabolic rate, and can quickly generate ethanol and CO₂. When the ratio was 2:1, *S.cerevisiae* dominated sugar decomposition, but the presence of non-*S.cerevisiae* could delay substrate depletion[34] and maintain the dynamic balance of sugars and amino acids required for the formation of Amadori compounds. If the proportion of *Saccharomyces cerevisiae* is too high (such as >2:1), excessive substrate consumption will inhibit the formation of Amadori compounds. In addition, non-*Saccharomyces* may decompose complex carbohydrates (such as Polysaccharides) or proteins, release amino acids (such as Gama-aminobutyric acid, Alanine), and provide additional substrates for the Amadori reaction. If the proportion of non-*Saccharomyces cerevisiae* is too high (1:2), its metabolic activity is too strong, which may lead to premature depletion of the substrate and inhibit the formation of Amadori compounds. Comparing the content of two Amadori compounds in Msalais fermented by four yeast strains at different proportions of *Saccharomyces cerevisiae* and non-*Saccharomyces cerevisiae*, the content of two Amadori compounds in Msalais fermented by Y2 and Y4 at a ratio of 2:1 was the highest. Therefore, Y2 and Y4 were selected as strains for mixed fermentation, and the fermentation ratio of 2:1 was the best level.

3.5. Response Surface Optimization Experiment of Fermentation Process

3.5.1. Response Surface Experimental Design and Results

According to the results of the above single factor experiments, four fermentation process single factors (yeast addition, fermentation temperature, fermentation time, ratio of *Saccharomyces cerevisiae* to non-*Saccharomyces cerevisiae*) affecting the content of Fru-Pro and Fru-Asp were selected for response surface design[35]. The results are as follows : Table 14.

Table 14. Response surface experimental design and results.

Serial number	A The ratio of <i>Saccharomyces cerevisiae</i> to non- <i>Saccharomyces cerevisiae</i>	B yeast inoculation amount (%)	C fermentation temperature (°C)	D Fermentation time (all day)	Fru-Pro (g/L)	Fru-Asp (g/L)
1	-1	0	0	-1	0.2351	0.0185
2	-1	0	0	1	0.2342	0.0183
3	1	1	0	0	0.1862	0.0114
4	1	0	0	1	0.2154	0.0175
5	0	1	-1	0	0.1712	0.0078
6	-1	0	1	0	0.1760	0.0083
7	0	0	0	0	0.2389	0.0190
8	1	0	1	0	0.1723	0.0081
9	0	0	0	0	0.2435	0.0191
10	0	0	1	1	0.1818	0.0107
11	0	-1	0	1	0.1918	0.0131
12	1	0	-1	0	0.1708	0.0075
13	0	0	1	-1	0.1599	0.0052
14	0	0	-1	1	0.1603	0.0058
15	0	-1	-1	0	0.1662	0.0063
16	0	0	0	0	0.2438	0.0201
17	-1	-1	0	0	0.1799	0.0088
18	1	0	0	-1	0.2002	0.0144
19	0	1	0	1	0.1896	0.0127
20	0	1	1	0	0.1673	0.0071
21	-1	0	-1	0	0.1946	0.0131

22	-1	1	0	0	0.2198	0.0180
23	1	-1	0	0	0.1845	0.0109
24	0	0	-1	-1	0.2018	0.0163
25	0	-1	0	-1	0.1884	0.0124
26	0	0	0	0	0.2674	0.0213
27	0	-1	1	0	0.1543	0.0045
28	0	0	0	0	0.2980	0.0218
29	0	1	0	-1	0.2307	0.0180

3.5.2. Model Establishment and Significance Analysis

The variance analysis of the content test results of Fru-Pro and Fru-Asp was performed using Design-Expert 13 software. The quadratic polynomial regression equation obtained by is: $Y1(\text{Fru-Pro})=0.2583-0.0092A+0.0083B-0.0044C-0.0036D-0.0095AB+0.0050AC+0.0040AD+0.0020BC-0.0111BD+0.0158CD-0.0219A^2-0.0393B^2-0.0584C^2-0.0194D^2$; $Y2(\text{Fru-Asp})=0.0203-0.0013A+0.0015B-0.0010C-0.0005D-0.0022AB+0.0013AC+0.0008AD+0.0001BC-0.0015BD+0.0040CD-0.0023A^2-0.0052B^2-0.0089C^2-0.0012D^2$. The R^2 values of the two models were 0.9064 and 0.9650, and the R^2_{Adj} values were 0.8128 and 0.9300, respectively. The results show that the fitting degree of the regression equation model is good, and the regression equation is representative. Therefore, the two models can better reflect the relationship between various factors and response values in the fermentation process of Msalais and predict the optimal process conditions[36]. The results of variance analysis of regression model are shown in Table 15 and Table 16.

Table 15. Analysis of variance for Fru-Pro regression model.

Source of variance	Quadratic sum	Degree of freedom	Mean square	F ratio	P Value	Significance
model	0.0321	14	0.0023	9.68	<0.0001	**
A	0.0010	1	0.0010	4.27	0.0578	
B	0.0008	1	0.0008	3.50	0.0826	
C	0.0002	1	0.0002	0.9992	0.3345	
D	0.0002	1	0.0002	0.6503	0.4335	
AB	0.0004	1	0.0004	1.54	0.2351	
AC	0.0001	1	0.0001	0.4263	0.5244	
AD	0.0001	1	0.0001	0.2735	0.6092	
BC	0.0000	1	0.0000	0.0675	0.7988	
BD	0.0005	1	0.0005	2.09	0.1703	
CD	0.0010	1	0.0010	4.24	0.0586	
A ²	0.0031	1	0.0031	13.11	0.0028	**
B ²	0.0100	1	0.0100	42.21	<0.0001	**
C ²	0.0222	1	0.0222	93.51	<0.0001	**
D ²	0.0024	1	0.0024	10.26	0.0064	**
Residual	0.0033	14	0.0002			
Lack of Fit	0.0009	10	0.0001	0.1384	0.9947	
Pure Error	0.0025	4	0.0006			
Cor Total	0.0354	28				
R ²	0.9064					
R ² _{Adj}	0.8128					

Note : “*” indicates significant difference (P<0.05) ; “***” indicated that the difference was extremely significant (P<0.01).(Table 16 is the same).

Table 16. Analysis of variance for Fru-Asp regression model.

Source of variance	Quadratic sum	degree of freedom	mean square	f ratio	P Value	significance
model	0.0008	14	0.0001	27.56	< 0.0001	**
A	0	1	0	9.4	0.0084	**
B	0	1	0	13.83	0.0023	**
C	0	1	0	6.11	0.0268	*
D	3.52E-06	1	3.52E-06	1.76	0.2053	
AB	0	1	0	9.48	0.0082	**
AC	6.76E-06	1	6.76E-06	3.39	0.087	
AD	2.72E-06	1	2.72E-06	1.36	0.2623	
BC	6.25E-08	1	6.25E-08	0.0313	0.8621	
BD	8.41E-06	1	8.41E-06	4.21	0.0593	*
CD	0.0001	1	0.0001	32.07	< 0.0001	**
A ²	0	1	0	16.59	0.0011	*
B ²	0.0002	1	0.0002	86.52	< 0.0001	**
C ²	0.0005	1	0.0005	260.18	< 0.0001	**
D ²	0	1	0	5.05	0.0412	*
Residual	0	14	2.00E-06			
Lack of Fit	0	10	2.15E-06	1.34	0.4169	
Pure Error	6.41E-06	4	1.60E-06			
Cor Total	0.0008	28				
R ²	0.9650					
R ² Adj	0.9300					

From the results of Table15 and Table 16, it can be seen that the F value of the Fru-Pro content model is 9.68, and the P value is less than 0.0001. The F value of the Fru-Asp content model is 27.56, and the P value is also less than 0.0001. The two models are highly significant on the surface, so the validity of the model can be confirmed. At the same time, the values of the missing fit terms were 0.1384 and 1.34, respectively, and the P value was greater than 0.05, which was not significant, which means that the influence of unknown factors on the test was relatively small[37].

The quadratic terms A², B², C² and D² all had extremely significant effects on the content of Fru-Pro (P< 0.01), while the first term A and B, interaction AB and CD and quadratic terms B² and C² had extremely significant effects on the content of Fru-Asp, and the first term C and quadratic terms A² and D² showed significant effects (P<0.05). Among them, the quadratic term C² is the most influential factor in both models. It can be seen from the F value that the order of influence of the four factors on the Fru-Pro content is : A>B>C>D, that is, the proportion of *Saccharomyces cerevisiae* to non-*Saccharomyces cerevisiae* > yeast addition > fermentation temperature > fermentation time. The order of influence on the Fru-Asp content is B>A>C>D, that is, yeast addition > *Saccharomyces cerevisiae* to non-*Saccharomyces cerevisiae* ratio > fermentation temperature > fermentation time[38].(Modify printing errors)

3.5.3. Response Surface Analysis

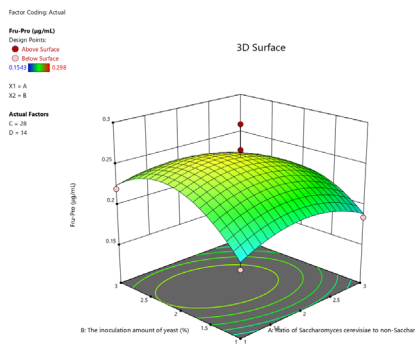
In order to explore the interaction of the four factors and the conditions for the highest content of Amadori compounds, according to the regression equation and variance analysis table, the response surface analysis diagram and contour map of each factor were drawn by Design-Expert 13 software.

The effects of four factors on the content of Fru-ProThe response surface analysis is as follows : Figure 11 (a) shows that the slope of the response surface is gentle, and the contour is elliptical. When the proportion of *Saccharomyces cerevisiae* and non-*Saccharomyces cerevisiae* is low or high, the content of Fru-Pro increases first and then decreases with the increase of yeast addition, indicating

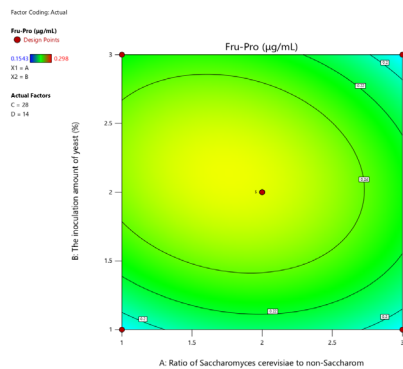
that the interaction between the two is obvious. In Figure 11 (b), the slope of the response surface was steep, and the contour line was oval. When the ratio of *Saccharomyces cerevisiae* to non-*Saccharomyces cerevisiae* was low or high, the content of Fru-Pro increased first and then decreased with the increase of fermentation temperature, indicating that the interaction between the two was significant. In Figure 11 (c), the slope of the response surface was relatively flat, and the contour line was approximately circular. When the ratio of *Saccharomyces cerevisiae* to non-*Saccharomyces cerevisiae* was different, the content of Fru-Pro increased first and then decreased with the prolongation of fermentation time, indicating that the interaction between the two was not significant. In Figure 11 (d), the slope of the response surface was steep, and the contour line was approximately circular. When the yeast addition amount was low or high, the content of Fru-Pro increased first and then decreased with the increase of fermentation temperature, indicating that the interaction between the two was not significant. In Figure 11 (e), the slope of the response surface was gentle, and the contour was elliptical. When the amount of yeast added was different, the content of Fru-Pro increased first and then decreased with the increase of fermentation time, indicating that the interaction between the two was not significant. In Figure 11 (f), the slope of the response surface was steep, and the contour line was oval. When the fermentation temperature was low or high, the content of Fru-Pro increased first and then decreased with the increase of fermentation time, indicating that the interaction between the two was significant.

Figure 11 (g) showed that the slope of the response surface was gentle, and the contour line was oval. When the ratio of *Saccharomyces cerevisiae* to non-*Saccharomyces cerevisiae* was low or high, the content of Fru-Asp increased first and then decreased with the increase of yeast addition, indicating that the interaction between the two was significant. In Figure 11 (h), the slope of the response surface was steep, and the contour line was oval. When the ratio of *Saccharomyces cerevisiae* to non-*Saccharomyces cerevisiae* was low or high, the content of Fru-Asp increased first and then decreased with the increase of fermentation temperature, indicating that the interaction between the two was significant. In Figure 11 (i), the slope of the response surface was relatively flat, and the contour line was approximately circular. When the ratio of *Saccharomyces cerevisiae* to non-*Saccharomyces cerevisiae* was different, the content of Fru-Asp increased first and then decreased with the prolongation of fermentation time, indicating that the interaction between the two was not significant. In Figure 11 (j), the slope of the response surface was steep, and the contour line was approximately circular. When the yeast addition amount was low or high, the content of Fru-Asp increased first and then decreased with the increase of fermentation temperature, indicating that the interaction between the two was not significant. In Figure 11 (k), the slope of the response surface was gentle, and the contour was elliptical. When the amount of yeast added was different, the content of Fru-Asp increased first and then decreased with the increase of fermentation time, indicating that the interaction between the two was significant. In Figure 11 (m), the slope of the response surface was steep, and the contour line was oval. When the fermentation temperature was low or high, the content of Fru-Asp increased first and then decreased with the increase of fermentation time, indicating that the interaction between the two was significant.

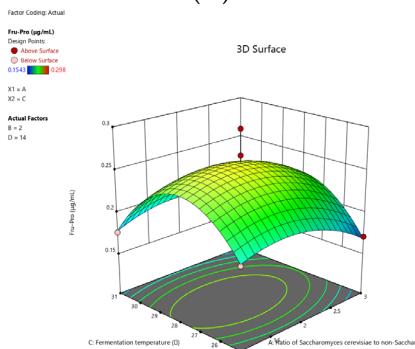
The most significant interaction was fermentation temperature and time (Figure 11 f, m), followed by yeast ratio and temperature (Figure 11 b, h). When optimizing the process, the coupling conditions of temperature (28°C) and time (14 days) should be determined first by response surface method, and the suboptimal parameters of ratio (2:1) and addition amount (2%) should be combined to maximize the content of Fru-Pro and Fru-Asp.



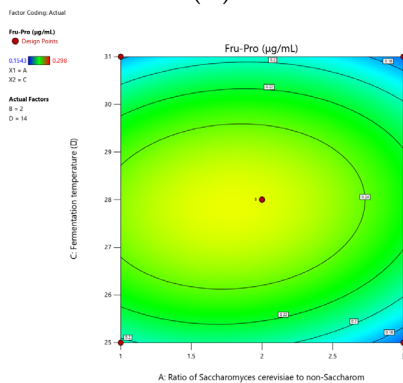
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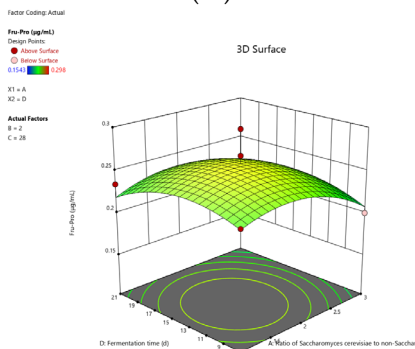
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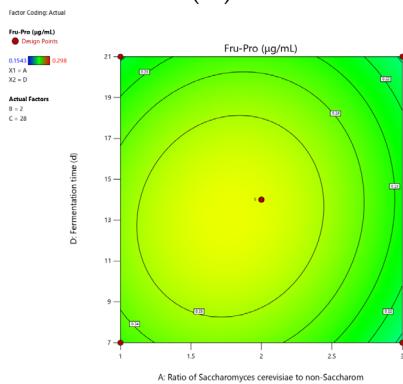
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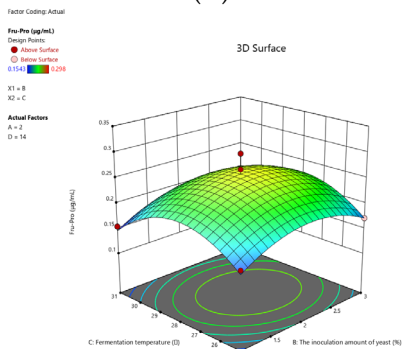
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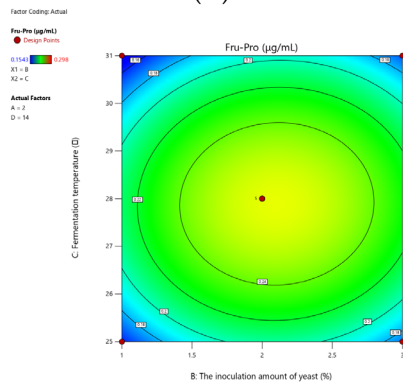
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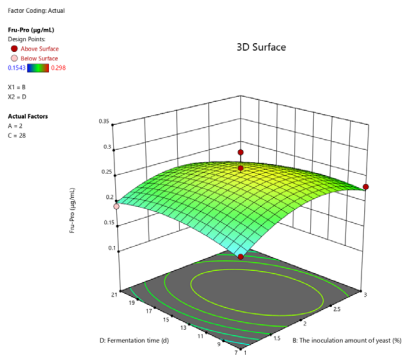
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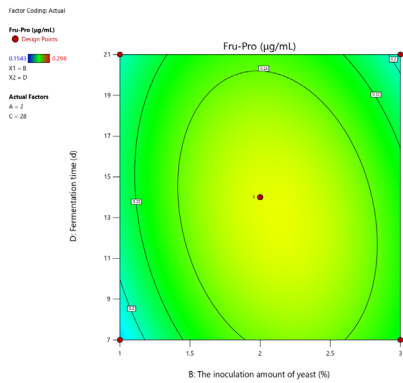
(d1)



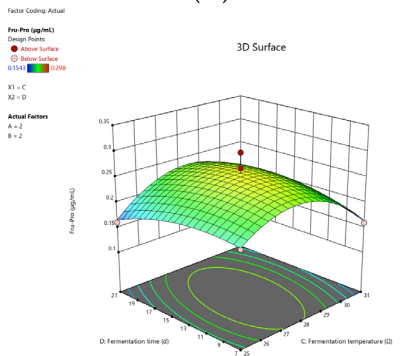
(d2)



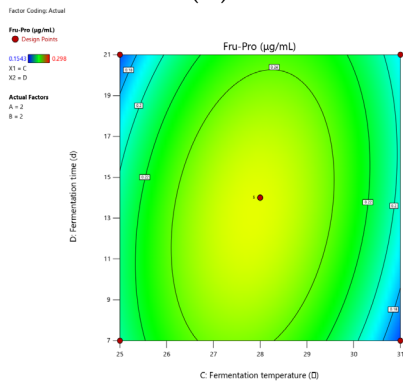
(e1)



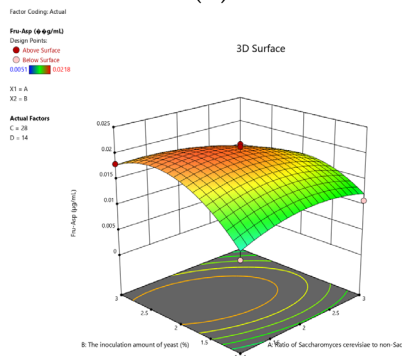
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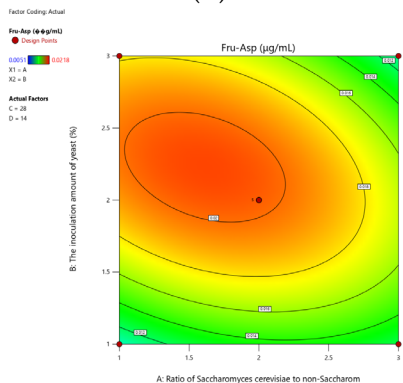
(f1)



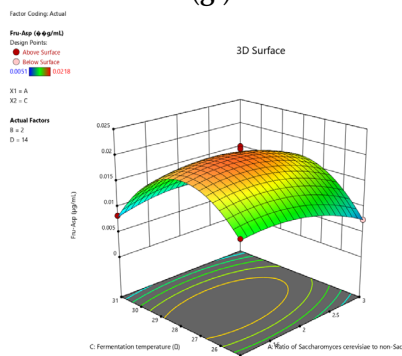
(f2)



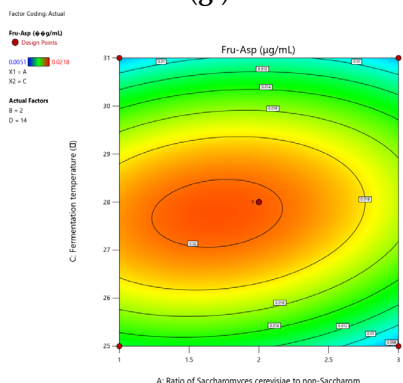
(g1)



(g2)



(h1)



(h2)

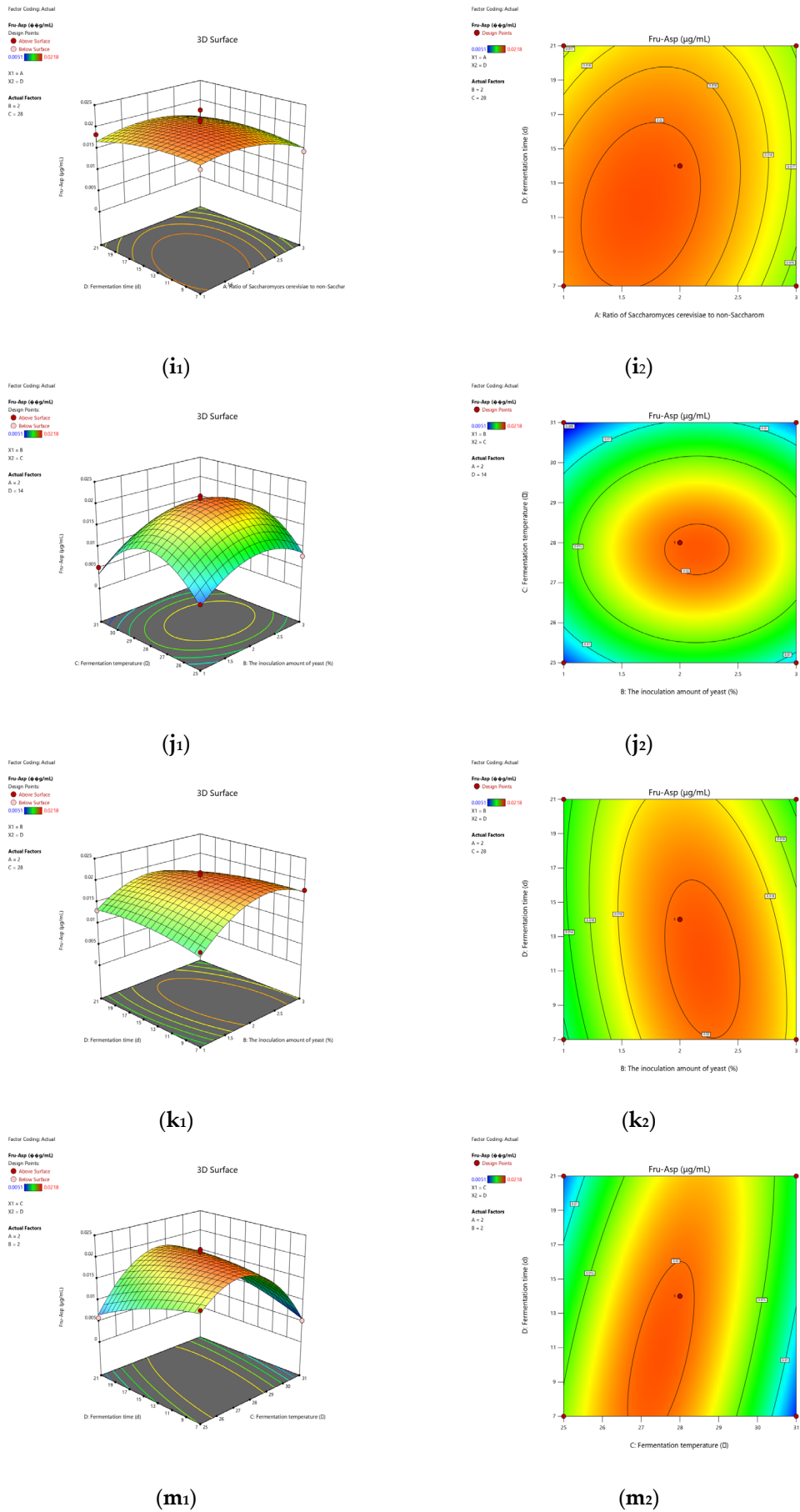


Figure 11. The response surface diagram and contour map of the interaction between the four factors on the content of Fru-Pro and Fru-Asp.

3.5.4. Experimental Results of Optimal Process Conditions

The experimental data were optimized and predicted by Design-Expert 13 software[35]. The fermentation process with the highest content of Amadori compounds was determined as the ratio of *Saccharomyces cerevisiae* to non-*Saccharomyces cerevisiae* 2:1, yeast addition amount 2%, fermentation temperature 28°C, fermentation time 14d. Under these conditions, the content of Fru-Pro was 0.2980g/L, and the content of Fru-Asp was 0.0196g/L.

3.6. Antioxidant Activity

3.6.1. DPPH Free Radical Scavenging Ability and ABTS Free Radical Scavenging Ability

A series of standard solutions with gradient changes in the concentration range of 0-40 µg / mL were prepared with Trolox as the reference standard substance, and their absorbance values were measured and linear regression equations were established[25]. The linear regression equation between DPPH free radical scavenging rate (%) and Trolox concentration (µg/mL) was $y = 2.3538x - 0.4904$, and the correlation coefficient $R^2 = 0.9993$. The linear regression equation between ABTS radical scavenging rate (%) and Trolox concentration (µg/mL) was $y = 2.1485x - 0.382$, and the correlation coefficient $R^2 = 0.9994$. The standard curve is shown in Figure 11. The DPPH and ABTS free radical scavenging abilities were expressed by Trolox equivalent[39]. The DPPH free radical scavenging rates of sample A, sample B and sample C were 116.37 ± 1.79 µmol Trolox/sample, 63.36 ± 1.20 µmol Trolox/L sample and 106.46 ± 3.31 µmol Trolox/L sample, respectively. Compared with sample B and sample C, the DPPH free radical scavenging ability of sample A increased by 53.01 µmol Trolox/L sample and 9.91 µmol Trolox/L sample, respectively. The ABTS free radical scavenging rates were 142.51 ± 1.98 µmol Trolox / sample, 74.28 ± 2.11 µmol Trolox/L sample, and 121.22 ± 1.93 µmol Trolox/L sample, respectively. Compared with sample B and sample C, the ABTS free radical scavenging ability of sample A increased by 68.23 µmol Trolox/L sample and 21.29 µmol Trolox/L sample, respectively. During the boiling process of grape juice, as the boiling time becomes longer, the content of vitamin C, reducing sugar, soluble protein, flavonoids and other substances in grape juice will be significantly reduced[40]. Vitamin C and flavonoids are easily oxidized and decomposed, and the content will decrease significantly during heating[41]. The content of reducing sugar will increase first and then decrease. The reason may be that heat treatment will lead to the precipitation of soluble sugar in grape juice, and polysaccharides will also hydrolyze into monosaccharides, thereby increasing the content of reducing sugar in grape juice. Subsequently, reducing sugar is added to the Maillard reaction, and the reaction rate is higher than the formation rate of reducing sugar, resulting in a decrease in reducing sugar content[42]. Protein will be hydrolyzed into peptide chains and free amino acids due to heat treatment, resulting in a decrease in its content[43]. At the same time, the generated free amino acids will undergo Maillard reaction with reducing sugars. Therefore, it can be inferred that the above substances are not the factors that enhance the antioxidant capacity of Msalais, and the significant increase in the content of Amadori compounds during cooking can be used as the main factor for the improvement of antioxidant capacity.

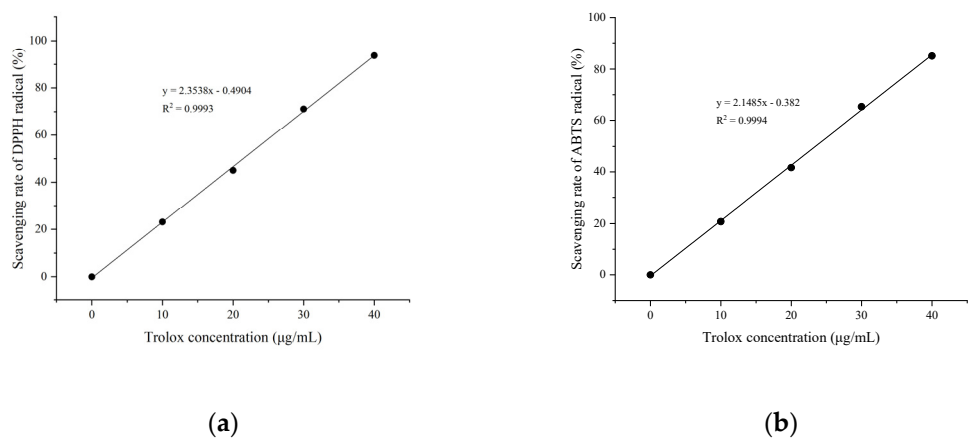


Figure 12. Trolox standard curve of DPPH free radical scavenging and ABTS free radical scavenging.

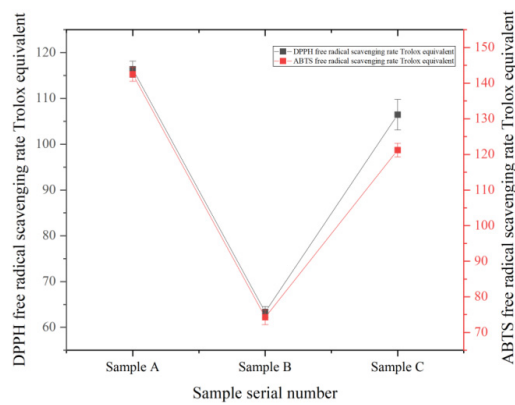


Figure 13. DPPH free radical scavenging ability of three different samples.

3.6.2. Determination of Total Oxygen Free Radical Reduction Capacity (ORAC)

A series of standard solutions with gradient changes in the concentration range of 10-50µg/mL were prepared with Trolox as the reference standard substance[44]. The absorbance values of 35 cycles were measured and the ΔAUC was calculated according to the formula to establish a linear regression equation. The linear regression equation between ΔAUC and Trolox concentration (µg/mL) was $y = 0.1925x - 0.5923$; $R^2 = 0.9997$. The total oxygen radical reduction ability of sample A, sample B and sample C were $132.74 \pm 6.36 \mu\text{mol Trolox/L sample}$, $72.62 \pm 8.19 \mu\text{mol Trolox/L sample}$, $100.29 \pm 9.38 \mu\text{mol Trolox/sample}$. Compared with sample B and sample C, the total oxygen free radical reduction ability of sample A increased by $60.12 \mu\text{mol Trolox/L sample}$ and $32.45 \mu\text{mol Trolox/L sample}$, respectively.

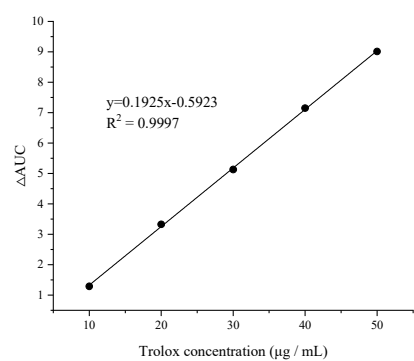


Figure 14. The Trolox standard curve of ΔAUC.

Table 17. ORAC of 3 different samples.

Name	AUC	ΔAUC	Trolox equivalent
Trolox concentration 10μg/mL	84.83	1.29	\
Trolox concentration 20μg/mL	86.36	3.33	\
Trolox concentration 30μg/mL	87.74	5.13	\
Trolox concentration 40μg/mL	89.08	7.15	\
Trolox concentration 50μg/mL	90.74	9.01	\
Sample A	87.29±0.31	5.80±0.31	132.74±6.36
SampleB	84.40±0.39	2.91±0.39	72.62±8.19
SampleC	85.73±0.45	4.24±0.45	100.29±9.38
Blank sample	81.49	\	\

4. Conclusion

Two Amadori compounds were detected by HPLC-ELSD (high performance liquid chromatography-evaporative light detector), One strain of *Saccharomyces cerevisiae* and one strain of non-*Saccharomyces cerevisiae* were screened out, which could increase the content of Amadori compounds by fermentation. The fermentation process was optimized by single factor experiment and response surface optimization experiment to develop Msalais rich in Amadori compounds. The optimum fermentation process was obtained as follows : fermentation temperature 28°C, fermentation time 14d, ratio of *Saccharomyces cerevisiae* Y4 to non-*Saccharomyces cerevisiae* Y2 2:1, yeast inoculation amount 2% (V/V). The content of Fru-Pro and Fro-Asp was 0.2867±0.0115g/L and 0.0203±0.0014g/L, respectively, which was higher than that of Fru-Pro (0.2165±0.0022g/L) and Fro-Asp (0.0177±0.0008g/L) of Msalais Amadori from Miandu distillery. Li[45] optimized the production process of pear paste, and the content of Amadori compounds could be increased by prolonging the heating time. The content of Fru-Asp in the self-made pear paste was 885.99±5.19 mg/g. Compared with the commercial pear paste on the market, its content increased by 14.79% ; the content of Fru-Pro detected in commercial pear paste was 445.20±3.46 mg/g. The results showed that optimizing the processing technology of the product could effectively increase the content of Amadori compounds and improve the quality of the product. According to the comparison of the antioxidant activity of the self-made Msalais and the commercial Msalais from Miandu Distillery, the DPPH scavenging ability, ABTS scavenging ability and ORAC Trolox equivalent of the self-made Msalais were 116.37±1.79 μmol Trolox/L sample, 142.51±1.98 μmol Trolox/sample, 132.74±6.36 μmol Trolox/sample, respectively. Compared with Msalais, the antioxidant activity of Msalais increased by

45.55%, 47.88% and 45.29% respectively. The traditional fermentation method of Msalais is natural fermentation. Natural fermentation is a complex process involving the interaction of multiple microorganisms, which has instability and potential health risks. The yeasts screened in this study can increase the content of Amadori compounds in Msalais to improve the quality of Msalais. Compared with natural fermentation, the fermentation process is more convenient to control and eliminate potential health risks. Therefore, the fermentation of Msalais by specific yeasts has great application prospects. The results of this study can also provide a theoretical basis for the development of new products. Although this study has preliminarily constructed the optimization system of Msalais process and realized the directional enrichment of target components, there are still many directions worthy of further exploration: Only two Amadori compounds, Fru-Pro and Fru-Asp, were analyzed. Other Amadori compounds and other Maillard reaction products were not analyzed. The research has limitations. In the future, non-targeted methods (such as HPLC-MS and NMR) can be used for more comprehensive analysis of Amadori compounds, as well as exploring potential volatile components and toxic by-products. The regulation mechanism of key enzymes (such as aldose reductase, glycosyltransferase, etc.) on the formation of Amadori compounds in the dynamic process of Maillard reaction can be further analyzed by molecular biology methods; through in vitro antioxidant/anti-inflammatory activity evaluation and animal model experiments, the physiological effects of Amadori compounds with high content in Msalais were clarified. The continuous boiling equipment and intelligent fermentation control system can be explored to promote the transformation of laboratory results into industrial production. Combined with metabolomics and sensory omics technology, the correlation model between Amadori compounds and Msalais flavor quality was established to achieve accurate regulation of product quality.

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