

Original Research Paper

# Isolation and Screening of Wine Yeasts from Grapes in Yalu River Valley China for Fermentation Performance

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**Abstract:** In order to further explore the diversity of yeast in grape brewing in Yalu River Valley, northern China and to better exploit and utilize the yeast resources in a grape producing area, we investigated soil, grape residue after juice extraction and naturally fermented grape juice samples collected from grape plantations in the Yalu River Valley in this study. Twelve yeast strains were identified using colony and cell morphology and molecular biology. Three *Saccharomyces cerevisiae* strains from different sources, EN2, BZ1 and EZ2, were selected to determine their acid, Sodium dioxide (SO<sub>2</sub>), glucose and ethanol tolerance. The results showed that EN2 exhibited significant acid and SO<sub>2</sub> tolerance, while BZ1 and EZ2 exhibited significant glucose and ethanol tolerance. These three *S. cerevisiae* strains showed good growth and fermentation characteristics and could be used as development strains for brewing wine. Results of this study could be useful in breeding, wine yeast-strain research and wine product development.

**Keywords:** Wine, Yeast Strain, Acid Tolerance, Alcohol Tolerance, Ice Wine, Regional Characteristics

## Introduction

Yeasts are important microorganisms in wine brewing that convert the sugar in grape juice to ethanol, carbon dioxide and other flavor-producing substances, such as fusels, aldehydes, acids and esters (Romano *et al.*, 2008; Vernocchi *et al.*, 2011). The yeast metabolites in wine fermentation significantly affect the flavor components, color and taste of wines; therefore, the types and characteristics of various yeast strains are important in wine production (Moreira *et al.*, 2008; Ye *et al.*, 2013; Lleixà *et al.*, 2016). Choosing a suitable yeast with good fermentation performance for winemaking ensures the proper development of unique qualities and characteristics of grape raw materials. Current research primarily focuses on the screening and use of yeasts from various wine-producing areas (Heard, 1999; Nikolaou *et al.*, 2006; Martínez *et al.*, 2007). Compared with commercial active dry *Saccharomyces cerevisiae*, fermentation using local *S. cerevisiae* strains from grape-producing areas is more conducive to the formation and development of varieties and styles of regional wines and in overcoming the wine-homogenization phenomenon. In addition, studies have shown that non-*S. cerevisiae* participate in wine fermentation and can produce some aromatic components to enhance the aroma and positively affect

the taste and quality of wine (Čuš and Jenko, 2013; Dashko *et al.*, 2015; Maturano *et al.*, 2015).

It is difficult for commercial yeasts to effectively represent the flavor of wine from different regions, especially ice wine, which can limit the production and development of wine from different regions to a certain extent. The Yalu River Valley, for example Beibinghong, is rich in raw grapes for ice wine brewing. Strict requirements are recommended for *S. cerevisiae* when brewing ice wine due to the high sugar and high acid within the raw materials and the low temperature environment during the fermentation process. Therefore, it is necessary to develop new *S. cerevisiae* according to the characteristics of the ice grape (Shen *et al.*, 2020). The Yalu River Valley is located at 41° N latitude in northern China, the same latitude as the Rhone Valley in Bordeaux, France and the Napa Valley in California, United States; it is the “golden latitude” for grape cultivation in China (Liu *et al.*, 2017). It is also an important location for ice wine. The superior geographical environment of the Yalu River Valley provides unique natural conditions for the growth of grapes and suitable conditions for screening and acquisition of high-quality yeast germplasm resources. In this study, soil, grape residue after juice extraction and naturally fermented grape juice samples were collected from grape plantations in the Yalu River Basin. A

preliminary study was conducted on the fermentation performance of the isolated and identified yeast strains.

## Materials and Methods

### Materials

#### Media and Chemicals

Yeast extract Peptone Dextrose (YPD) containing 2% glucose, 2% peptone, 1% yeast extract and 2% agar sterilized at 121°C for 20 min was used for the yeast screening and slant media (Nevoigt *et al.*, 2000).

The yeast identification medium was Wallerstein Laboratory (WL) agar medium containing 0.5% yeast extract, 0.5% tryptone, 5% glucose, 2% agar, 0.055% potassium dihydrogen phosphate, 0.0425% potassium chloride, 0.0125% calcium chloride, 0.00025% ferric chloride, 0.0125% magnesium sulfate, 0.00025% manganese sulfate and 0.0022% bromocresol green, pH 6.5 sterilized at 121°C for 20 min (Di Maio *et al.*, 2011).

The basic yeast tolerance medium contained 2% glucose, 2% peptone and 1% yeast extract sterilized at 121°C for 20 min.

The agarose gel was obtained from Biowest Technology Co., Ltd. The Taq DNA polymerase and dNTP were purchased from TransGen Biotech Co., Ltd. The DNA extraction and agarose-gel DNA recovery kits were purchased from TianGen Biotech Co., Ltd. The standard molecular weight DNA marker DL-3000 was purchased from Solarbio Science and Technology Co., Ltd. The methylene blue, glucose, peptone, yeast extract and agar were analytically pure reagents purchased from Sinopharm Chemical Reagent Co., Ltd.

#### Soil and Yeast Isolate Samples

The soil samples were collected from the Beibinghong grape plantation in the Yalu River Valley in northern China. The grape residue samples after juicing and the naturally fermented grape juice samples were obtained from Beibinghong, Shuanghong and Gongniang No.1 grapes.

### Methods

#### Isolation and Screening of Yeast Strains from Plantation Soil Samples

One-gram soil samples were aseptically weighed and placed in a flask with 100 mL sterile water, in a constant-temperature incubator (MJ-70-I, Yiheng Shanghai, China) and oscillated at 28°C for 30 min. One milliliter of the mixture was added to 9 mL sterile water for a 10<sup>-7</sup> gradient dilution, from which 0.1 mL aliquots of varying concentrations were added to YPD medium and cultivated at 28°C for 2-3 days. Colonies with different morphologies were inoculated onto the media using the continuous streaking method and slant-stored at 4°C.

#### Yeast Isolation from Grape Residue After Juicing

After juicing, approximately one-gram samples of grape residue were aseptically weighed and the yeast isolates were used to inoculate the soil samples. Morphologically different colonies were inoculated onto media plates using the continuous streaking method, purified and slant-stored at 4°C.

#### Isolation with Yeast from Naturally Fermented Grape Juice

One milliliter of grape naturally fermentation juice was added to 9 mL sterile water for gradient dilution to 10<sup>-7</sup>, from which 0.1 mL aliquots of varying concentrations were added to YPD medium and cultivated at 28°C for 2-3 days. Morphologically different colonies were inoculated onto media plates using the continuous streaking method, purified and slant-stored at 4°C.

#### Yeast Strain Screening on WL Sedium

The strains obtained from the preliminary screening were inoculated onto WL medium using the continuous streaking method and cultured at 28°C for 3-7 days. The colony color and morphology were observed and recorded.

#### Microscopic Observation of Yeast Strains

A light microscope (CX33, Olympus, Japan) was used to screen the yeast strains. A small amount of yeast was mixed with 0.1% methylene blue staining solution to stain the yeast cells; after 2-3 min of staining, the cell morphology was observed under microscope with 16×40 times.

#### Molecular Biological Identification of Yeast

The yeast strain genomic DNA was extracted as a template using a DNA extraction kit. The forward primer NLI (5'-GCATATCAATAAGCGGAGGAAAAG-3') and reverse primer NL4 (5'-GGTCCGTGTTTCAAGACGG-3') were used to amplify the yeast 26S rDNA D1/D2 region. The PCR-amplified fragments were sequenced by Shenggong Bioengineering Co., Ltd (Shanghai) and the sequences were compared with the GenBank database using BLAST software.

#### Determination of *S. cerevisiae* Strain Fermentation Performance and Acid Tolerance

Based on the results of strain identification, combined with the pretests and references, three *S. cerevisiae* strains, BZ1, EN2 and EZ2, from different sources were selected for fermentation performance tolerance testing (Pallmann *et al.*, 2001; Zhang *et al.*, 2016). The three strains were inoculated onto YPD liquid media with pH values of 2.0, 2.5, 3.0, 3.5 and 4.0 and 2% inoculum volume. The samples were incubated at 28°C for 24 h and

the absorbance value was measured at 600 nm wavelength using a spectrophotometer (7200, Unico, China).

#### *Determination of S. cerevisiae Sodium Dioxide Tolerance*

Sodium bisulfite (NaHSO<sub>3</sub>) was added to five YPD liquid media to bring the Sodium dioxide (SO<sub>2</sub>) concentrations to 100, 150, 200, 250 and 300 mg/L. The activated yeast strains BZ1, EN2 and EZ2, were incubated with a 2% inoculum, cultured at 28°C for 24 h and the absorbance was determined.

#### *Determination of S. cerevisiae Strain Sugar Tolerance*

YPD liquid media containing 10, 15, 20, 25, 30 and 35% glucose were prepared. The activated strains BZ1, EN2 and EZ2, were inoculated with a 2% inoculum. The absorbance was measured after culturing for 24 h.

#### *Determination of S. cerevisiae Alcohol Tolerance*

YPD liquid media with ethanol concentrations of 10, 12, 14, 16 and 18% (v/v) were prepared. The activated strains BZ1, EN2 and EZ2, were inoculated with a 2% inoculum volume. The samples were incubated at 28°C for 24 h and the absorbance value was measured.

## Results

### *Yeast Strain Screening*

In this study, 23 yeast strains were isolated from various Yalu River Valley grape plantation samples and screened. Four strains were isolated from soil samples, nine from the grape residue after juice extraction and ten strains from naturally fermented grape juice. The sources and strain numbers of the isolated strains are listed in Table 1.

### *Identification of Yeast Strains on WL Medium*

The 23 yeast strains were inoculated onto WL medium and cultured at 28°C for 4 days, of which 12 conformed to the colony morphology of yeast on WL medium. The morphological characteristics of 12 strains are shown in Fig. 1. One strain was isolated from a soil sample, six from the grape residue after juice extraction and five from naturally fermented grape juice. The yeast strain number, sample source and basic strain morphology are shown in Table 2.

### *Microscopic Observation of Yeast*

The 12 yeast strains were examined under a microscope and identified by the morphological

characteristics of their WL-medium colonies. The yeast cells were approximately spherical or oval-shaped, without pseudohyphae or spores. The morphology showed single, opposite, or group, single-terminal or double terminal budding. The microscopic morphology of the strains was consistent with yeast morphological characteristics (Garde-Cerdán *et al.*, 2007).

### *26S rDNA Identification of Yeast Strains*

The D1/D2 region of the yeast strain 26S rDNA was amplified by PCR and the homology of the gene sequences of each strain was compared with the GenBank database. The results showed that among the 12 strains, there were 10 *S. cerevisiae*, 1 *Meyerozyma guilliermondii* and 1 *Pichia membranifaciens*. The homology was 100 or 99%. The results of the 26S rDNA yeast strain identification are shown in Table 3.

### *S. cerevisiae Strain Acid Tolerance*

The acid tolerance of three *S. cerevisiae* strains, BZ1, EN2 and EZ2, was measured by inoculation onto YPD media with various pH levels. The results presented in Fig. 2 show that the growth of EN2 at pH 2.5 was close to that at pH 3 and slightly lower than at pH 4, while that of BZ1 and EZ2 were significantly affected by low pH. The results showed that EN2 exhibited significant acid resistance.

### *S. cerevisiae SO<sub>2</sub> Tolerance*

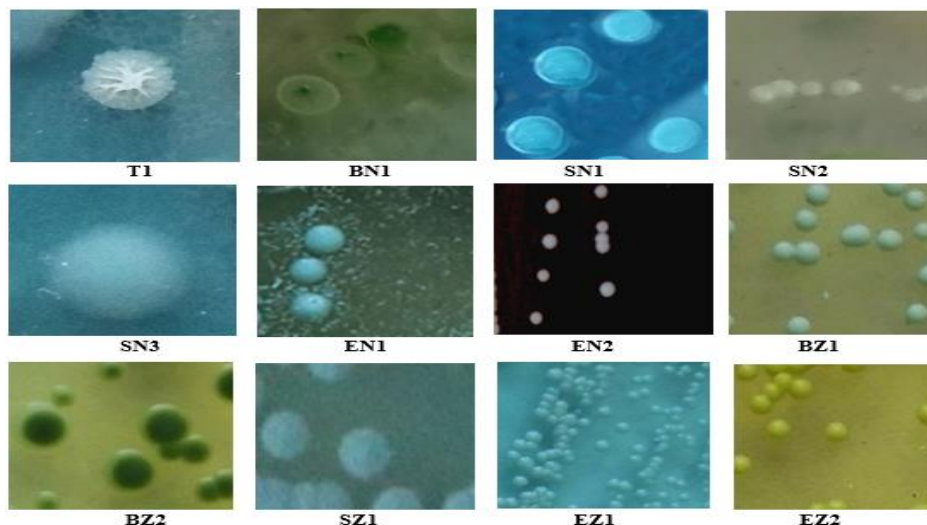
The results of the SO<sub>2</sub> tolerance test on the three strains are shown in Fig. 3. It is necessary to add an appropriate amount of SO<sub>2</sub> during wine production to inhibit harmful microorganisms, antioxidant activity and color changes (Maragatham and Panneerselvam, 2011). The ideal wine-making yeast ferments normally at a specific SO<sub>2</sub> concentration. Fig. 3 shows that when the media SO<sub>2</sub> concentration was 250 mg/L, EN2 growth was normal, while BZ1 and EZ2 decreased, indicating that EN2 possessed significant SO<sub>2</sub> tolerance.

### *S. cerevisiae Sugar Tolerance*

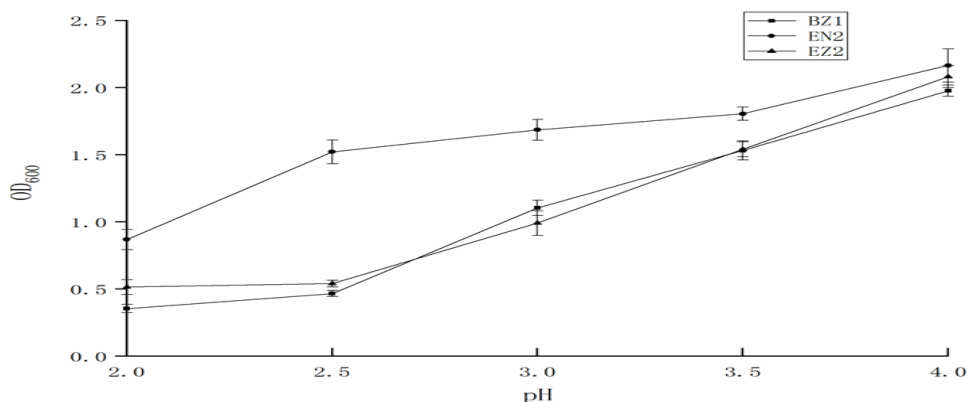
The glucose tolerance of the various *S. cerevisiae* strains is shown in Fig. 4. Increased media glucose concentration inhibited EN2 and EZ2 growth and at 30%, their growth was significantly inhibited, while that of BZ1 decreased slightly.

### *S. cerevisiae Alcohol Tolerance*

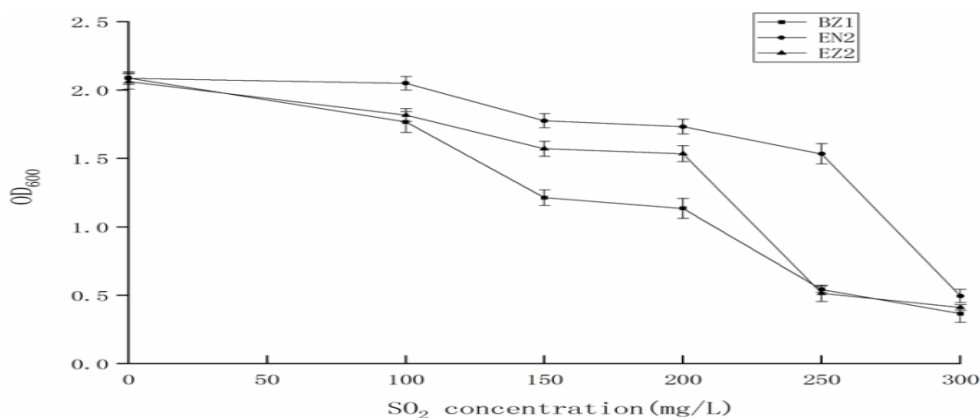
The results of the alcohol-tolerance tests on three *S. cerevisiae* strains are shown in Fig. 5. The growth of EN2 and BZ1 was significantly affected by increased media alcohol content, while EZ2 maintained normal growth at 16% alcohol concentration.



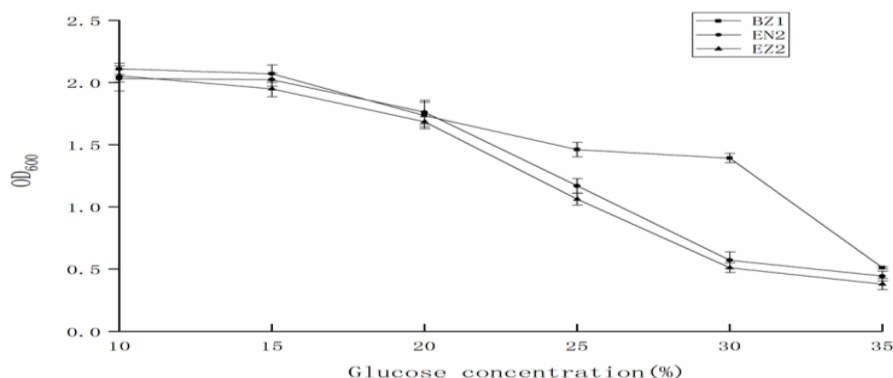
**Fig. 1:** Morphology of yeasts from various sources strain T1 was obtained from plantation soil samples and was not *S. cerevisiae*. Strains BN1, SN1, SN2, SN3, EN1 and EN2 were obtained from the residue after juicing samples of Beibinghong, Shuanghong and Gongniang No.1, respectively. Strains BZ1, BZ2, SZ1, EZ1 and EZ2 were obtained from the natural fermented grape juice samples of Beibinghong, Shuanghong and Gongniang No.1, respectively and SZ1 was not *S. cerevisiae*



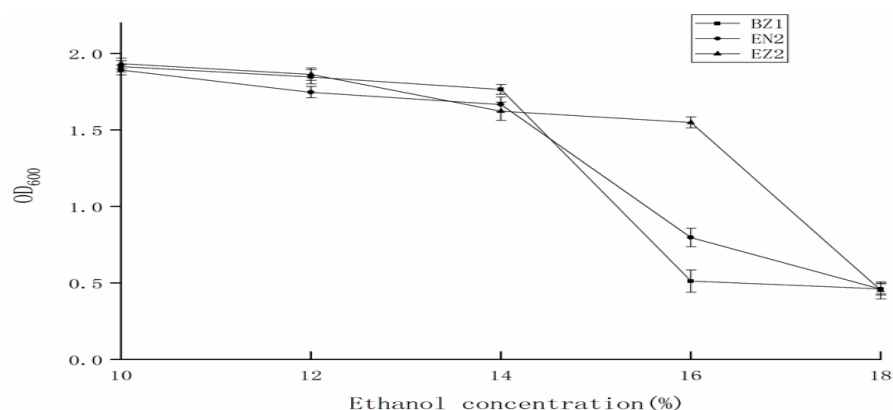
**Fig. 2:** *S. cerevisiae* strain acid tolerance the growth status of the three strains was different under variable acidity conditions and the strain EN2 could maintain good growth status under higher acidity conditions



**Fig. 3:** *S. cerevisiae* strain sodium dioxide tolerance during the process of wine brewing, the addition of SO<sub>2</sub> affects the growth of yeast. When the addition of SO<sub>2</sub> was 250 mg/L, the growth of strain EN2 was good. When the addition of SO<sub>2</sub> was 300 mg/L, the three yeast strains could not grow normally



**Fig. 4:** *S. cerevisiae* strain glucose tolerance a high concentration of sugar can inhibit the growth of *S. cerevisiae* and affect the fermentation process. When the concentration of glucose reached 30%, the growth of strain EN2 and EZ2 was inhibited, while strain BZ1 screened from Beibinghong juice maintained normal growth



**Fig. 5:** *S. cerevisiae* strain alcohol tolerance the tolerance of *S. cerevisiae* to alcohol directly affects its fermentation ability. Strains BZ1, EN2 and EZ2 could grow in the medium with 14% alcohol concentration. Strain EZ2 could still maintain growth when the alcohol concentration was 16%

**Table 1:** Yeast strains isolated from various sources

Sample Source		
Grape variety	Sample type	Strain number
-	Soil	T1, T2, TH1, TX2
Beibinghong	Grape residue after juice extraction	BN1, BN2
Shuanghong	Grape residue after juice extraction	SN1, SN2, SN3, SN4,
Gongniang No.1	Grape residue after juice extraction	EN1, EN2, EN3
Beibinghong	Naturally fermented grape juice	BZ1, BZ2, BZ3, BZ4
Shuanghong	Naturally fermented grape juice	SZ1, SZ2, SZ3
Gongniang No.1	Naturally fermented grape juice	EZ1, EZ2, EZ3

**Table 2:** Colony morphology of yeast strains isolated from different sources

Strain number	Sample source	Strain morphology
T1	soil	Milky white, convex at center, dry, irregular edges
BN1	Beibinghong grape residue after juice extraction	Dark green, convex in center, neat edges
SN1	Shuanghong grape residue after juice extraction	White, creamy, neat edges
SN2	Shuanghong grape residue after juice extraction	White, creamy, raised surface, neat edges
SN3	Shuanghong grape residue after juice extraction	White, dry, irregular edges
EN1	Gongning No.1 grape residue after juice extraction	Milky white, creamy, with raised surface and neat edges
EN2	Gongning No.1 grape residue after juice extraction	White with neat edges
BZ1	Beibinghong grape naturally fermented juice	White, raised surface, creamy, neat edges
BZ2	Beibinghong grape naturally fermented juice	Dark green, raised surface, creamy, neat edges
SZ1	Shuanghong grape naturally fermented juice	White, the surface is annular convex, dry, the edge is powdery
EZ1	Gongniang No.1 grape naturally fermented juice	White, spherical raised, neat edges, creamy
EZ2	Gongniang No.1 grape naturally fermented juice	Yellow-green, spherical convex, neat edges, creamy

**Table 3:** 26S rDNA identification of yeast strains

Strain number	Identification	Genebank serial number
T1	<i>Meyerozyma guilliermondii</i>	KX791362.1
BN1	<i>Saccharomyces cerevisiae</i>	MN648829.1
SN1	<i>Saccharomyces cerevisiae</i>	KY273294.1
SN2	<i>Saccharomyces cerevisiae</i>	LC496585.1
SN3	<i>Saccharomyces cerevisiae</i>	MT322857.1
EN1	<i>Saccharomyces cerevisiae</i>	MG641152.1
EN2	<i>Saccharomyces cerevisiae</i>	MH472657.1
BZ1	<i>Saccharomyces cerevisiae</i>	MT420738.1
BZ2	<i>Saccharomyces cerevisiae</i>	CP046092.1
SZ1	<i>Pichia membranifaciens</i>	MW009112.1
EZ1	<i>Saccharomyces cerevisiae</i>	CP046463.1
EZ2	<i>Saccharomyces cerevisiae</i>	MG017580.1

## Discussion

WL medium can be used to identify and differentiate between yeasts, primarily based on colony color and morphology. It has been reported that the general morphological characteristics of *S. cerevisiae* on WL medium are milky white to green, with spherical protuberances, smooth surface, opaque and creamy consistency (Nielsen and Arneborg, 2007). In this study, 12 yeast strains from various sources were screened and identified by WL medium colony observation,

Culture conditions had a great influence on the growth of yeast. For example, the pH value can change the ionization degree of media nutrients, affecting cellular nutrient absorption and ultimately affecting growth (Xue *et al.*, 2007).

Sugar is the substrate for alcohol production via fermentation and is the energy source for yeast; however, high sugar concentration can inhibit yeast growth, resulting in glucose repression and inhibition. Moreover, the high osmotic pressure at increased sugar concentrations leads to water loss from yeast cells and decreases their activity (Xu *et al.*, 2014). Several metabolic systems, including transmission pathways and molecular response mechanisms, regulate yeast osmotic pressure under high sugar stress (Jin *et al.*, 2020). The Beibinghong grape, developed in 2008, is a mountain hybrid with high cold resistance and yield. It is primarily cultivated in northeast China and is the most important ice-wine brewing mountain grape variety (Biasi *et al.*, 2014). Generally, the sugar content of grape juice used for winemaking is about 250 g/L. The ice-wine production environment requires yeast that can tolerate high sugar and acid concentrations and low temperature (GuoHuan *et al.*, 2017). The optimal alcohol content in industrial wine brewing is 10 - 13%; higher alcohol concentrations can inhibit yeast growth. EZ2 selected from naturally fermented Gongniang No.1 grape juice exhibited significant alcohol tolerance up to 16%.

## Conclusion

Yeast is the most important strain in the brewing industry. It is the soul of wine quality and has a big influence on the color, aroma and flavor of a wine. In this study, 12 yeast strains from various sources were screened and identified by WL medium colony observation, microscopic observation and 26S rDNA sequencing. The results of the fermentation test showed that EN2 exhibited significant acid and SO<sub>2</sub> tolerance and can tolerate certain acidity and SO<sub>2</sub> at the concentration of 250 mg/L. Strain BZ1 showed significant sugar tolerance, which can be fermented at a glucose concentration of 300 g/L. The results demonstrated that strain EZ2 had good alcohol tolerance and could still ferment at 16% alcohol volume fraction. The three strains have excellent fermentation performance and could be used as development strains for wine making. Further studies are required to investigate the wine-brewing performance of the yeasts to select strains that exhibit characteristic qualities of the grape-producing areas with better fermentation abilities.

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## Author's Contributions

**Xiaochun Yu:** Participated in the experimental procedure and was responsible for data analysis and manuscript preparation.

**Jing Xu:** Participated in the experimental design and contributed to the writing and revision of the manuscript.

**Yuan Peng Li:** Contributed to the collection of samples, screening and identification of strains.

**Xian Peng Li:** Contributed to the fermentation performance and tolerance experiments and participated in data analysis.

## Ethics

All authors read and approved the final version and are responsible for any ethical issue that may arise after the publication of this manuscript.

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