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Original article

The glycerol and ethanol production kinetics in low-temperature wine fermentation using *Saccharomyces cerevisiae* yeast strains

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Summary Increasing glycerol production in low-temperature wine fermentation is of concern for winemakers to improve the quality of wines. The objective of this study was to investigate the effect of 10 different *Saccharomyces cerevisiae* on the kinetics of production of glycerol, ethanol and the activities of glycerol-3-phosphate dehydrogenase (GPD) and alcohol dehydrogenase (ADH) in low-temperature fermentation. Ethanol production was influenced by temperature, and it was slightly higher at 13 °C than at 25 °C. Glycerol yields were significantly affected by both temperature and strains. More glycerol was produced at 25 °C than at 13 °C because the activity of GPD was higher at 25 °C than at 13 °C. Glycerol production of the different yeast strains was up to 3.19 and 3.18 g L^{-1} at 25 and 13 °C, respectively. Therefore, isolating the yeast strains with high glycerol production and adaptation to low-temperature fermentation is still the best method in winemaking.

Keywords ADH, glycerol, GPD, low-temperature fermentation, *Saccharomyces cerevisiae*.

Introduction

Low-temperature fermentation (10–15 °C) is widely used in winemaking to improve the characteristics of taste and aroma (García-Ríos *et al.*, 2017). The optimal growth temperature for *Saccharomyces cerevisiae* is 25 °C, whereas 13 °C is a restrictive temperature which could lead to fermentation stuck or sluggish (Bisson, 1999). Many studies have showed that *S. cerevisiae* seeks to accumulate glycerol to protect against freeze injury (Panadero *et al.*, 2006; Aguilera *et al.*, 2007; Pérez-Torrado *et al.*, 2016).

Glycerol is quantitatively the most important by-product of alcoholic fermentation, and it is the major end-product other than ethanol and CO_2. In yeasts, glycerol is generally synthesised via the two-step reduction of dihydroxyacetone phosphate by glycerol-3-phosphate dehydrogenase (GPD, EC 1.1.1.8) and glycerol-3-phosphatase (GPP, EC 3.1.3.21) (Pahlman *et al.*, 2001). Glycerol formation plays a major role in balancing the NADH/NAD intracellular ratio (Albers *et al.*, 1998) and in acting as an osmolyte that balances the high external osmotic pressure (Remize *et al.*, 2000). Although glycerol is a non-volatile compound with no aromatic properties, it makes a significant contribution to wine quality of mouthfeel by providing sweetness and fullness (Remize *et al.*, 1999). Much work has been done to elucidate the factors influencing glycerol yields, including temperature, yeast strain, inoculation level, sulfite concentration, sugar concentration, osmotic stress, nitrogen source and concentration, pH, aeration, grape variety and ripeness (Remize *et al.*, 2000). However, for the wineries, since the grape raw materials and fermentation methods are difficult to change, the best way to increase the glycerol yield is to use high glycerol-yield yeast strain.

In recent years, there have been two main approaches used in increasing the content of glycerol. One has been to use other *Saccharomyces* species (*S. uvarum*, *S. kudriavzevii* and *S. paradoxus*) or the yeast hybrids (*S. cerevisiae* × *S. uvarum* and *S. cerevisiae* × *S. kudriavzevii*) as they produce more glycerol than *S. cerevisiae* in low-temperature fermentation (González *et al.*, 2007; Arroyo-López *et al.*, 2010; Gamero *et al.*, 2013; Oliveira *et al.*, 2014; Pérez-Torrado *et al.*, 2015). The other approach has been genomic engineering technology used in the *Saccharomyces cerevisiae* to produce higher content of glycerol, such

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as the overexpression of the *GPD1* gene (Michnick *et al*., 1997), the deletion of one or several *ADH* genes coding for alcohol dehydrogenases (Drewke *et al*., 1990; Cordier *et al*., 2007) or *PDC1* and *PDC5* genes coding for pyruvate decarboxylases (Nevoigt & Stahl, 1996) and the modification of the ***Fps1*** protein which is involved in the efflux of glycerol (Wang *et al*., 2001).

However, it is well known that *Saccharomyces cerevisiae* still plays the main role in the winemaking process, and genetically modified yeast strains have not been accepted for use in the wine industry. There are many references that emphasise the *GPD1* and *ADH1* genes expression in the low-temperature fermentation; nevertheless, little is known about the kinetics of activities of GPD and ADH during low-temperature fermentation. Therefore, this study investigated the effect of 10 different *Saccharomyces cerevisiae* yeast strains widely used in Chinese wine industry on the kinetics of production of glycerol and ethanol and the activities of GPD and ADH protein in low-temperature fermentation.

Materials and methods

Yeast strains

Nine commercial yeast strains independently isolated and one laboratory-isolated yeast strain BH8 (Li *et al*., 2010) were used in this work (Table 1). These commercial yeast strains are widely used in Chinese wine factories. The wine yeast BH8 used in this study was isolated from spontaneously fermenting must of 'Beihong' red wine grape (*Muscat Hamburg* × *V. amurensis*), a variety cultivated by the institute of Botany, the Chinese Academy of Sciences, Beijing, China. This strain is able to complete microvinification in several natural musts and in the model synthetic medium (MSM) with good results (Li *et al*., 2010; Du *et al*., 2012). It has been identified as *Saccharomyces cerevisiae* by colour and colony topography on WL Nutrient Agar (Pallmann *et al*., 2001) as well as DNA sequence analysis (Data S1) conducted by the Institute of Microbiology, Chinese Academy of Sciences, Beijing, China.

Table 1 *Saccharomyces cerevisiae* strains used in this study

	Strains	Factories	Purposes
Y1	DV10	Lallemand, France	Champagne, white wine
Y2	R2	Lallemand, France	White wine
Y3	FREDDO	Erbslöh Geisenheim AG, Germany	White wine
Y4	AWRI R2	Marivin, Australia	White wine
Y5	CY3079	Lallemand, France	White wine
Y6	LVCB	DSM, the Netherlands	White wine
Y7	K1	Lallemand, France	White, red and ice wine
Y8	N°.7303	DSM, the Netherlands	Red wine
Y9	XR	Lamothe-abiet, France	Red wine
Y10	BH8	Laboratory	—

Fermentation experiments

In order to keep a constant composition for the study of wine fermentation and wine yeast metabolism, the model synthetic medium (MSM) as described by Marullo *et al*. (2004) was used in this study. This medium (pH 3.3) contained the following components (expressed in g L^{-1}): glucose (100 g), fructose (100 g), tartaric acid (3 g), citric acid (0.3 g), L-malic acid (0.3 g), $MgSO_4$ (0.2 g), KH_2PO_4 (2 g). Nitrogen sources were adjusted to 190 mg total N/L as $(NH_4)_2SO_4$ (0.3 g) and asparagine (0.6 g). Mineral salts (mg L^{-1}): $MnSO_4{\cdot}H_2O$ (4), $ZnSO_4{\cdot}7H_2O$ (4), $CuSO_4{\cdot}5H_2O$ (1), KI (1), $CoCl_2{\cdot}6H_2O$ (0.4), $(NH_4)_6Mo_7O_{24}{\cdot}4H_2O$ (1), H_3BO_3 (1). Vitamins (mg L^{-1}): mesoinositol (300), biotin (0.04), thiamin (1), pyridoxine (1), nicotinic acid (1), pantothenic acid (1), p-amino benzoic acid (1). Fatty acids (mg L^{-1}): palmitic acid (1), palmitoleic acid (0.2), stearic acid (3), oleic acid (0.5), linoleic acid (0.5), linoleic acid (0.2).

Before yeast inoculation, the medium was sterilised by filtration (nitrate cellulose membrane, 0.45 μm, Millipore) and supplemented with sulfur dioxide (20 mg L^{-1}) in accordance with enological treatments. The fatty acid mixture was prepared in ethanol solution and fixed by drying on cellulose (0.5 g L^{-1}) in order to obtain 200 NTU (nephelometric turbidity units).

Fermentations were conducted in 500-mL flasks with 400-mL medium and fitted with closures that enabled the carbon dioxide to escape and samples to be removed, but which excluded the atmospheric oxygen. The initial yeast inoculums were of 1×10^6 cells m/L from YPD medium [1% (w/v) yeast extract, 2% (w/v) peptone and 2%(w/v) glucose] overnight cultures.

The fermentations were conducted in triplicate at two different temperatures, 13 °C and 25 °C (used as control), without shaking. The maximal fermentation rate was the maximal weight loss caused by release of CO_2 versus fermentation day and was expressed as g of CO_2 L^{-1} day^{-1}. The weight loss of the fermentation process was monitored and the fermentation stopped when the CO_2 release was less than 0.2 g day^{-1}.

Density and viability measurement

The daily density was measured by weighing 5 mL of must. Cell viability was determined by two methods including microscopy and plating them on YPD agar

at adequate dilution. For the microscopy study, the yeast suspension was mixed (v/v) with a methylene blue solution (0.1 g L^{-1} of methylene blue, 20 g L^{-1} of trisodium citrate, in 1 L of a binary solution) at the same temperature as the cells suspension (Marañón *et al*., 1999). At least 300 cells were counted for each aliquot.

Analytical procedures

Glucose, fructose, trehalose (intracellular and extracellular), glycerol (intracellular and extracellular), succinic acid, acetic acid, ethanol were determined by high-performance liquid chromatography (HPLC) Waters 2695 with a Waters 2414 refractive index detector (Moreira *et al*., 2005). The supernatant was filtered through 0.22-μm pore-size nylon filters prior to loading on an Aminex HPX-87H column (300 × 7.8 mm; Bio-Rad, USA) used with 5 mM H_2SO_4 as mobile phase at 0.6 mL min^{-1}. The column was controlled at 65 °C.

Enzymatic assays

Cells were harvested by centrifugation at 6000 × ***g*** for 10 min at 4 °C, washed twice with 10-mL cold 20 mM Hepes pH 7.1. About 10-mg (dry mass) yeast cells were resuspended in this buffer for 30 min. Cells were disrupted with an ultrasonic crusher (Ningbo Scientz Research Institute of Instruments, Ningbo, China). Cells were then centrifuged (12 000 × ***g*** for 30 min at 4 °C) and the supernatant was used as crude enzyme solution.

The NAD^+-dependent glycerol-3-phosphate dehydrogenase is a key enzyme for glycerol formation in *S. cerevisiae* (Guadalupe-Medina *et al*., 2014). The activity of glycerol-3-phosphate dehydrogenase (GPD, EC 1.1.1.8) was determined as described (Guadalupe-Medina *et al*., 2014) with some modifications. The reaction solution consisted of 20 mM Hepes, pH 7.1, 20 mM KCl, 1 mM EDTA, 1 mM DTT, 5 mM dihydroxyacetonephosphate (DHAP) and 0.1 mM NADH. The reaction was initiated by adding DHAP. NADH depletion was monitored spectrophotometrically at 340 nm and 30 °C. The molar extinction coefficient ε_{340} is 6.2 × $10^3 M^{-1}$ cm^{-1}. One unit of enzyme activity (u) is defined as 1 μM of NADH consumed per minute.

Alcohol dehydrogenase (ADH) (EC 1.1.1.1) activity was determined in 50 mM KH_2PO_4, pH 8.0 in the presence of 1.0 mM NAD^+ and the reaction was started by the addition of 100 mM ethanol at 30 °C.

Protein concentration was determined using bovine serum albumin (BSA) as standard. The reagents dihydroxyacetone phosphate (DHAP), NADH, NAD^+, Hepes, DTT and BSA were all purchased from Sigma–Aldrich (China sector).

Statistical analysis

All the experiments were repeated at least three times. All values were presented as mean ± standard deviation (SD). An analysis of variance (ANOVA) was applied to the experimental data, which was subjected to double-factor analysis of variance (yeast strain/fermentation temperature). For all the statistical analyses components, differences were considered significant at $P < 0.001$. The significant differences were determined by Tukey tests. All statistical analyses were components using the software SAS (SAS Institute Inc., Cary, NC, USA) for Windows, version 8.02.

Results

Effect of different yeast strains on principal enological characteristics in low-temperature fermentation

After the fermentations were finished (final concentration of sugar <4 g L^{-1}), the results showed that both low-temperature fermentation and yeast strains affected the fermentation rate, the fermentation time, yeast growth and the concentration of metabolites (Table 2).

The length of fermentation at 13 °C (47–58 days) was longer than at 25 °C (14–23 days). However, the maximum population at 13 °C (2.1 × 10^8–2.5 × 10^8 cells mL^{-1}) was similar to that at 25 °C (2.3 × 10^8–2.9 × 10^8 cells mL^{-1}). Fermentation began more slowly at 13 °C than at 25 °C, which caused a delay in reaching the maximum population density. Once the maximum density was reached, it remained high throughout fermentation at 13 °C, whereas it declined at 25 °C, with considerable mortality at the end of fermentation (data not shown). There was less sugar residue at 13 °C (1.28–2.2 g L^{-1}) than at 25 °C (2.15–3.89 g L^{-1}), which might be due to the greater mortality by the end of fermentation at 25 °C. The final concentration of ethanol was higher at 13 °C (11.7%–12.1%) than at 25 °C (11.32%–11.83%).

In addition to glycerol, acetic acid and succinic acid also play a role in maintaining the redox balance (van Dijken & Scheffers, 1986). The concentrations of glycerol, acetic acid and succinic acid were significantly less at 13 °C than at 25 °C.

Trehalose is believed to protect cytosol components against stress such as heat shock, osmotic shock and starvation (Wiemken, 1990; Attfield *et al*., 1994). Trehalose was produced less at 13 °C than at 25 °C in all the 10 different yeast strains.

As estimated by variance analysis (Tukey test, $P < 0.001$), both low-temperature fermentation and different yeast strains influenced the production of metabolite components, including acetic acid, succinic acid, trehalose and glycerol; however, ethanol

Table 2 Effect of yeast strain and temperature on fermentation kinetics and principal metabolites

Yeast strains		Length of fermentation (days)	Maximal fermentation rate (g of CO_2 L^{-1} day^{-1})	Sugar residue (g L^{-1})	Maximal population (10^8 cells ml^{-1})	Day of maximal population	Final ethanol production (%, v/v)	Glycerol production (g L^{-1})*	Trehalose production (g L^{-1})	Acetic acid production (mg L^{-1})	Succinic acid production (mg L^{-1})
Y1	25°C	18	23.42 ± 0.53[a]	3.14 ± 0.3[a]	2.4 ± 0.22[a]	3	11.75 ± 0.07[a, ***]	8.91 ± 0.17[a, **]	1.36 ± 0.13[a]	807 ± 12[a]	1250 ± 23[a]
	13°C	48	4.45 ± 0.32[a]	1.45 ± 0.04[a]	2.1 ± 0.12[a]	10	11.91 ± 0.02[a, ***]	6.24 ± 0.03[a, **]	0.94 ± 0.01[a]	612 ± 23[a]	560 ± 34[a]
Y2	25°C	21	19.45 ± 0.47[a]	2.98 ± 0.19[a]	2.7 ± 0.15[a]	2	11.61 ± 0.24[a, ***]	8.34 ± 0.15[a, **]	1.42 ± 0.09[a]	936 ± 15[a]	1888 ± 24[a]
	13°C	48	4.32 ± 0.24[a]	1.32 ± 0.03[a]	2.2 ± 0.07[a]	7	11.75 ± 0.12[a, ***]	6.25 ± 0.13[a, **]	0.86 ± 0.02[a]	710 ± 34[a]	510 ± 42[a]
Y3	25°C	21	21.27 ± 0.86[a]	2.95 ± 0.16[a]	2.6 ± 0.11[a]	3	11.55 ± 0.03[a, ***]	8.85 ± 0.41[a, **]	1.51 ± 0.04[a]	859 ± 21[a]	1600 ± 54[a]
	13°C	55	3.75 ± 0.15[a]	1.78 ± 0.14[a]	2.1 ± 0.1[a]	13	11.86 ± 0.28[a, ***]	6.09 ± 0.11[a, **]	1.02 ± 0.09[a]	652 ± 34[a]	480 ± 25[a]
Y4	25°C	14	26.52 ± 0.65[a]	2.15 ± 0.15[a]	2.4 ± 0.2[a]	2	11.83 ± 0.02[a, ***]	8.4 ± 0.46[a, **]	1.3 ± 0.06[a]	829 ± 21[a]	1485 ± 32[a]
	13°C	55	6.52 ± 0.12[a]	1.35 ± 0.13[a]	2.2 ± 0.13[a]	10	12.1 ± 0.05[a, ***]	6.98 ± 0.03[a, **]	1.07 ± 0.02[a]	624 ± 34[a]	795 ± 43[a]
Y5	25°C	18	20.4 ± 0.74[a]	3.03 ± 0.17[a]	2.9 ± 0.24[a]	2	11.54 ± 0.24[a, ***]	8.49 ± 0.24[a, **]	1.3 ± 0.05[a]	872 ± 12[a]	1005 ± 21[a]
	13°C	56	5.72 ± 0.34[a]	1.78 ± 0.14[a]	2.5 ± 0.12[a]	7	11.7 ± 0.2[a, ***]	6.33 ± 0.15[a, **]	1.01 ± 0.02[a]	501 ± 23[a]	412 ± 23[a]
Y6	25°C	18	23.63 ± 0.45[a]	2.72 ± 0.14[a]	2.3 ± 0.07[a]	3	11.5 ± 0.28[a, ***]	7.98 ± 0.09[a, **]	1.21 ± 0.12[a]	938 ± 21[a]	1172 ± 23[a]
	13°C	58	2.53 ± 0.12[a]	1.28 ± 0.08[a]	2.2 ± 0.04[a]	10	11.77 ± 0.06[a, ***]	6.66 ± 0.17[a, **]	0.83 ± 0.02[a]	730 ± 12[a]	662 ± 24[a]
Y7	25°C	23	14.45 ± 0.75[a]	2.84 ± 0.13[a]	2.7 ± 0.18[a]	3	11.32 ± 0.04[a, ***]	7.44 ± 0.24[a, **]	1.38 ± 0.05[a]	955 ± 13[a]	1475 ± 57[a]
	13°C	48	5.34 ± 0.32[a]	1.32 ± 0.03[a]	2.1 ± 0.16[a]	10	11.78 ± 0.05[a, ***]	5.29 ± 0.03[a, **]	0.76 ± 0.02[a]	720 ± 21[a]	808 ± 32[a]
Y8	25°C	23	16.12 ± 0.67[a]	3.79 ± 0.21[a]	2.8 ± 0.12[a]	2	11.44 ± 0.05[a, ***]	9.44 ± 0.5[a, **]	1.52 ± 0.1[a]	867 ± 18[a]	1509 ± 46[a]
	13°C	57	5.13 ± 0.21[a]	1.8 ± 0.03[a]	2.5 ± 0.13[a]	13	11.82 ± 0.03[a, ***]	6.49 ± 0.1[a, **]	1 ± 0.03[a]	660 ± 34[a]	475 ± 14[a]
Y9**	25°C	16	19.62 ± 0.77[a]	3.89 ± 0.23[a]	2.4 ± 0.21[a]	3	11.46 ± 0.07[a, ***]	9.08 ± 0.78[a, **]	1.29 ± 0.04[a]	745 ± 12[a]	1375 ± 59[a]
	13°C	55	4.67 ± 0.14[a]	2.2 ± 0.09[a]	2.2 ± 0.09[a]	10	11.78 ± 0.17[a, ***]	5.36 ± 0.11[a, **]	0.81 ± 0.02[a]	538 ± 23[a]	685 ± 32[a]
Y10	25°C	23	18.35 ± 0.11[a]	3.65 ± 0.11[a]	2.6 ± 0.12[a]	3	11.59 ± 0.11[a, ***]	10.63 ± 0.52[a, **]	1.15 ± 0.06[a]	790 ± 32[a]	1234 ± 98[a]
	13°C	47	6.43 ± 0.09[a]	1.76 ± 0.04[a]	2.3 ± 0.1[a]	7	11.7 ± 0.11[a, ***]	8.47 ± 0.57[a, **]	0.81 ± 0.04[a]	603 ± 32[a]	807 ± 32[a]

[a]Mean value and SD for three independent fermentation.
*Significant difference in glycerol production at both temperatures by different yeast strains $P < 0.001$.
**Significant difference in glycerol production with same yeast strain at different temperatures $P < 0.001$.
***Significant difference in ethanol production with same yeast strain at different temperatures $P < 0.05$.

production was only affected by the fermentation temperature. The concentrations of acetic acid, succinic acid, trehalose and glycerol were significantly lower at 13 °C than at 25 °C, whereas ethanol production was increased slightly (1.2%–4.06%) at 13 °C compared with that at 25 °C.

Effect of low-temperature fermentation on alcohol production and ADH activity kinetics during wine fermentation

The final production of ethanol was higher at 13 °C than at 25 °C for all yeast strains (Table 2). Yeast strain Y7 exhibited the greatest difference in ethanol production at the two temperatures, so yeast strain Y7 was selected for studying the influence of fermentation temperature on ethanol production and ADH activity kinetics.

Fermentations with yeast strain Y7 at both temperatures were done with an initial concentration of sugar of 200 g L^{-1} (Fig. 1a). After fermentation for 3 days at 25 °C, the sugar consumption rate was significantly slow, the sugar residue was 2.84 g L^{-1} and the concentration of ethanol was 11.32% at the end of fermentation. The fermentation rate at 13 °C attained the maximum after 8 days, the sugar residue was 1.32 g L^{-1} and the concentration of ethanol was 11.78% at the end of fermentation. The length of fermentation was 1.09-fold longer at 13 °C than at 25 °C and the yield of ethanol was increased slightly (0.46%) at 13 °C, which was the biggest difference among all the yeast strains.

Figure 1b showed the kinetics of enzyme activity (ADH) during fermentation with yeast strain Y7 at 13 °C and at 25 °C. At 25 °C, the activity of ADH reached a maximum after 3 days and then decreased rapidly. On the contrary, the activity of ADH reached a high level at about the 7th day and remained high until the end of fermentation at 13 °C (13 days). The activity of ADH was significantly lower at 13 °C than

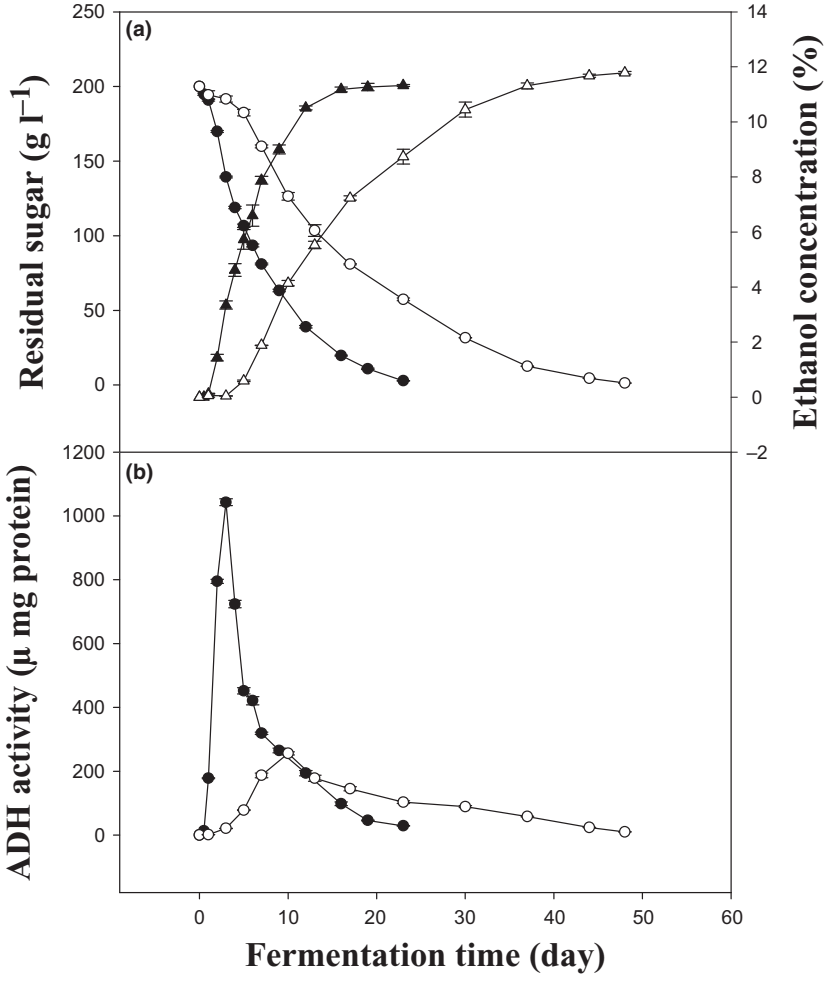

Figure 1 The kinetics of residual sugar, ethanol production and ADH activity with Y7 during fermentation. Symbols for a: ▲, 25 °C ethanol production; △, 13 °C ethanol production; ●, 25 °C residual sugar; ○, 13 °C residual sugar. Symbols for b: ●, 25 °C ADH activity; ○, 13 °C ADH activity. The error bars in the figure indicate standard errors from three independent samples.

at 25 °C, and this corresponded with the fermentation rate.

Effect of low-temperature fermentation and different yeast strains on glycerol production and GPD activity kinetics during wine fermentation

Glycerol production was influenced by both of the fermentation temperature and the different yeast strain. The significant differences of the effect of yeast strains and temperature on glycerol production are shown in Table 3 (two interaction factors). Yeast strains Y7 and Y10 were observed to have the greatest difference in glycerol yield among all the yeast strains. Yeast strain Y9 exhibited the greatest difference in glycerol yield at both of fermentation temperatures, so yeast strains Y7, Y9 and Y10 were selected for studying the influence of both of fermentation temperature and different yeast strains on glycerol production and GPD activity kinetics.

The kinetics of glycerol yield and the activity of GPD at 25 °C were investigated with yeast strains Y7 and Y10 and are shown in Fig. 2a. After 7 days, the rate of glycerol accumulation at 25 °C slowed significantly, and the amount of glycerol produced at 25 °C in the first 7 days was 74.1% and 89.2% of the total glycerol production for the yeast strains Y10 and Y7, respectively. The final glycerol production was 10.63 g L^{-1} and 7.44 g L^{-1} at the end of fermentation at 25 °C with yeast strains Y10 and Y7, respectively.

Table 3 Significant differences in glycerol production with double-factor analysis of variance (yeast strain/fermentation temperature (Two factors with interaction, Tukey test, $P < 0.001$)

Yeast strains	Glycerol (g L^{-1})[a]	Significance	
Y10	9.55 ± 0.54[b]	A	
Y8	7.965 ± 0.35[b]	B	
Y4	7.69 ± 0.41[b]	B	C
Y1	7.575 ± 0.14[b]	D	C C C
Y3	7.47 ± 0.32[b]	D	C C C
Y5	7.41 ± 0.2[b]	D	C
Y6	7.32 ± 0.1[b]	D	
Y2	7.295 ± 0.14[b]	D	
Y9	7.22 ± 0.45[b]	D	
Y7	6.365 ± 0.2[b]	E	

[a]Mean of glycerol production at both temperatures.
[b]Mean and SD for three independent fermentations.

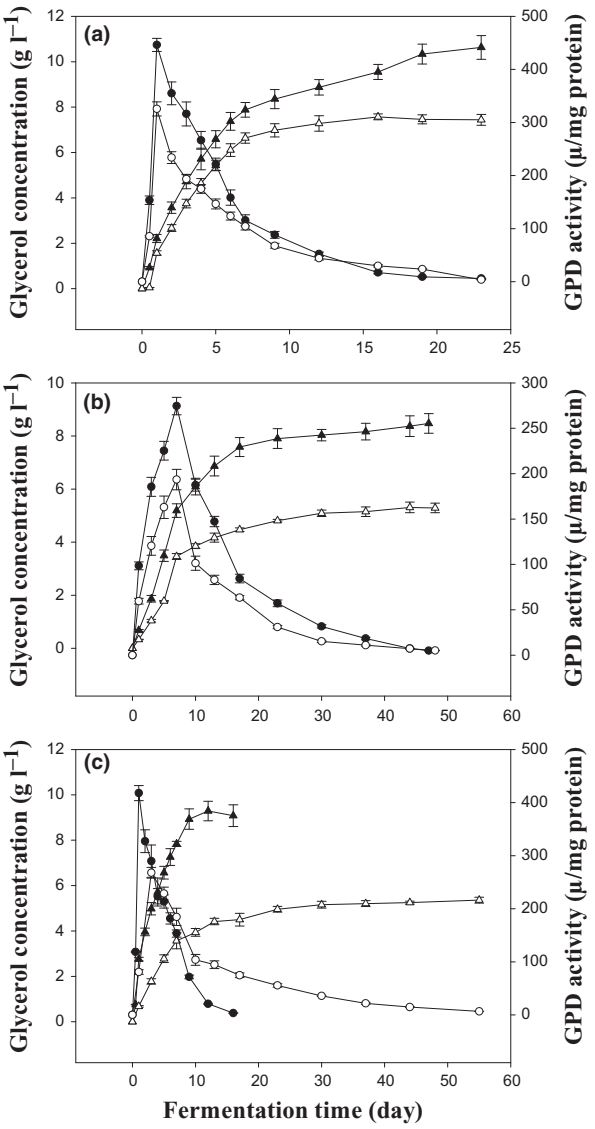

Figure 2 The kinetics of glycerol production and GPD activity with Y9, Y7 and Y10 during fermentation. Symbols for a: ▲, 25 °C glycerol production Y10; △, 25 °C glycerol production Y7; ●, 25 °C GPD activity Y10; ○, 25 °C GPD activity Y7. Symbols for b: ▲, 13 °C glycerol production Y10; △, 13 °C glycerol production Y7; ●, 13 °C GPD activity Y10; ○, 13 °C GPD activity Y7. Symbols for c: ▲, 25 °C glycerol production Y9; △, 13 °C glycerol production Y9; ●, 25 °C GPD activity Y9; ○, 13 °C GPD activity Y9. The error bars in the figure indicate standard errors from three independent samples.

The ratio of glycerol production between yeast strains Y10 and Y7 at 25 °C was 1.43, which is the greatest difference among all the yeast strains tested. The activity of GPD at 25 °C reached a peak on the 2nd day for both of yeast strains and then decreased dramatically. The activity of GPD was significantly higher in Y10 than that in Y7 at 25 °C.

In contrast, after 7 days, the rate of glycerol accumulation by yeast strains Y10 and Y7 at 13 °C was significantly slow, and the amount of glycerol produced in the first 7 days was 61.3% and 65.4% of the total glycerol production by strains Y10 and Y7 at 13 °C, respectively (Fig. 2b). The total glycerol production at the end of fermentation at 13 °C was 8.47 g L^{-1} and 5.29 g L^{-1} for strains Y10 and Y7, respectively. The ratio of glycerol production between yeast strains Y10 and Y7 at 13 °C was 1.60, which was the greatest difference among the yeast strains tested. The GPD activity of yeast strains Y10 and Y7 at 13 °C reached a maximum on the 4th day. The maximal value of GPD activity was delayed at 13 °C compared with that at 25 °C. The activity of GPD was significantly higher in Y10 than that in Y7 at 13 °C.

The kinetics of glycerol accumulation and the activity of GPD during wine fermentation with yeast strain Y9 at 13 °C and at 25 °C are showed in Fig. 2c. After 7 days, the rate of glycerol accumulation was significantly slowed at both temperatures. The contents of glycerol produced in the first 7 days at 25 °C and 13 °C were 86.3% and 66.6% of the total glycerol production, respectively. The final glycerol productions at the end of fermentation were 9.08 g L^{-1} and 5.36 g L^{-1} at 25 °C and at 13 °C, respectively. Decreasing the fermentation temperature from 25 °C to 13 °C resulted in a 69% decrease in glycerol yield, which was the biggest difference among the yeast strains tested. The activity of GPD at 25 °C and at 13 °C reached a maximum on the 2nd day and the 4th day, respectively. Decreasing the fermentation temperature from 25 °C to 13 °C resulted in a significant decrease (87%) in the activity of GPD, which was the greatest difference among the yeast strains tested.

As already illustrated by previous research, most of the glycerol production and the peak of GPD activity were produced in the initial fermentation process at both temperatures. These results were in agreement with the requirement of glycerol production to counteract the hyperosmotic stress at the beginning of vinification (Van Dijck *et al*., 2000). These results also showed that glycerol production was positively correlated to GPD activity. Compared with the influence of fermentation temperature on glycerol production, the glycerol yield was also significantly affected by different yeast strains. The difference in glycerol contents among all the yeast strains tested was up to 3.19 g L^{-1} (42.8%) and 3.18 g L^{-1} (60.1%) during fermentation at 25 °C and 13 °C, respectively.

Discussion

In this work, we studied the influence of 10 different *Saccharomyces cerevisiae* on the production of ethanol and glycerol and the activities of ADH and GPD in low-temperature fermentation. Decrease in the fermentation temperature from 25 °C to 13 °C prolonged the fermentation time and increased cell viability. This is because the gene *Ole1* is upregulated in cold stress to increase the fatty acid unsaturation in the yeast cell, which improves ethanol tolerance and increase cell viability (Aguilera *et al*., 2007). When the yeast cells were exposed to the high osmostress, the Hog1 MAPK is activated and mediates an adaptive response by increasing glycerol production. The results showed that more than 60% of glycerol was produced in the initial fermentation (7 days) at 25 °C and at 13 °C in response to osmotic stress. Glycerol production was influenced by both fermentation temperatures and yeast strains and more glycerol was produced at 25 °C than at 13 °C. This was because the activities of GPD were significantly higher at 25 °C than at 13 °C.

Ethanol production was only affected by the fermentation temperature and it was produced slightly higher at 13 °C than at 25 °C. The results showed that ethanol production is not required to be considered in isolating wine yeast stains. What we need to focus on in the screening yeast strains is just the factors affecting the wine quality such as glycerol production, aroma and colour. Our results agree with those of Torija *et al*. (2003). This was probably due to the fermentation at 13 °C with few sugar residues and higher population yeast cells at the end of fermentation (Table 2). Another reason is that temperature affected not only the fermentation kinetics but also yeast metabolism. In our results, the main secondary products (glycerol and succinic acid) were increased as the temperature increased (Table 2). Therefore, the ethanol yields were increased as the temperature decreased in our results.

In contrast to earlier observations on high and low glycerol-producing yeast strains (Remize *et al*., 2000), high glycerol production was not correlated to production of acetate or succinate, which could be explained by the fact that other metabolites (in particular, acetoin and 2, 3-butanediol) participate in the adjustment of the intracellular redox balance.

The normal concentration of glycerol usually produced by *Saccharomyces cerevisiae* in wine is in the range of 4–9 g L^{-1} (Remize *et al*., 2000). The interesting result in this study was that the amount of glycerol was 10.63 and 8.47 g L^{-1} with yeast strain Y10 at 25 and 13 °C. This glycerol production was higher than the average of glycerol yield, especially in the low temperature condition. The high osmolarity glycerol (HOG) signalling pathway is well studied in yeast (de Nadal *et al*., 2011). The osmostress is mainly sensed by two upstream mechanisms (Sln1 and Sho1). Finally, the kinase Hog1 is activated and regulates the transcription expression by recruiting the transcription factors. In 2006, it has been shown that Hog1 kinase

could be activated under cold stress throughout the 'SLN1-YPD1-SSK1' signal pathway and the glycerol production increased (Panadero *et al*., 2006). Further research is required to determine which transcription factors were recruited by Hog1 and which genes were regulated by Hog1 in the cold stress condition. Compared with the other nine commercial yeast strains and the other results of articles (Remize *et al*., 1999; Torija *et al*., 2003; Arroyo-López *et al*., 2010), the yeast strain BH8 exhibited the highest glycerol yield at both temperatures. This result demonstrates that the yeast strain BH8 was sensitive to cold stress and Hog1 was activated more than the other yeast strains. Finally, the gene *GPD1* was significantly upregulated and more GPD activity and glycerol were produced. In addition, the difference of glycerol yields between different yeast strains was up to 3.19 g L^{-1} (42.8%) and 3.18 g L^{-1} (60.1%) during fermentation at 25 °C and 13 °C, respectively. Compared with the other methods in increasing the glycerol yield, such as *Saccharomyces* species, the yeast hybrids and the genomic engineering technology, the results showed that isolating the yeast strains with high glycerol production and adaptation to the low-temperature fermentation was still the best method in winemaking.

Conclusions

The total amount of ethanol produced was affected by the fermentation temperature; more ethanol was obtained compared to fermentation at 13 °C in that at 25 °C. Glycerol was influenced by both fermentation temperature and yeast strains. Compared with the effect of fermentation temperature on glycerol production, the glycerol yield was also significantly affected by different yeast strains. Moreover, the yeast strain BH8 exhibited the highest glycerol production at 25 and 13 °C. It is very valuable to study the factors influencing the quality of wine using yeast strain BH8 such as colour, aroma and polyphenol in further research. Because *Saccharomyces cerevisiae* is still predominant in winemaking, thus from this research, we can conclude that isolating *Saccharomyces cerevisiae* yeast strains with high glycerol production and adaptation to the low-temperature fermentation are still the best methods in winemaking.

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Supporting Information

Additional Supporting Information may be found in the online version of this article:

Data S1. The rDNA ITS sequencing results of *Saccharomyces cerevisiae* yeast cell BH8