OENO One
VINE AND WINE OPEN ACCESS JOURNAL
By iVES International Viticulture & Enology Society

DOI: https://doi.org/10.20870/oeno-one.2023.57.1.7238

OPEN ACCESS

ORIGINAL RESEARCH ARTICLE

Continuous fed-batch strategy decreases acetic acid production and increases volatile ester formation in wines under high-gravity fermentation

Haixia Deng[1, 2], Meiyu Wang[1, 2] and Erhu Li[1, 2*]

[1] College of Food Science and Technology, Huazhong Agricultural University, Wuhan 430070, Hubei, China
[2] Key Laboratory of Environment Correlative Dietology, Ministry of Education, Huazhong Agricultural University, Wuhan, Hubei, 430070, China

*correspondence:
erhuli@mail.hzau.edu.cn

Associate editor:
Hervé Alexandre

Received:
29 October 2022
Accepted:
21 February 2023
Published:
28 March 2023

This article is published under the **Creative Commons licence** (CC BY 4.0).

Use of all or part of the content of this article must mention the authors, the year of publication, the title, the name of the journal, the volume, the pages and the DOI in compliance with the information given above.

ABSTRACT

High sugar fermentation elevates acetic acid levels in wines, which can be avoided by applying the continuous fed-batch strategy. In this study, yeast gene expressions and wine volatile compounds were evaluated by quantitative real-time PCR (RT-qPCR) and gas chromatograph mass spectrometry (GC-MS) in high-gravity (HG, 320 g/L sugars) fermentations with different batch strategies. The acetic acid concentration in continuous fed-batch fermentation wine was reduced by 51.69 %, compared with that in whole-batch fermentation wine. The acetyl-CoA synthase gene (*ACS2*) expression was up-regulated, whereas the glycerol-3-phosphate dehydrogenase gene (*GPD1*) expression was down-regulated on day 3 and day 7 during the continuous fed-batch fermentation. The volatile ester concentration in continuous fed-batch fermentation wine was 36.74 % higher than that in whole-batch fermentation wine. Overall, the continuous fed-batch strategy can modulate the expression of yeast genes involved in acetic acid metabolism and can increase volatile esters in wine under high sugar fermentation. Our findings provide a reference for the application of a continuous fed-batch strategy in high-sugar fermentation so as to improve the quality of the wine.

KEYWORDS: fermentation strategy, acetic acid production, *Saccharomyces cerevisiae*, sugar osmotic pressure, gene regulation, volatile aroma

INTRODUCTION

In recent years, the global average temperature has been increasing continuously (Malhi *et al*., 2021), which results in extremely high sugar concentrations in harvested grapes (Mira de Orduna, 2010). In winemaking, this high-sugar grape juice leads to a high osmotic stress, thus, inducing *S. cerevisiae* to produce more by-products, such as glycerol and acetic acid (Pigeau and Inglis, 2005). For example, in Botrytised wine (Bely *et al*., 2005) and Icewine (Pigeau *et al*., 2007), the initial sugar concentrations are usually above 30 °Brix, which triggers *S. cerevisiae* to produce over 10 g/L glycerol and over 1.2 g/L acetic acid (Bely *et al*., 2003; Heit *et al*., 2018). Acetic acid is the major volatile acid in wine, with its normal content ranging from 0.2 to 0.6 g/L, and the excessive content will seriously affect the sensory quality of the final products (Vilela-Moura *et al*., 2011). Once its concentration exceeds 0.8 g/L, there will be an obvious bitter and rancidity flavour (Vilela, 2017). According to European legislation, the maximum limit of volatile acidity (expressed as acetic acid) should be 1.2 g/L in wine and 2.1 g/L in Icewine, respectively. In addition, a large amount of acetic acid has been reported to inhibit yeast cell activity and fermentation efficiency (Guaragnella *et al*., 2011; Sousa *et al*., 2013). Therefore, it is essential to solve the problem of excessive acetic acid formation during high-sugar fermentations, especially in the production of speciality wines such as Icewine and late-harvest wines.

High sugar stress triggers yeast to produce more glycerol so as to achieve osmotic balance inside and outside yeast cells (Liu *et al*., 2021), which is accompanied by the oxidation of NADH to NAD^+ (Jain *et al*., 2011). Glycerol-3-phosphate dehydrogenase is the key rate-limiting enzyme for glycerol production, which is mainly encoded by the *GPD1* of yeast (Remize *et al*., 2003) (Figure 1).

Glycerol production often leads to an increase in acetic acid produced via the pyruvate dehydrogenase (PDH) bypass pathway during high-sugar fermentation (Pigeau *et al*., 2007). In fact, acetic acid production results from the balance by *S. cerevisiae* of excess NAD^+ produced from glycerol formation (Yang *et al*., 2017) (Figure 1). This PDH bypass pathway involves the following three major components and some key regulatory genes: (1) Pyruvate is catalysed by pyruvate decarboxylase to form acetaldehyde. The 80 %–90 % activity of pyruvate decarboxylase is derived from Pdc1p, mainly encoded by *PDC1* (Milanovic *et al*., 2012). The *PDC1* knockout of *S. cerevisiae* has been reported to decrease acetic acid production by 57.14 % (Curiel *et al*., 2016); (2) Acetaldehyde is oxidised into acetic acid by acetaldehyde dehydrogenase, accompanied by the reduction of NAD^+ into NADH. The main regulation gene of this oxidation is *ALD6,* with $NADP^+$ as a cofactor (Meaden *et al*., 1997). Several studies have shown that *ALD6* deletion of *S. cerevisiae* significantly reduces acetic acid production (Eglinton *et al*., 2002; Saint-Prix *et al*., 2004); and (3) Acetic acid combines with coenzyme A under the catalysis of acetyl-CoA synthase to form acetyl-CoA. *ACS2* mainly encodes acetyl-CoA synthase, and it plays an important role in acetic acid production (Van den Berg *et al*., 1996). *ACS2* overexpression of *S. cerevisiae* significantly reduces acetic acid production in sake fermentation (Akamatsu *et al*., 2000).

Reducing sugar concentration by the fed-batch approach during alcoholic fermentation can effectively increase yeast viability, promote ethanol production (Lemos *et al*., 2020), and decrease acetic acid formation. Compared with whole-batch fermentation, the continuous fed-batch

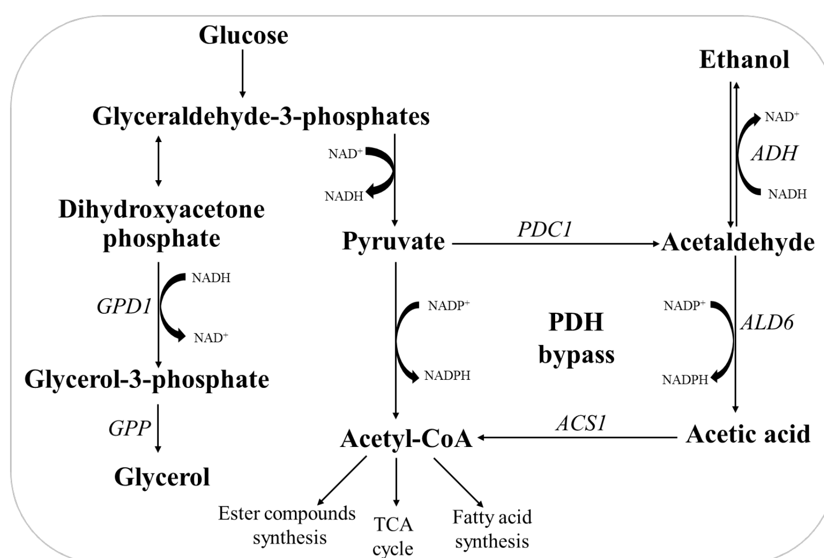

FIGURE 1. Main genes involved in PDH bypass and glycerol synthesis pathway in *S. cerevisiae* (Heit *et al*., 2018; Sadoudi *et al*., 2017).

PDH for coding pyruvate dehydrogenase, *ADH* for coding alcohol dehydrogenase, *PDC1* for coding pyruvate decarboxylase, *ALD6* for coding acetaldehyde dehydrogenase, *ACS2* for coding acetyl-CoA synthase, *GPD1* for coding glycerol-3-phosphate dehydrogenase, and *GPP* for coding glycerol 3-phosphatase.

approach has been confirmed to significantly reduce acetic acid production by 80 % in high-gravity fermentation (343.3 g/L sugar) (Frohman and Mira de Orduna, 2013). However, the concerning metabolism and gene expression of *S. cerevisiae* in high-gravity grape juice fermentation based on a continuous fed-batch strategy remain largely unknown, and the effect of this strategy on volatile compounds of the wine is still unclear, which hinders the application of this fermentation strategy.

In this study, whole-batch fermentation under high-gravity conditions was used as a control, and the relationships between acetic acid production and gene expressions (*PDC1*, *ALD6*, *ACS2,* and *GPD1*) involved in glycerol synthesis and PDH bypass were investigated during continuous fed-batch fermentation. In addition, the effects of the continuous fed-batch strategy on the volatile compounds of the wines were also studied. Our findings will provide a theoretical basis for the application of a continuous fed-batch strategy in high-gravity fermentation.

MATERIALS AND METHODS

1. Grape juice preparation

A flash-pasteurised grape juice (Vidal) from the Shangri-La wine region (Yunnan, China) was used for both whole-batch and fed-batch fermentations with combined sugar (glucose and fructose) concentration of 210 g/L and a pH of 2.9. The titratable acidity was 6.4 g/L, expressed as tartaric acid. The acetic acid content was 0.02 g/L. Fermentation substrates with different initial sugar concentrations were prepared by adding equal amounts of D-glucose and D-fructose to reach final sugar concentrations of 320, 400, and 450 g/L. 250 mg/L of $(NH_4)_2SO_4$ was added as supplemented nitrogen source.

2. Microorganism

Commercial *S. cerevisiae* strain RV002 was provided by Angel Yeast Co. Ltd. (Yichang, Hubei, China). The 1 g of dry yeast was added into 100 mL sterile water and rehydrated at 40 °C for 15 min to obtain yeast suspension for all the subsequent fermentations.

3. Fermentations

All fermentations were conducted in 500 mL glass bottles containing 400 mL grape juice. The grape juice was inoculated with 10 mL yeast suspension. The bottles were sealed with suitable airlocks to prevent air ingress during fermentation. Fermentation was carried out at 20 °C, and it did not stop until the termination of sugar consumption or until the sugar concentration was below 3 g/L. All the fermentations were conducted by using the previously reported method with some modifications (Frohman and Mira de Orduna Heidinger, 2018). The normal-gravity (NG, 210 g/L sugars) and high-gravity groups (HG, 320 g/L sugars) were established to investigate the osmotic stress of grape juice. The grape juices were fermented by whole-batch and two fed-batch strategies. Whole-batch fermentation was started with all the 400 mL juice as starting materials.

The continuous fed-batch fermentation was started with 50 mL juice. For the NG group, after 48-hour fermentation, the remaining 350 mL juice was continuously fed within 48 h at 0.12 mL/min by a peristaltic pump (Meiyingpu Instrument Manufacturing Co., Ltd., Shanghai, China). For the HG group, after 72-hour fermentation, the remaining 350 mL juice was added continuously within 72 h at 0.08 mL/min.

The intermittent fed-batch fermentation was also started with 50 mL juices. Under the NG condition, the remaining 350 mL juice was fed at two-time points (48 h and 72 h) at 8.7 mL/min for 20 min (per time point). Under the HG condition, the remaining 350 mL juice was fed at two-time points (96 h and 144 h) at 8.7 mL/min for 20 min (per time point).

Samples (5 mL) of whole-batch and continuous fed-batch fermentations were collected on day 3 (exponential phase of yeast growth), day 7 (mid-stationary phase of yeast growth), and day 12 (late stationary phase of yeast growth) for subsequent gene expression analysis. The remaining samples were collected every 24 h and stored at –20 °C for subsequent analysis. All the fermentation and sample analyses were carried out in triplicates.

4. Sample analysis

Yeast growth was quantified by means of optical density at 600 nm (OD_{600nm}). The titratable acidity, sugar content, and ethanol content were detected according to GB/T 15038-2006 (Chinese National Standard). Specifically, the titratable acidity was determined by titration with 0.1 M NaOH to reach a final pH of 8.2 and expressed as g/L of tartaric acid. The sugar content was determined by the 3,5-dinitrosalicylic acid method, and the ethanol content was measured by an alcoholmeter.

Acetic acid and glycerol were determined enzymatically using commercial test kits according to the manufacturer's instructions (Megazyme, Ireland).

5. RNA extraction, cDNA synthesis, and quantitative real-time PCR (RT-qPCR)

5.1. RNA extraction and cDNA synthesis

Total RNA extraction was performed using a commercial Yeast RNA Kit (Aidlab Biotechnologies Co., Ltd., Beijing, China) with slight modifications. The yeast cells were harvested by centrifugation (Baiyang Medical Devices Co., Ltd., Beijing, China) at 1000 g for 5 min at 4 °C. After centrifugation, yeast cells were added into 350 µL extraction buffer containing 50 mg glass beads. Then, cells were disrupted using a vortex instrument (Qilin Bell Instrument Manufacturing Co., Ltd., Haimen, China) for 5 min, followed by chilling on ice for 5 min. This cell disruption treatment step was repeated three times. The obtained cell suspension was centrifuged at 13,000 g for 3 min at 4 °C. RNA was precipitated from the suspension with 700 µL of 70 % (v/v) ethanol. The RNA purification and extraction were performed according to the manufacturer's instructions (Aidlab Biotechnologies Co., Ltd., Beijing, China).

TABLE 1. Genes and primers used for RT-qPCR.

Gene name	NCBI Gene ID	Forward and reverse primers (5′- 3′)
PDC1	850733	CTTACGCCGCTGATGGTTA GGCAATACCGTTCAAAGCAG
ALD6	856044	TCTCTTCTGCCACCACTGAA CCTCTTTCTCTTGGGTCTTGG
ACS2	850846	ATTGGTCCTTTCGCCTCAC GCTGTTCGGCTTCGTTAGA
GPD1	851539	TTTTGCCCCGTATCTGTAGC TGGACACCTTTAGCACCAACT
PGK1	850370	GGTAACACCGTCATCATTGG AAGCACCACCACCAGTAGAGA

The extracted RNA was quantified by measuring absorbance at 260 nm using an ultra-micro spectrophotometer (Nanodrop). Two µg of total RNA was treated with 5 U of DNase (Thermo Fisher Scientific, France). Then, cDNAs were synthesised using the TRUEScript cDNA Synthesis Kit (Aidlab Biotechnologies Co. Ltd., Beijing, China) as described by the manufacturer. RT-qPCR was employed to determine the presence or absence of chromosomal DNA contamination.

5.2. Primers design

The primers (of target and housekeeping reference genes) used for RT-qPCR were designed using the free online Primer 5.0 software (Table 1) with a length of about 18–22 bp, a G/C content of over 50 %, and a melting temperature (Tm) of about 60 °C. The size of PCR products ranged from 90 to 120 bp. Oligo analyser 1.0.3.0 software was used to control the formation of secondary structure and dimer. Primer specificity and PCR product size were calculated based on the whole genome of *S. cerevisiae* strain ES288C. *PGK1* (Table 1) was used as a housekeeping reference gene since its expression was independent of growth conditions (Sadoudi *et al*., 2017).

5.3. RT-qPCR assays

RT-qPCR was performed in a 20 µL reaction system containing 2.0 µL of cDNA, 10.0 µL of Master Mix (Universal), 1.0 µL of primer (forward and reverse, 7.0 pmol/µL), and 7.0 µL of Rnase-free water. The RT-qPCR program was as follows: pre-denaturation at 95 °C for 30 s, 40 cycles of denaturation at 95 °C for 20 s, annealing at 60 °C for 20 s, and extension at 72 °C for 10 s, followed by a final extension at 72 °C for 5 min. Afterwards, a melting curve was constructed by raising the temperature from 65 to 95 °C at 0.05 °C/s to test PCR specificity, chromosomal DNA contamination, and primer dimers.

The relative expression of a given gene was calculated using the method (Schmittgen and Livak, 2008). The results were normalised by using the reference gene *PGK1* (Table 1). The amount of sample target RNA was adjusted to a control target RNA.

$\Delta C_T = C_T$ gene of target - C_T reference gene

$\Delta\Delta C_T = \Delta C_T$ of sample - ΔC_T of control

Relative expression level = $2^{-\Delta\Delta C_T}$

In the above formulae, the control indicated the target RNA of *S. cerevisiae* in whole-batch fermentation under the HG condition, and the sample referred to the target RNA of *S. cerevisiae* in continuous fed-batch fermentation under the HG condition.

In this study, significant down-regulation or up-regulation of gene expression was defined as their relative expression levels at least two-fold lower or higher than the control group, as previously described (Desroche *et al*., 2005).

6. Determination of volatile compounds

The volatile compounds were extracted using a manual solid-phase microextraction (SPME) device equipped with a 50/30 µm DVB/CAR/PDMS fibre (Supelco, Bellefonte, PA, USA). The 5 mL of wine was put into a 10 mL vial. The 20 µL of cyclohexanone (0.946 mg/mL) was added to the vial as an internal standard. The vial was sealed, and the mixture solution was equilibrated in a 50 °C water bath for 10 min, and then the volatile compounds were headspace extracted at 60 °C for 40 min and then thermally desorbed in the injection port at 250 °C for 5 min.

Volatile compounds were detected using gas chromatography-mass spectrometry (GC-MS) with an Agilent 7890 gas chromatograph and quadrupole mass selective detector 5977A (Santa Clara, CA, USA). The mass spectral ionisation temperature was set as 230 °C, and the mass spectrometer voltage was set as 70 eV. The *m*/*z* of mass spectra ranged from 30 to 550 amu/sec.

The volatile compounds separation was performed on a DB-WAX column (30 m × 320 µm × 0.25 µm). The injector temperature was set at 250 °C. The temperature was programmed as follows: at 40 °C for 3 min, and then

heated to 160 °C (held for 2 min) at 3 °C/min, and finally increased to 220 °C (maintained for 3 min) at 8 °C/min. Volatile components were identified based on MS libraries (NIST 14) and semi-quantified, referring to the internal standard.

7. Statistical analysis

Statistical analysis was performed using SPSS statistics software (V.17.0, SPSS Inc., Chicago, IL, USA). The Duncan test and independent sample t-test were conducted to determine the statistical significance between groups. The diagrams were plotted by OriginPro software (V.8.5, Southampton, MA, USA).

RESULTS AND DISCUSSION

1. Fermentation kinetics during whole-batch fermentations with different initial sugar concentrations

In fermentation with an initial sugar concentration of 210 g/L, sugar was completely consumed within 10 days. Conversely, high-gravity groups with initial sugar concentrations of 320–450 g/L exhibited 44.67–164.67 g/L residual sugar after 16 day of fermentation (Figure 2, Table 2).

Acetic acid is mainly produced by *S. cerevisiae* for the redox balance in the early stage of alcoholic fermentation (Vilela-Moura *et al*., 2011). In the 210 g/L initial sugar group, acetic acid content was increased slowly in the first 6 days and then gradually plateaued at 0.41 g/L. In contrast, in the 320–450 g/L initial sugar groups, acetic acid content was increased rapidly, resulting in 1.06–1.62 g/L acetic acid concentration in final wines (Figure 2, Table 2). Our results were consistent with the previous report that *S. cerevisiae* can produce more acetic acids in high-sugar fermentation (Kallitsounakis and Catarino, 2020; Kelly *et al*., 2020). The increased acetic acid may be due to the metabolic regulation of *S. cerevisiae* driven by hyperosmotic stress.

Overall, acetic acid, glycerol, and titratable acidity in the fermented wines were significantly increased with increasing initial sugars of grape juice. In addition, increasing the initial sugar concentration of the juice had a negative impact on the fermentation rate, resulting in higher residual sugars and lower ethanol levels in final wines (Table 2). As previously reported, fermentation rate and yeast activity during fermentation were decreased with increasing wort sugar concentration (Yu *et al*., 2012).

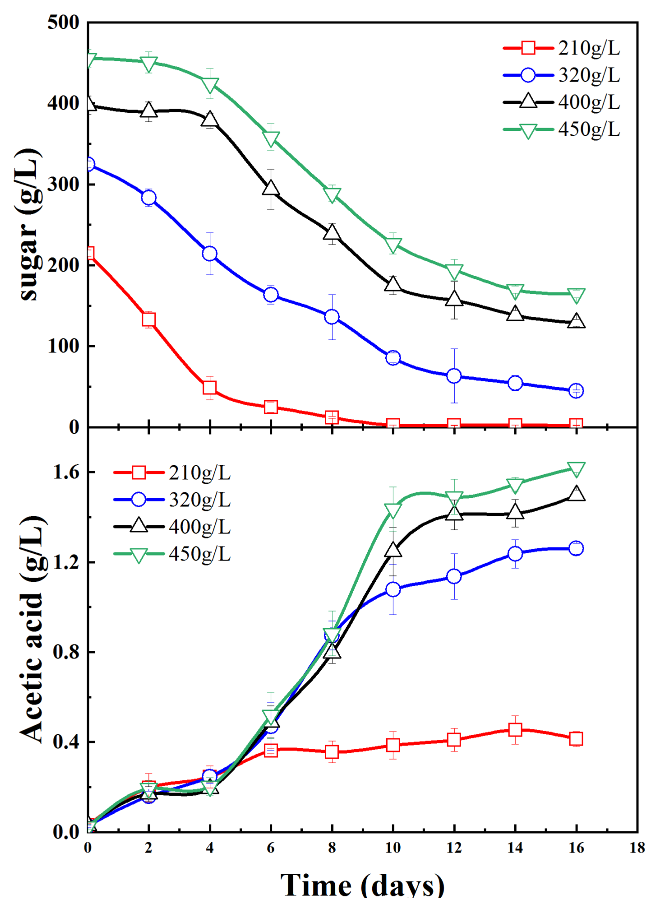

FIGURE 2. Sugar consumption and acetic acid production during whole-batch fermentations.

2. Fermentation kinetics during whole-batch, intermittent fed-batch, and continuous fed-batch fermentations

Under the NG condition, the yeast biomass of the continuous fed-batch group and the intermittent fed-batch group were higher than that of the whole-batch group (Figure 3, NG). Whole-batch and continuous fed-batch fermentations showed similar sugar consumption patterns with the sugar exhausted on day 4 (Figure 3, NG). In contrast, sugar was completely consumed on day 6 in intermittent fed-batch fermentation since a large amount of juice was suddenly fed at 48 h and

TABLE 2. Physicochemical indexes in final wines fermented by whole-batch strategy.

Initial sugar (g/L)	Residual sugar (g/L)	Titratable acidity (g/L)	Acetic acid (g/L)	Glycerol (g/L)	Ethanol (%v/v)
210	2.37 ± 0.11[d]	6.10 ± 0.35[c]	0.41 ± 0.04[d]	5.40 ± 0.23[d]	12.20 ± 0.21[a]
320	44.67 ± 1.08[c]	7.10 ± 0.26[b]	1.06 ± 0.03[c]	8.10 ± 0.25[c]	11.50 ± 0.30[b]
400	129.00 ± 3.74[b]	9.03 ± 0.25[a]	1.49 ± 0.03[b]	9.80 ± 0.40[b]	11.50 ± 0.07[b]
450	164.67 ± 4.14[a]	9.13 ± 0.38[a]	1.62 ± 0.03[a]	12.2 ± 0.49[a]	9.30 ± 0.15[c]

Data are expressed as the mean ± standard deviation (n = 3). The lower-case letters in the same column indicate significant difference at $p < 0.05$.

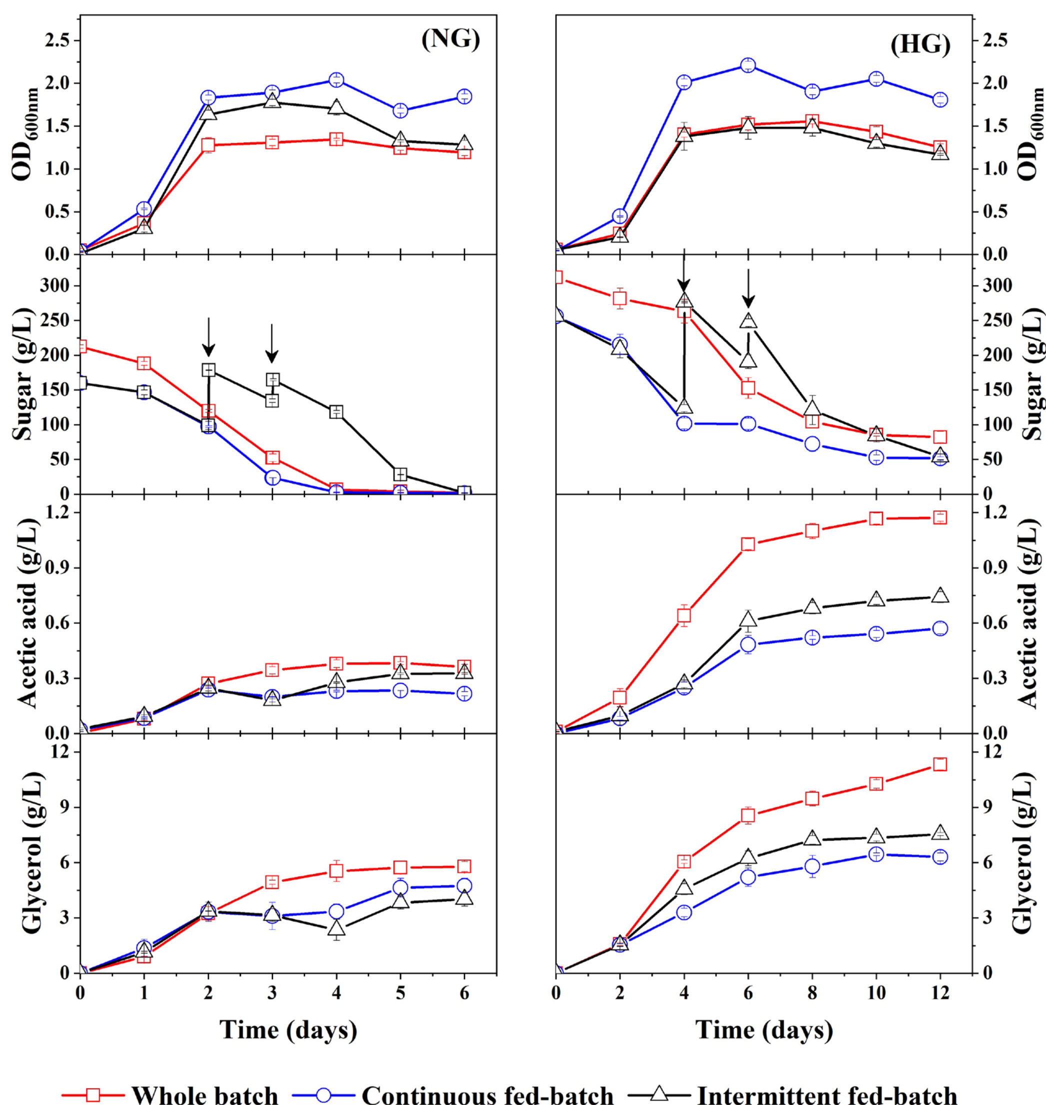

FIGURE 3. Kinetics of yeast growth, sugar, acetic acid, and glycerol contents during fermentations by three strategies under normal-gravity (NG, 210 g/L sugars) and high-gravity (HG, 320 g/L sugars) conditions.

The arrow (↓) points to time points of juice feeding in intermittent fed-batch fermentations.

72 h (Figure 3, NG). Three fermentation strategies exhibited similar acetic acid production patterns within the first 2 days, and then acetic acid was consumed from 48 h to 72 h (feeding phase) in two fed-batch fermentations under NG condition (Figure 3, NG). Consequently, final acetic acid contents in continuous fed-batch fermentation and intermittent fed-batch fermentation were 43.24 % and 10.81 % lower, respectively, than that in the whole-batch fermentation (Table 3). Additionally, whole-batch fermentation showed a faster glycerol production rate and higher final glycerol level compared with two fed-batch fermentations (Table 3).

Under the HG condition, the yeast biomass of the continuous fed-batch was significantly higher than that of the whole-batch and intermittent fed-batch group (Figure 3, HG). About half of the initial sugar was consumed during two fed-batch fermentations in the first 4 days, while only 15.63 % sugar was consumed in whole-batch fermentation (Figure 3, HG). In addition, in continuous fed-batch fermentation, the juice feeding rate was consistent with the sugar consumption rate, which was evidenced by the fact that the sugar concentration stayed at about 100 g/L within 4–6 days (Figure 3, HG). The final residual sugar level was significantly lower in the two fed-batch fermentations than in whole-batch fermentation ($p < 0.05$). The formation pattern of acetic acid was similar in all the fermentations, but continuous fed-batch fermentation exhibited the minimum final acetic acid level, which was 22.97 % lower than intermittent fed-batch fermentation, and 51.69 % lower than whole-batch fermentation (Table 3). Similarly, the glycerol was produced rapidly throughout whole-batch fermentation, resulting significant considerably higher glycerol content (11.80 g/L) in final wines, which was 46.61 % and 36.10 % higher than continuous fed-batch fermentation (6.30 g/L) and intermittent fed-batch fermentation (7.54 g/L), respectively (Table 3).

Under both NG and HG conditions, different fermentation strategies had no significant effect on titratable acidity and

TABLE 3. Physicochemical indexes of final wines fermented by three different strategies under NG and HG conditions.

Condition (g/L)	Fermentation strategy	Residual sugar (g/L)	Titratable acidity (g/L)	Acetic acid (g/L)	Glycerol (g/L)	Ethanol (%v/v)
NG	Whole-batch	1.8 ± 0.02[c]	5.7 ± 0.04[b]	0.37 ± 0.01[c]	5.78 ± 0.19[c]	12.5 ± 0.19[b]
	Continuous fed-batch	1.6 ± 0.42[c]	5.9 ± 0.10[b]	0.21 ± 0.17[d]	4.50 ± 0.26[d]	12.8 ± 0.10[b]
	Intermittent fed-batch	1.9 ± 0.08[c]	6.6 ± 0.05[b]	0.33 ± 0.02[d]	4.74 ± 0.28[d]	12.6 ± 0.32[b]
HG	Whole-batch	82.10 ± 8.20[a]	9.80 ± 0.02[a]	1.18 ± 0.19[a]	11.8 ± 0.29[a]	14.9 ± 0.09[a]
	Continuous fed-batch	51.50 ± 2.50[b]	8.30 ± 0.03[a]	0.57 ± 0.02[c]	6.30 ± 0.22[b]	15.2 ± 0.12[a]
	Intermittent fed-batch	53.80 ± 4.00[b]	8.60 ± 0.16[a]	0.74 ± 0.03[ab]	7.54 ± 0.08[b]	14.8 ± 0.02[a]

Data are expressed as the mean ± standard deviation (n = 3). The lower-case letters in the same column indicate significant difference at $p < 0.05$.

ethanol levels in final wines. However, compared with whole-batch fermentation, the continuous fed-batch fermentation significantly decreased the residual sugar, acetic acid, and glycerol levels in the final wines ($p < 0.05$) (Table 3).

One previous study has reported that batch feeding of grape musts can alleviate the hyperosmotic stress response of *S. cerevisiae* during alcoholic fermentation (Frohman and Mira de Orduna Heidinger, 2018). Consistently, this research showed that fed-batch strategies reduced hyperosmotic stress products (acetic acid and glycerol) of *S. cerevisiae* under both normal and high-gravity fermentation. These results were in line with the report by Frohman and Mira de Orduna (2013) that the automated fed-batch technique can lower acetic acid, glycerol, and acetaldehyde production during fermentation of Riesling must (191.9 g/L sugar) with or without Mg addition. Furthermore, our results showed no significant difference in ethanol levels in wines among the three fermentation strategies. This appeared to be inconsistent with one previous report that fed-batch fermentation achieved higher bioethanol production efficiency compared to batch fermentation under agitation and vortex formation with total glucose concentrations up to 260 g/L (Chang *et al*., 2018). One explanation may be that the effect of fed-batch fermentation on ethanol production is related to the distribution of major carbon fluxes such as glycerol and biomass amount. Further assessment of carbon flux balance will be helpful for the optimisation of fermentation conditions.

3. *PDC1*, *ALD6*, *ACS2*, and *GPD1* expressions during continuous fed-batch fermentation

Previous data suggested that continuous fed-batch fermentation showed slow acetic acid production kinetics and low final acetic acid levels under HG conditions. In this context, the relative expression levels of four genes (*PDC1*, *ALD6*, *ACS2,* and *GPD1*) involved in acetic acid metabolism in *S. cerevisiae* were detected. Samples were taken from both continuous fed-batch and whole-batch fermentation conditions on day 3, day 7, and day 12 and was compared the gene expression profile between the two fermentation conditions (Figure 4).

On day 3 (exponential phase of yeast growth) and day 7 (mid-stationary phase of yeast growth), the expression of *ACS2* during the continuous fed-batch fermentation was significantly higher (3.78 fold) and (2.16 fold) than that during the whole-batch fermentation, respectively. In contrast, *GPD1* expression was significantly down-regulated, which was only 35 %–38 % of the control group. On day 12 (late stationary phase of yeast growth), no significant difference in expression levels of *ACS2* and *GPD1* was observed between the continuous fed-batch fermentation and control group (Figure 4).

It has been reported that *ACS2* overexpression of *S. cerevisiae* reduced acetic acid production during sake fermentation (Akamatsu *et al*., 2000), based on which, we speculated that significant up-regulation of *ACS2* expression of *S. cerevisiae* in the early and middle stages of continuous batch fermentation might be the reasons for the less acetic acid production compared with whole-batch fermentation in the present study. However, another study reported that *ACS2* overexpression of *S. cerevisiae* had no significant effect on acetic acid production during simulated grape juice medium fermentation (Remize *et al*., 2000). These different findings may be attributed to the possibility that the regulation effect of *ACS2* in *S. cerevisiae* on acetic acid production is related to the fermentation substrate. The expression of *GPD1* is required for yeast cell growth under high osmolarity (Albertyn *et al*., 1994), and it is induced by hyperosmotic stress through the so-called HOG (high osmolarity glycerol) signalling pathway (Babazadeh *et al*., 2014; Patel *et al*., 2014). In addition, *GPD1* of *S. cerevisiae* was up-regulated during Icewine fermentation, resulting in higher glycerol and acetic acid levels in final wines compared to diluted Icewine

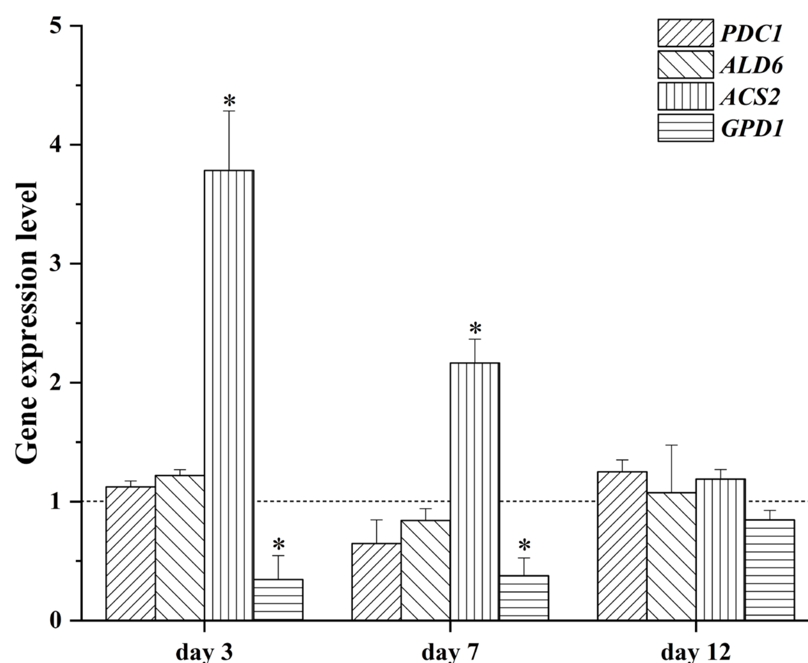

FIGURE 4. *PDC1*, *ALD6*, *ACS2*, and *GPD1* expression levels in *S. cerevisiae* during continuous fed-batch fermentation under HG condition.

Yeast samples of both continuous fed-batch and whole-batch fermentations were collected on day 3, day 7, and day 12. The gene expression levels were calculated as a differential fold change in continuous fed-batch *versus* whole-batch (control). The asterisk (*) indicates that significant difference in gene expression levels between continuous fed-batch fermentation and whole-batch fermentation.

fermentation (Pigeau and Inglis, 2005). Therefore, in this study, down-regulation of the *GPD1* in *S. cerevisiae* may be one reason for low glycerol and acetic acid levels in final wines during continuous fed-batch fermentation under HG conditions.

PDC1 and *ALD6* expressions of *S. cerevisiae* exhibited no obvious difference between continuous fed-batch fermentation and whole-batch fermentation at all three time points (Figure 3). This result was inconsistent with some previous reports that the knockout of *PDC1* and *ALD6* in yeast lowered acetic acid production (Curiel *et al*., 2016; Eglinton *et al*., 2002; Saint-Prix *et al*., 2004). Therefore, low acetic acid production during continuous fed-batch fermentation may be independent of gene expression of *PDC1* and *ALD6*. *ALD3* is another important NAD^+-dependent aldehyde dehydrogenase isoform, which was upregulated 14.6-fold in yeast cells fermenting Icewine juice compared to diluted juice (Heit *et al*., 2018). Further studies could focus on assessing the expression of *ALD3* and redox balance during continuous fed-batch under high-gravity fermentation.

4. Volatile compounds and odour activity values (OAVs) in final wines from whole-batch and continuous fed-batch fermentation

The volatile compounds of whole-batch and continuous fed-batch wines under HG conditions are listed in Table 4. A total of 21 compounds, including 7 esters, 8 alcohols, and 6 acids, were detected in whole-batch fermentation wine, whereas a total of 25 compounds, including 10 esters, 9 alcohols, and 6 acids, were detected in continuous fed-batch fermentation wine. The predominant components in these two wines were propanol, propyl methanol, and isoamyl alcohol. The continuous fed-batch fermentation increased the concentrations of esters in the wines by 56.06 % and decreased the concentrations of acids by 23.24 %, compared with the traditional whole-batch fermentation. Hexanoic acid was the only unique component detected from the whole-batch fermentation wine. In contrast, continuous fed-batch fermentation wine exhibited some unique volatile components, including ethyl 9-decenoate, phenethyl acetate and ethyl laurate, and geraniol. As the important aroma component of wines, ethyl acetate in continuous fed-batch fermentation wine was about three times as much as that in the whole-batch fermentation wine.

Volatile components are important for the quality and characteristics of wine (Petronilho *et al*., 2020). In this study, the volatile components in whole-batch and continuous fed-batch fermentation wines were compared under high-gravity conditions. Isoamyl alcohol was one of the dominant alcohols in both whole-batch fermentation wines and continuous fed-batch fermentation wines, which was consistent with previous reports that isoamyl alcohol was one of the major alcohols in whole-batch fermentation wines (Nurgel *et al*., 2004). There are two main groups of flavour-active esters in wine, including ethyl esters and acetate esters. The ethyl esters comprise an alcohol group (ethanol) and an acid group (short- to medium-chain fatty acids), and the acetate esters comprise an acid group (acetate) and an alcohol group which is either ethanol or a higher alcohol (Sumby *et al*., 2010). Previous reports have shown that higher alcohols mainly derive from amino acid metabolism, while a recent study proved that

TABLE 4. Major volatile compounds and OAVs in wines from whole-batch and continuous fed-batch fermentations under the HG condition.

	Components	Concentration (mg/L)		Odour threshold (mg/L)	OAVs		Aroma characteristics
		Whole-batch	Continuous fed-batch		Whole-batch	Continuous fed-batch	
Esters	Ethyl acetate	13.50 ± 1.10^{b}	42.31 ± 3.40^{a}	7.50	1.80	5.64	fruity, strawberry
	Ethyl butyrate	1.01 ± 0.00^{b}	1.17 ± 0.60^{a}	0.02	50.50	58.50	fruity, strawberry
	Ethyl decanoate	20.31 ± 1.40^{a}	25.30 ± 4.50^{a}	2.00	10.16	12.65	fruity, fatty
	Ethyl 2-methyl-butyrate	12.30 ± 1.20^{b}	17.50 ± 1.30^{a}	0.20	61.50	87.50	fruity, pineapple
	Ethyl 9-decenoate	n.d.	0.91 ± 0.00	0.30	0.00	3.03	cheese
	Amyl propionate	1.25 ± 0.90^{a}	1.01 ± 0.10^{a}	0.03	41.67	33.67	-
	Diethyl succinate	10.13 ± 3.00^{b}	15.33 ± 1.50^{a}	200.00	0.05	0.08	grape
	Phenylethyl acetate	n.d.	0.26 ± 0.00	0.25	0.00	1.04	fruity, roses
	Ethyl heptanoate	0.87 ± 0.10^{a}	1.10 ± 0.20^{a}	0.22	3.95	5.00	pineapple
	Ethyl laurate	n.d.	2.21 ± 0.10	0.10	0.00	22.10	fruity, flower fragrance
	Subtotal	66.57 ± 7.70^{b}	103.89 ± 11.70^{a}				
Alcohols	Propanol	69.03 ± 12.60^{a}	63.43 ± 1.10^{b}	50.00	1.38	1.27	alcohol
	2-Phenylethanol	37.07 ± 1.40^{a}	36.08 ± 0.40^{b}	14.00	2.65	2.58	roses, honey
	Propyl methanol	67.06 ± 0.10^{b}	87.02 ± 3.10^{a}	200.00	0.34	0.44	-
	Hexanol	2.49 ± 0.10^{b}	3.20 ± 0.20^{a}	7.00	0.36	0.46	herbal
	Isoamyl alcohol	82.07+0.30^{a}	79.08 ± 1.90^{b}	300.00	0.27	0.26	fruity, alcohol
	Heptanol	52.02+0.20^{a}	41.09 ± 2.00^{b}	2.50	20.81	16.44	oily
	Geraniol	n.d.	3.56 ± 0.10	0.40	0.00	11.40	roses
	Neroli tertiary alcohol	0.14 ± 0.00^{b}	0.22 ± 0.00^{a}	0.70	0.20	0.31	-
	3-methyl butanol	0.10 ± 0.00^{b}	0.96 ± 0.30^{a}	0.60	0.17	1.60	malty
	Subtotal	309.98 ± 14.70^{a}	314.64 ± 9.10^{a}				
Acids	Octanoic acid	47.08 ± 4.00^{a}	38.03 ± 2.00^{b}	0.50	94.16	76.06	cheese
	Hexanoic acid	0.60 ± 0.00	n.d.	0.02	30.00	0.00	rancid, sweaty
	Valeric acid	n.d.	0.22 ± 0.00	0.03	0.00	7.33	-
	Decanoic acid	28.01 ± 0.30^{a}	26.00 ± 0.60^{b}	1.00	28.01	26.00	fatty, rancid
	Benzoic acid	1.24 ± 0.40^{a}	1.35 ± 0.30^{a}	1.20	1.03	1.13	-
	Cis-3-hexenoic acid	7.10 ± 0.00^{a}	1.71 ± 0.50^{b}	0.40	16.63	4.28	green
	2-methylpropionic acid	5.80 ± 0.30^{a}	1.30 ± 0.10^{b}	0.20	29.00	6.50	rancid
	Subtotal	89.38 ± 5.00^{a}	68.61 ± 3.50^{b}				
Total		466.38 ± 27.40^{a}	461.14 ± 24.30^{a}				

Concentrations of volatile compounds are expressed as the mean ± standard deviation (n = 3). The lower-case letters in the same column indicate significant difference at $p < 0.05$. "n.d." indicates not detected. Threshold odour and aroma characteristics of volatile components are obtained from published literature (Cai *et al.*, 2014; Callejon *et al.*, 2008; Dein *et al.*, 2021; Escudero, *et al.*, 2007; Ferreira *et al.*, 2002; Jiaming *et al.*, 2016; Lu *et al.*, 2022; Niimi *et al.*, 2020).

more than 90 % of the higher alcohols (and their acetate ester derivatives) were derived from intermediates produced by the central carbon metabolism using ^{13}C-isotope labelling-based analysis during wine fermentation (Rollero *et al*., 2017). Our data showed that the concentration of both ethyl esters and acetate esters in continuous fed-batch fermentation wine was significantly higher than that in whole-batch fermentation wine. We speculated that up-regulation of *ACS2* expression increased acetyl-CoA production and promoted fatty acid synthesis in continuous fed-batch fermentation. Moreover, ester synthesis in *S. cerevisiae* has been reported to contribute to the detoxification of fatty acids (Legras *et al*., 2010). Moreover, the increased acetyl-CoA production in yeast under the continuous fed-batch conditions would support increased cell growth and reproduction. Further investigation of enzyme activity and flux distribution will help to elucidate the effect of continuous fed-batch strategy on the production of fermentative aromas during wine fermentation.

The OAV is calculated by dividing the mean concentration of volatile compound concentration by the odour threshold (Bowen and Reynolds, 2012). If OAV > 1, the volatile compound is considered to be above its sensory threshold and is said to contribute to the aroma of the product. The higher OAV, the more contribution volatile compounds make to the aroma of the product (Arcari *et al*., 2017; Welke *et al*., 2014). In this study, 15 and 21 volatile compounds were above their sensory threshold (OAV > 1) in whole-batch fermentation wine and continuous fed-batch fermentation wine, respectively, indicating aromas produced from continuous fed-batch fermentation wine were richer than that from whole-batch fermentation wine. Esters play a crucial role in wine-berry fruit aromas (Escudero *et al*., 2007). In this research, continuous fed-batch fermentation wine has been found to have higher OAVs than whole-batch fermentation wine for most ester compounds. Especially, OAVs of ethyl acetate, ethyl 9-decenoate, phenylethyl acetate, ethyl laurate in continuous fed-batch fermentation wine were over three times as high as those of whole-batch fermentation wine (Table 4). These esters contributed to the flower, fruity, and fatty aromas in wine (Cai *et al*., 2014; Wang *et al*., 2016; Lu *et al*., 2022). The OAVs of hexanoic acid, cis-3-hexenoic acid, and 2-methylpropionic acid in whole-batch fermentation wine were over four times as high as those of continuous fed-batch fermentation wine (Table 4), which might lead to more green, sweaty, and rancid flavours of wine (Cai *et al*., 2014; Dein *et al*., 2021; Ferreira *et al*., 2002).

CONCLUSIONS

In very high-gravity juice fermentations such as Icewine and late harvest wines, the acetic acid and glycerol production were significantly increased with increasing initial sugar concentration. A continuous fed-batch strategy could effectively reduce acetic acid production during high-gravity fermentation, and this may be related to the *ACS2* up-regulation and *GPD1* down-regulation in *S. cerevisiae*. In addition, a continuous fed-batch fermentation strategy was beneficial to increase the volatile esters and may enhance flower, fruity, and fatty aromas in wine, but future studies on how these compounds alter the sensory profile of the resulting wines will be needed. This study provides a theoretical reference for the application of a continuous fed-batch strategy in high-gravity juice fermentation. Further evaluation of the suitability of this technique in a winery will require the automation of sugar determination and feeding rate adaptation, as well as special fermenters with online detection probes.

ACKNOWLEDGEMENTS

This research was financially supported by Fundamental Research Funds for the National Natural Science Foundation of China [32272294]. The authors thank the anonymous reviewers for their valuable advice.

REFERENCES

Akamatsu, S., Kamiya, H., Yamashita, N., Motoyoshi, T., Goto-Yamamoto, N., Ishikawa, T., Okazaki, N., & Nishimura, A. (2000). Effects of aldehyde dehydrogenase and acetyl-CoA synthetase on acetate formation in sake mash. *Journal of Bioscience and Bioengineering, 90* (5), 555-560. https://doi.org/10.1016/S1389-1723(01)80040-2

Albertyn, J., Hohmann, S., Thevelein, J. M., & Prior, B. A. (1994). *GPD1*, which encodes glycerol-3-phosphate dehydrogenase, is essential for growth under osmotic stress in *Saccharomyces cerevisiae*, and its expression is regulated by the high-osmolarity glycerol response pathway. *Molecular and Cellular Biology, 14* (6), 4135-4144. https://doi.org/10.1128/mcb.14.6.4135

Arcari, S. G., Caliari, V., Sganzerla, M., & Godoy, H. T. (2017). Volatile composition of Merlot red wine and its contribution to the aroma: optimization and validation of analytical method. *Talanta, 174*, 752-766. https://doi.org/10.1016/j.talanta.2017.06.074

Babazadeh, R., Furukawa, T., Hohmann, S., & Furukawa, K. (2014). Rewiring yeast osmostress signalling through the MAPK network reveals essential and non-essential roles of Hog1 in osmoadaptation. *Scientific Reports, 4*. https://doi.org/10.1038/srep04697

Bely, M., Masneuf-Pomarede, I., & Dubourdieu, D. (2005). Influence of physiological state of inoculum on volatile acidity production by *Saccharomyces cerevisiae* during high sugar fermentation. *Journal International des Sciences de la Vigne et du Vin, 39* (4), 191-197. https://doi.org/10.20870/oeno-one.2005.39.4.886

Bely, M., Rinaldi, A., & Dubourdieu, D. (2003). Influence of assimilable nitrogen on volatile acidity production by *Saccharomyces cerevisiae* during high sugar fermentation. *Journal of Bioscience & Bioengineering, 96* (6), 507-512. https://doi.org/10.1016/s1389-1723(04)70141-3

Bowen, A. J., & Reynolds, A. G. (2012). Odor potency of aroma compounds in riesling and vidal blanc table wines and icewines by gas chromatography-olfactometry-mass spectrometry. *Journal of Agricultural and Food Chemistry, 60* (11), 2874-2883. https://doi.org/10.1021/jf203314j

Cai, J., Zhu, B.-Q., Wang, Y.-H., Lu, L., Lan, Y.-B., Reeves, M. J., & Duan, C.-Q. (2014). Influence of pre-fermentation cold maceration treatment on aroma compounds of cabernet sauvignon wines

fermented in different industrial scale fermenters. *Food Chemistry, 154*, 217-229. https://doi.org/10.1016/j.foodchem.2014.01.003

Callejon, R. M., Lourdes Morales, M., Silva Ferreira, A. C., & Troncoso, A. M. (2008). Defining the typical aroma of sherry vinegar: sensory and chemical approach. *Journal of Agricultural and Food Chemistry, 56* (17), 8086-8095. https://doi.org/10.1021/jf800903n

Chang, Y. H., Chang, K. S., Chen, C. Y., Hsu, C. L., Chang, T. C., & Jang, H. D. (2018). Enhancement of the efficiency of bioethanol production by *Saccharomyces cerevisiae* via gradually batch-wise and fed-batch increasing the glucose concentration. *Fermentation-Basel, 4* (2). https://doi.org/10.3390/fermentation4020045

Curiel, J. A., Salvado, Z., Tronchoni, J., Morales, P., Rodrigues, A. J., Quiros, M., & Gonzalez, R. (2016). Identification of target genes to control acetate yield during aerobic fermentation with *Saccharomyces cerevisiae*. *Microbial Cell Factories, 15*. https://doi.org/10.1186/s12934-016-0555-y

Dein, M., Kerley, T., & Munafo, J. P. (2021). Characterization of odorants in a 10-year-old riesling wine. *Journal of Agricultural and Food Chemistry, 69* (38), 11372-11381. https://doi.org/10.1021/acs.jafc.1c04196

Desroche, N., Beltramo, C., & Guzzo, J. (2005). Determination of an internal control to apply reverse transcription quantitative PCR to study stress response in the lactic acid bacterium *oenococcus oeni*. *Journal of Microbiological Methods, 60* (3), 325-333. https://doi.org/10.1016/j.mimet.2004.10.010

Eglinton, J. M., Heinrich, A. J., Pollnitz, A. P., Langridge, P., Henschke, P. A., & Lopes, M. D. (2002). Decreasing acetic acid accumulation by a glycerol overproducing strain of *Saccharomyces cerevisiae* by deleting the *ALD6* aldehyde dehydrogenase gene. *Yeast, 19* (4), 295-301. https://doi.org/10.1002/yea.834

Escudero, A., Campo, E., Farina, L., Cacho, J., & Ferreira, V. (2007). Analytical characterization of the aroma of five premium red wines. Insights into the role of odor families and the concept of fruitiness of wines. *Journal of Agricultural and Food Chemistry, 55* (11), 4501-4510. https://doi.org/10.1021/jf0636418

Ferreira, V., Ortin, N., Escudero, A., Lopez, R., & Cacho, J. (2002). Chemical characterization of the aroma of Grenache rosé wines: aroma extract dilution analysis, quantitative determination, and sensory reconstitution studies. *Journal of Agricultural and Food Chemistry, 50* (14), 4048-4054. https://doi.org/10.1021/jf0115645

Frohman, C. A., & Mira de Orduna, R. (2013). Cellular viability and kinetics of osmotic stress associated metabolites of *Saccharomyces cerevisiae* during traditional batch and fed-batch alcoholic fermentations at constant sugar concentrations. *Food Research International, 53* (1), 551-555. https://doi.org/10.1016/j.foodres.2013.05.020

Frohman, C. A., & Mira de Orduna Heidinger, R. (2018). The substratostat - an automated near-infrared spectroscopy-based variable-feed system for the continuous alcoholic fed-batch fermentation of high gravity grape must at static sugar concentrations. *OENO One, 52* (4), 279-289. https://doi.org/10.20870/oeno-one.2018.52.4.2199

Guaragnella, N., Antonacci, L., Passarella, S., Marra, E., & Giannattasio, S. (2011). Achievements and perspectives in yeast acetic acid-induced programmed cell death pathways. *Biochemical Society Transactions, 39*, 1538-1543. https://doi.org/10.1042/bst0391538

Heit, C., Martin, S. J., Yang, F., & Inglis, D. L. (2018). Osmoadaptation of wine yeast (*Saccharomyces cerevisiae*) during Icewine fermentation leads to high levels of acetic acid. *Journal of Applied Microbiology, 124* (6), 1506-1520. https://doi.org/10.1111/jam.13733

Jain, V. K., Divol, B., Prior, B. A., & Bauer, F. F. (2011). Elimination of glycerol and replacement with alternative products in ethanol fermentation by *Saccharomyces cerevisiae*. *Journal of Industrial Microbiology & Biotechnology, 38* (9), 1427-1435. https://doi.org/10.1007/s10295-010-0928-x

Kallitsounakis, G., & Catarino, S. (2020). An overview on botrytized wines. *Ciencia e Tecnica Vitivinicola, 35* (2), 76-106. https://doi.org/10.1051/ctv/20203502076

Kelly, J. M., Van Dyk, S. A., Dowling, L. K., Pickering, G. J., Kemp, B., & Inglis, D. L. (2020). *Saccharomyces uvarum* yeast isolate consumes acetic acid during fermentation of high sugar juice and juice with high starting volatile acidity. *OENO One, 54* (2), 199-211. https://doi.org/10.20870/oeno-one.2020.54.2.2594

Legras, J. L., Erny, C., & Le, J. C. (2010). Activation of two different resistance mechanisms in *Saccharomyces cerevisiae* upon exposure to octanoic and decanoic acids. *Applied and Environmental Microbiology, 76*, 7526-7535. https://doi.org/10.1128/aem.01280-10

Lemos, D. A., Sonego, J. L. S., Cruz, A. J. G., & Badino, A. C. (2020). Improvement of ethanol production by extractive fed-batch fermentation in a drop column bioreactor. *Bioprocess and Biosystems Engineering, 43* (12), 2295-2303. https://doi.org/10.1007/s00449-020-02414-5

Liu, G. L., Bi, X. Y., Tao, C. L., Fei, Y. T., Gao, S. J., Liang, J. L., & Bai, W. D. (2021). Comparative transcriptomics analysis of *Zygosaccharomyces mellis* under high-glucose stress. *Food Science and Human Wellness, 10* (1), 54-62. https://doi.org/10.1016/j.fshw.2020.05.006

Lu, L., Mi, J., Chen, X., Luo, Q., Li, X., He, J., Zhao, R., Jin, B., Yan, Y., & Cao, Y. (2022). Analysis on volatile components of co-fermented fruit wines by *Lycium ruthenicum* murray and wine grapes. *Food Science and Technology, 42*. https://doi.org/10.1590/fst.12321

Malhi, G. S., Kaur, M., & Kaushik, P. (2021). Impact of climate change on agriculture and its mitigation strategies: a review. *Sustainability, 13* (3). https://doi.org/10.3390/su13031318

Meaden, P. G., Dickinson, F. M., Mifsud, A., Tessier, W., Westwater, J., Bussey, H., & Midgley, M. (1997). The *ALD6* gene of *Saccharomyces cerevisiae* encodes a cytosolic, Mg^{2+}-activated acetaldehyde dehydrogenase. *Yeast, 13* (14), 1319-1327. https://doi.org/10.1002/(sici)1097-0061(199711)13:14<1319::Aid-yea183>3.0.Co;2-t

Milanovic, V., Ciani, M., Oro, L., & Comitini, F. (2012). *Starmerella bombicola* influences the metabolism of *Saccharomyces cerevisiae* at pyruvate decarboxylase and alcohol dehydrogenase level during mixed wine fermentation. *Microbial Cell Factories, 11*. https://doi.org/10.1186/1475-2859-11-18

Mira de Orduna, R. (2010). Climate change associated effects on grape and wine quality and production. *Food Research International, 43* (7), 1844-1855. https://doi.org/10.1016/j.foodres.2010.05.001

Niimi, J., Guixer, B., & Splivallo, R. (2020). Odour active compounds determined in the headspace of yellow and black plum wines (*Prunus domestica* L.). *Lwt-Food Science and Technology, 130*. https://doi.org/10.1016/j.lwt.2020.109702

Nurgel, C., Pickering, G. J., & Inglis, D. L. (2004). Sensory and chemical characteristics of Canadian ice wines. *Journal of the Science of Food and Agriculture, 84* (13), 1675-1684. https://doi.org/10.1002/jsfa.1860

Patel, A. K., Bhartiya, S., & Venkatesh, K. V. (2014). Analysis of osmoadaptation system in budding yeast suggests that regulated degradation of glycerol synthesis enzyme is key to near-perfect adaptation. *Systems and Synthetic Biology, 8* (2), 141-154. https://doi.org/10.1007/s11693-013-9126-2

Petronilho, S., Lopez, R., Ferreira, V., Coimbra, M. A., & Rocha, S. M. (2020). Revealing the usefulness of aroma networks to explain wine aroma properties: a case study of Portuguese wines. *Molecules, 25* (2). https://doi.org/10.3390/molecules25020272

Pigeau, G. M., Bozza, E., Kaiser, K., & Inglis, D. L. (2007). Concentration effect of Riesling Icewine juice on yeast performance and wine acidity. *Journal of Applied Microbiology, 103* (5), 1691-1698. https://doi.org/10.1111/j.1365-2672.2007.03397.x

Pigeau, G. M., & Inglis, D. L. (2005). Upregulation of *ALD3* and *GPD1* in *Saccharomyces cerevisiae* during Icewine fermentation. *Journal of Applied Microbiology, 99* (1), 112-125. https://doi.org/10.1111/j.1365-2672.2005.02577.x

Remize, F., Andrieu, E., & Dequin, S. (2000). Engineering of the pyruvate dehydrogenase bypass in *Saccharomyces cerevisiae*: role of the cytosolic Mg^{2+} and mitochondrial K^{+} acetaldehyde dehydrogenases Ald6p and Ald4p in acetate formation during alcoholic fermentation. *Applied and Environmental Microbiology, 66* (8), 3151-3159. https://doi.org/10.1128/aem.66.8.3151-3159.2000

Remize, F., Cambon, B., Barnavon, L., & Dequin, S. (2003). Glycerol formation during wine fermentation is mainly linked to Gpd1p and is only partially controlled by the HOG pathway. *Yeast, 20* (15), 1243-1253. https://doi.org/10.1002/yea.1041

Rollero, S., Mouret, J. R., Bloem, A., Sanchez, I., Ortiz-Julien, A., Sablayrolles, J. M.. & Dequin, S. (2017). Quantitative ^{13}C-isotope labelling-based analysis to elucidate the influence of environmental parameters on the production of fermentative aromas during wine fermentation. *Microbial Biotechnology, 10*, 1649-1662. https://doi.org/ 10.1111/1751-7915.12749

Sadoudi, M., Rousseaux, S., David, V., Alexandre, H., & Tourdot-Marechal, R. (2017). *Metschnikowia pulcherrima* influences the expression of genes involved in PDH bypass and glyceropyruvic fermentation in *Saccharomyces cerevisiae*. *Frontiers in Microbiology, 8*. https://doi.org/10.3389/fmicb.2017.01137

Saint-Prix, F., Bonquist, L., & Dequin, S. (2004). Functional analysis of the *ALD* gene family of *Saccharomyces cerevisiae* during anaerobic growth on glucose: the $NADP^{+}$-dependent Ald6p and Ald5p isoforms play a major role in acetate formation. *Microbiology-Sgm, 150*, 2209-2220. https://doi.org/10.1099/mic.0.26999-0

Schmittgen, T. D., & Livak, K. J. (2008). Analyzing real-time PCR data by the comparative C_T method. *Nature Protocol, 3* (6), 1101-1108. https://doi.org/10.1038/nprot.2008.73

Sousa, M., Duarte, A. M., Fernandes, T. R., Chaves, S. R., Pacheco, A., Leao, C., Corte-Real, M., & Sousa, M. J. (2013). Genome-wide identification of genes involved in the positive and negative regulation of acetic acid-induced programmed cell death in *Saccharomyces cerevisiae*. *Bmc Genomics, 14*. https://doi.org/10.1186/1471-2164-14-838

Sumby, K. M., Grbin, P. R., & Jiranek, V. (2010). Microbial modulation of aromatic esters in wine: current knowledge and future prospects. *Food Chemistry, 121* (1), 1-16. https://doi.org/10.1016/j.foodchem.2009.12.004

Van den Berg, M., Jong-Gubbels, P. D., Kortland, C. J., Dijken, J., & Steensma, H. Y. (1996). The two acetyl-coenzyme a synthetases of *Saccharomyces cerevisiae* differ with respect to kinetic properties and transcriptional regulation. *Journal of Biological Chemistry*, 271 (46), 28953-28959. https://doi.org/10.1074/jbc.271.46.28953

Vilela-Moura, A., Schuller, D., Mendes-Faia, A., Silva, R. D., Chaves, S. R., Sousa, M. J., & Corte-Real, M. (2011). The impact of acetate metabolism on yeast fermentative performance and wine quality: reduction of volatile acidity of grape musts and wines. *Applied Microbiology and Biotechnology, 89* (2), 271-280. https://doi.org/10.1007/s00253-010-2898-3

Vilela, A. (2017). Biological demalication and deacetification of musts and wines: can wine yeasts make the wine taste better? *Fermentation-Basel*, *3*(4), 51-Article No.: 51. https://doi.org/10.3390/fermentation3040051

Wang, J., Capone, D. L., Wilkinson, K. L., & Jeffery, D. W. (2016). Chemical and sensory profiles of rose wines from Australia. *Food Chemistry, 196*, 682-693. https://doi.org/10.1016/j.foodchem.2015.09.111

Welke, J. E., Zanus, M., Lazzarotto, M., & Zini, C. A. (2014). Quantitative analysis of headspace volatile compounds using comprehensive two-dimensional gas chromatography and their contribution to the aroma of Chardonnay wine. *Food Research International, 59*, 85-99. https://doi.org/10.1016/j.foodres.2014.02.002

Yang, F., Heit, C., & Inglis, D. L. (2017). Cytosolic redox status of wine yeast (*Saccharomyces cerevisiae*) under hyperosmotic stress during Icewine fermentation. *Fermentation-Basel, 3* (4), 61. https://doi.org/10.3390/fermentation3040061

Yu, Z., Zhou, M., Li, H., Zhao, H., Zhang, Q., Wang, C., & Li, H. (2012). A comparative study on physiological activities of lager and ale brewing yeasts under different gravity conditions. *Biotechnology and Bioprocess Engineering, 17* (4), 818-826. https://doi.org/10.1007/s12257-011-0658-6.