Effects of *Saccharomyces cerevisiae* quorum sensing signal molecules on ethanol production in bioethanol fermentation process

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Abstract : In this study, the concentrations of *Saccharomyces cerevisiae* quorum sensing signal molecules (QSMs) were determined, not to mention the exploration of the effects of exogenous *S. cerevisiae* QSMs on the sole fermentation of *S. cerevisiae* and co-fermentation of *S. cerevisiae* and *Lactobacillus plantarum*. The results showed that the concentrations of three signal molecules (2-phenylethanol (2-PE), tyrosol and tryptophan) produced by *S. cerevisiae* increased with a higher bacteria density, which

tends to become stable up to 118.02, 32.05 and 1.93 mg/L respectively when cultivating for 144 hours. Among the three signaling molecules, only 2-PE promoted the ethanol production capacity of *S. cerevisiae*. The ethanol concentration of the sole fermentation of *S. cerevisiae* loaded with 120 mg/L 2-PE reached 3.2 g/L in 9 hours, which was 58.7% higher than that of the group without 2-PE addition. Moreover, 2-PE reduced the negative impact of *L. plantarum*on *S. cerevisiae*. Within12 hours of the co-fermentation of *L. plantarum*and*S. cerevisiae*, the ethanol concentration in the co-fermentation group loaded with2-PE reached 5.6 g/L, similar to that in the group fermenting with sole *S. cerevisiae*, and the yeast budding rate was also restored to 28.51%. qRT-PCR results of *S. cerevisiae* which was in sole fermentation with 2-PE addition for 9 hours showed that the relative expression levels of ethanol dehydrogenase gene *ADH1* in *S. cerevisiae* decreased by 25% and the relative expression levels of *MLS1*, *CIT2*, *IDH1*,*CIT1* decreased by 26%, 30%, 22%,18%, respectively, meant that the glyoxylic and tricarboxylic acid cycles were greatly inhibited, which promotes the accumulation of ethanol. The results of this study provide basic data for using QSMs more than antibiotics in the prevention of contamination during the industrialized bioethanol production.

Keywords: *Saccharomyces cerevisiae*; quorum sensing signaling molecules; bioethanol; 2-phenylethanol; *Lactobacillus plantarum*

1. Introduction

Bioethanol, a renewable green energy which is of great significance in

alleviating energy pressure, improving environmental problems and achieving global sustainable development. Nowadays, 95% fuel ethanol is produced by microbial fermentation, and bioethanol has become the largest fermentation product in the world (Mohapatra et al., 2019). However, with the development of society and the growth of population, the demand for ethanol production has increased dramatically. The first generation of bioethanol production using food crops as raw materials has failed to meet the current demand for ethanol (Ahorsu et al., 2018).The second generation of bioethanol production using non-food crops such as lignocellulose as raw materials has developed accordingly (Scully and Orlygsson, 2015; Rastogi and Shrivastava, 2017). While the efficient fermentation process of the second generation needs to be established. More and more studies are devoted to finding ways for the higher bioethanol production (Aro, 2016; Sharma et al., 2020).

S. cerevisiae is considered to be the most widely used strain in the industrial bioethanol production due to its short fermentation period, simple growing conditions and large capacity of ethanol production(Widiastuti et al., 2011). In the fermentation process, *S. cerevisiae* converts sugar into ethanol via the glycolysis pathway. Although a high ethanol yield can be obtained, other by-products, such as glycerol, certain acids, lipids and so on, are also produced in the fermentation process(A. and G., 2013). The synthesis of these by-products consumes some raw materials and decreases the yield of ethanol to a certain degree. Kong et al.(2007) constructed two recombinant strains of *S. cerevisiae* by deleting the *GPD2* gene and the over expression of *GLT1*gene, which reduced the formation of glycerol and successfully

increased the ethanol yield. However, in the process of industrial bioethanol fermentation, it is more troubling that bacterial contamination often occurs. Among them, lactic acid bacteria(LAB) are found to be the key bacteria (Brexó and Sant Ana, 2017; Patrick et al., 2019).It has been reported that chronic contamination of LAB would lead to the reduction of sugar, which is an important substrate for the yeast to produce ethanol. The acute contamination of LAB in the bioethanol fermentation process could inhibit the growth of yeast and cause the fermentation to fail via excreting acetic acid, lactic acid and other by-products (Makanjuola et al., 1992; Beckner et al., 2011; Roach et al., 2013). As was reported, Josepg et al. (2015)had isolated 768 species of miscellaneous bacteria in eight ethanol fermentation plants, of which 92% are *Lactobacillus*.Dellias et al.(2018) had isolated contaminant species of *Lactobacillus* from industrial and pilot-scale fermentation processes, among which *Lactobacillus casei*, *Lactobacillus fermentum* and *L. plantarum* were the most abundant strains. So far, *L. plantarum* has been widely used in the study of LAB contamination in the bioethanol fermentation process (Dong et al., 2015; Liu et al., 2017; Li et al., 2018). Particularly, Li et al. (2018)selected *L. plantarumATCC8014* as the target strain which was also used in our experments to simulate LAB contamination in the industrial bioethanol fermentation process. Studies have shown that contaminative *L. plantarum* not only secreted lactic acid to lower the pH value in the fermenter but also competed with *S. cerevisiae* for nutrients and living space (Cui et al., 2015). In addition, Dong et al. (2015)showed that the contaminative *L. plantarum* inhibited the glycolytic pathway of *S. cerevisiae*, resulting in a reduced

ethanol yield. In a contaminated fermenter, the production efficiency of bioethanol can be reduced by about 30% (Brexó and Sant Ana, 2017). Hence, it is necessary to conduct extensive antibacterial methods to avoid the LAB contamination in bioethanol fermentation. So far, adding antibiotics is the most common method to mitigate the LAB contamination in the ethanol fermentation process (Patrick et al., 2019). However, the application of antibiotics promotes the growth of drug-resistant bacteria, and the residual antibiotics in the fermentation broth are toxic to the environment and human beings(Muthaiyan et al., 2011a; Rich et al., 2018). Other prevention methods, such as using bacteriophage or bacteriocins to inhibit the growth of LAB, are infeasible in practice due to the complicated operations and high costs (Muthaiyan et al., 2011b; Worley-Morse et al., 2015). Therefore, it is crucial to find a safe, effective and environmentally friendly method for the prevention of LAB contamination in order to seek sustainable development of the bioethanol industry.

In recent years, quorum sensing has been widely investigated for industrial food preservation, pathogen infection prevention, biofilm processing in wastewater etc (Castillo-Juárez et al., 2015; Siddiqui et al., 2015; Idalina et al., 2020). Quorum sensing is a mechanism of communications among microorganisms, and QSMs are secreted into the environment during the growth of microorganisms. When the concentrations of signal molecules reach the threshold, the expression levels of related genes are regulated, thereby changing the cell behaviors (Fuqua et al., 1994; Christopher and Bonnie, 2005; Atkinson and Williams, 2009), such as bacterial luminescence, biofilm synthesis, and the expression of virulence factors (Benjamin et al., 2018; Paluch et al.,

2020). Similarly, quorum sensing also exists in the growing of *S. cerevisiae*. Hao and Gerald (2006) found that *S. cerevisiae* cells secrete aromatic alcohols as QSMs, which can stimulate their morphogenesis under nitrogen-poor conditions. Also, Zupan et al. (2013) found that 2-PE, tryptophan (TrpOH) and tyrosol (TyrOH) working as signaling molecules existed in the yeast fermenter. According to the results of quorum sensing kinetics, the concentrations of these aromatic alcohols reach peak at the exponential growing phase, and then begin to decrease sharply in the stationary growing phase(Zupan et al., 2013). Recently, more and more studies on the application of *S. cerevisiae* QSMs are focused. Nath et al. (2020) studied the effect of TyrOH on the fermentation performance of *S. cerevisiae*, and the results showed that TyrOH could protect the growth of *S. cerevisiae* under the ethanol mediated oxidative stress. Huang and Reardon(2021) found that2-PE, TrpOH and TyrOH could increase the ethanol production capacity of *S. cerevisiae JAY 270*. However, the effects of exogenous QSMs of *S. cerevisiae* on the performances of bioethanol fermentation processes contaminated with *L. plantarum* has not been reported. Compared to antibiotics, QSMs are safer and friendly to the environment. Altering yeast cell behaviors and thus increasing the ethanol production by adding QSMs provides a new strategy for industrial bioethanol fermentation.

This paper aims to explore the effects of *S. cerevisiae* QSMs on ethanol production in the bioethanol fermentation process. We've selected three typical signal molecules of *S. cerevisiae* (2-PE, TyrOH and TrpOH) and studied their effects on the growth, morphology and ethanol production capacity of *S. cerevisiae* in the sole

fermentationof *S. cerevisiae* and co-fermentation of *S. cerevisiae* and *L. plantarum*, respectively. Moreover, quantitative real-time PCR (qRT-PCR) was used to determine the expression levels of related genes, revealing the changes of the ethanol production pathway in *S. cerevisiae*whenexogenous2-PEwas applied.

2. Materials and methods

2.1 Strains and QSMs

Saccharomyces cerevisiae GIM2.188 and *Lactobacillus plantarum ATCC8014* used in this study were both purchased from Guangdong Microbial Conservation Culture Collection Center. 2-PE, TyrOH and TrpOH were purchased from ANPEL Laboratory Technologies Inc.(Shanghai) with a purity of 97.9%, 98.8% and 99.9%, respectively.

2.2 Media and culture conditions

Saccharomyces cerevisiae GIM2.188 was cultured in the Yeast-Peptone-Dextrose (YPD) medium (2% peptone, 2% glucose, 1% yeast extract, based on w/v) at 28℃, 180rpm (revolutions per minute)in a shaking incubator.*Lactobacillus plantarumATCC8014* was cultured in the de Man-Rogosa-Sharpe (MRS) medium (1% peptone, 1% beef extract, 0.5% yeast extract, 0.2% diammonium hydrogen citrate, 2% glucose, 0.5% sodium acetate, 0.2% potassium phosphate, 0.058% magnesium sulfate, 0.025% manganese sulfate, based on w/v, 0.1% (v/v) Tween 80) at 37℃.

2.3 Sole fermentation experiment

S. cerevisiae was activated from frozen stocks by cultivation on YPD plates, and then inoculated to 50 mL YPD medium to pre-culture at 28℃, 180 rpm for 12 h. The pre-cultured *S. cerevisiae*was inoculated to 180mLYPD medium with the density of 4×10^5 cells/mLcountingby a blood cell plate. The control group (sole fermentation of *S. cerevisiae,* SC) and experimental groups (sole fermentation of *S. cerevisiae*loaded with QSMs,SC + QSMs) were set up. All groups were cultured at 28℃, 180 rpm for 30 h. Each group was performed in triplicate.

2.4 Co-fermentation experiment

Lactobacillus plantarum ATCC8014 is widely used to simulate the LAB contamination in bioethanol fermentation processes(Dong et al., 2015; Liu et al., 2017; Li et al., 2018). In this study, we also selected *Lactobacillus plantarum ATCC8014* as a target strain. It was inoculated into the fermenter culturing with sole *S. cerevisiae*atthe concentration of 1×10^8 cells/mL to simulate the contaminationsituation of *L. plantarum*in the industrial bioethanol fermentation process. A control group (sole fermentation of *S. cerevisiae,* SC), a contaminated group (co-fermentation of *S. cerevisiae* and *L. plantarum*, SC + LP) and a restored group (co-fermentation of *S. cerevisiae* and *L. plantarum* loaded with QSMs, SC + LP + QSMs) were set up. All groups were cultured at 28℃, 180 rpm for 30 h. Each group was run in triplicate.

2.5 Analysis methods

2.5.1 UPLC-MS/MS determination of the concentrations of *S. cerevisiae* quorum sensing signal molecules

Ultra performance liquid chromatography tandem mass spectrometry (ACQUITY UPLC-MS/MS) was used to quantitatively detect QSMs in *S. cerevisiae*. The system was composed of ultra performance liquid chromatography (ACQUITY QSM) (Waters, US) and triple quadrupole mass spectrometry (XEVO-TQD) (Waters, US), equipped with QSM quaternary solvent manager, FTN automatic sampler and CH-A column temperature box. The components were separated on an ACQUITY UPLC BEH C18 column (1.7 μm, 2.1×50 mm). The column temperature was 40℃, and the mobile phase consisted of HPLC grade pure water (0.1% formic acid) and HPLC grade acetonitrile solution (0.1% formic acid). The mobile phase was pumped at a flow rate of 0.300 mL/min, and the linear gradient was used. The initial solution was HPLC grade pure water (0.1% formic acid)/HPLC grade acetonitrile solution (0.1% formic acid) (85/15, v/v). After 6 min, the amount of acetonitrile solution with 0.1% formic acid increased to 45%, and was maintained for 1 min, and than returned to the initial solution at the 8 min. The working conditions of mass spectrometry are:electrospray ionization (ESI+), the capillary voltage of 2.5kV, the ion source temperature of 150℃, the desolvation gas temperature of 400℃, the flow rate of desolvation gas at 600 L/h, the flow rate of cone gas of 50L/h .The detection method of mass spectrometry was multiple reaction monitoring (MRM). The main parameters

of mass spectrometry conditions are listed in TableS1.

The quantitative methods of determining *S. cerevisiae* QSMs were using internal standard curves (Gori et al., 2011; Verbrugghe et al., 2018).The original stock solutions of 1200 mg/L 2-PE, TyrOH and TrpOH were prepared respectively by using methanol as the solvent, and then the original stock solutions were respectively mixed with methanol to obtain the solutions of 250 mg/L 2-PE, 128 mg/L TyrOH and 12.8 mg/L TrpOH. Each solution of 400μL was diluted to 4mL with a mixture of ultrapure water/methanol (75/25, v/v), and named as Solution 1. 2.8mL Solution 1 was added to 1.2mL ultrapure water/methanol (75/25, v/v) to be Solution 2. 2.8mL Solution 2was added to 1.2mL ultrapure water/methanol (75/25, v/v) to get Solution 3, and so on. Solution 1 to Solution 12 were prepared to make the standard curves of 2-PE, TyrOH and TrpOH. The results were shown in Table S2.

The fermentation broth was sampled at an interval of 36 h, and centrifuged at 8000 rpm for 10 min. The supernatant was filtered through a filter (nylon 66, 0.22 μm×13 mm). The samples were stored at -25℃. Before testing, the samples were diluted up to 5-foldby using ultra pure water/methanol (75/25, v/v), and 0.5 mg/L 2-methylindole was added to all samples as an internal standard substance. The recovery efficiencies of 2-PE, TyrOH and TrpOH were shown in Table S3.

2.5.2 Determination of the concentrations of ethanol and glucose

The fermentation broth was centrifuged at 8000 rpm for 10 min and filtered with a 0.22μm filter to obtain the cell-free fermentation supernatant. The concentrations of glucose and ethanol in the supernatant were measured by using 3,5-dinitrosalicylic

acid (DNS) and potassium dichromate colorimetric, respectively(G., 2002; Peng et al., 2019).

2.5.3 Yeast growing curves and cell morphology

When the fermentation broth was diluted to 5-fold, the OD_{600nm} value was measured by a spectrophotometer. The growth curve of yeast was described in the way of X-axis of time and Y-axis of OD_{600nm}. Morphological characteristics of yeast cells were observed by an optical microscope (Mshot MD50-B, China) at 400 times magnification. The budding rate was calculated according to the following formula(Zhang, 2017), by counting at least 500 cells in a random field.

$$\text{Budding rate} = \frac{\text{Number of budding yeast cells}}{\text{Total yeast cells number}} \times 100\%$$

2.5.4 RNA extraction, cDNA synthesis and qRT-PCR analysis

The expression levels of genes encoding carbohydrate and rate-limiting enzymes for the energy metabolism were applied to investigate the effect of 2-PE on the ethanol production pathway in *S. cerevisiae.* According to the results of the sole fermentation experiment, cells were collected at 9 h in the groups of SC and SC + 120 mg/L 2-PE, respectively. After the supernatant was removed by centrifugation, the cells were quickly frozen in liquid nitrogen for 15 min, and then stored at -80℃ for RNA extraction. Total RNA was extracted using the Total RNA Extraction Kit (Tiangen, China). DNA was removed using DNase I working solution (10 μL DNase I stock solution + 70 μL buffer RDD), and the extracted RNA was stored at -70℃. The concentration and purity of RNA were detected by ultraviolet absorption and

RNA integrity was detected by denaturing agarose gel electrophoresis (Table S4 and Figure S4). The cDNA was reversely transcribed using Prime Script TM RT reagent Kit with gDNA Eraser (TaKaRa, China). Genomic DNA removal reactions were listed inTableS5, and the cDNA was stored in a refrigerator at -20℃ for further use. Primers designed by Primer Premier 6were listed in Table 1. qRT-PCR was performed on Applied Biosystems ABI 7500 (Applied Biosystems, USA). The amplification efficiencies of primers were in the range of 90%~105%. The total volume of the reaction was 20 μL, including 2 μL cDNA, 10 μL 2×SYBR Green Master Mix (TaKaRa), 0.5 μL forward and reverse primers, and 7 μL sterile ddH2O. The amplification was running in the procedure of initial denaturation at 95℃ for 10 min followed by 40 cycles of denaturation at 95℃for 15 s and annealing at 60℃ for 1 min. The melting curve of each PCR product was determined to ensure the specific amplification of the target gene (Figure S5). The data were analyzed by $2^{-\triangle\triangle CT}$method(Livak and Schmittgen, 2001).

Table.1 The primer information of genes

Primer name		Sequences (5′to 3′)
HXK2	F	GGTTCCGTTTACAACAGATACCCAG
	R	CAATAACAGCGGCACCAGCA
PFK1	F	ATGCCACCGCTAAATCCCACT
	R	CCAGCCATCAAGGCCAACC
PYK1	F	CAACGCCAGAAAGTCCGAAGA
	R	GTGGTTTGGTGGGATTGGGTAG
ADH1	F	GGACTTCTTCGCCAGAGGTTTG
	R	CGTATCTACCAACGATTTGACCCTT
ZWF1	F	CCCGTGTCCTACCCCACTTG
	R	TTTCCCGAAATGTAGCTGACCAT
ICL1	F	ACACTAACGCTTTAGCTGTCCATAACTT

	R	CCATCGTCCATTTCCCTCTGC
MLS1	F	GACTCGGGCTCCTATCATCTGG
	R	CCCTGTGATTTCCGTTGACCTAT
CIT2	F	TTCCCGGTTATGGTCATGCTG
	R	GTTCAGTCAATACGCCAGGTGC
IDH1	F	ATGAATCCGTCCCTGGTGTAGTG
	R	TGGCGAAGTCAAAGGCAAATC
CIT1	F	CCGTGTTAGACCCCGAAGAAGG
	R	AGGTATTTCACCAGTCAAAAGCAACC
KGD1	F	CACCCATTCCCATTTGCTCAG
	R	TGTGTATGCCCACGAACCCAT
ACT1	F	RCTCCAATGAACCCTAAATCAAACAG
	R	CGGAAGAGTACAAGGACAAAACG

2.6 Statistical analysis

All statistical analyses were performed using SPSS 22.0, and one-way or two-way analysis of variance (ANOVA) and two-tailed t test were used to determine the significant differences between groups. $P<0.05$ was considered to be significantly different.

3. Results and discussion

3.1 Concentrations of quorum sensing signal molecules in *S. cerevisiae* fermentation process

The concentrations of QSMs (2-PE, TyrOH and TrpOH) in *S. cerevisiae* fermentation process increased with the growing of cells (Figure1), which was in consist with the report that the production of QSMs was positively correlated to the cell density(Deborah, 2006). Among the three signaling molecules, 2-PE had the highest concentration. After fermenting for 144 h, the concentration of 2-PE yielded a

peak of 118.02 mg/L, which was 3.7-fold and 61.2-foldhigher than TyrOH and TrpOH, respectively. As reported by Deli et al. (2021),2-PE has a positive effect on the biofilm formation of *S. cerevisiae*, and the biofilm working as a barrier could provide a stable internal environment for yeast cells to cope with adverse environment, such as ethanol stress and nutrient insufficiency.

The concentration of TyrOH was lower than 2-PE, but higher than TrpOH (Figure1). Within the cultivation for 144 h, the concentration of TyrOH reached 32.05 mg/L. Being different to the other two signaling molecules, the concentration of TyrOH was still gradually increasing at a rate of 25.8% after 144 h. Nath et al.(2020)had shown that TyrOH can protect *S. cerevisiae* from the ethanol stress, and reduce the lag time and induce mycelium formation during cell proliferation as well. Compared to 2-PE and TyrOH, TrpOH had a lowest concentration. Within the cultivation of144 h, the content of TrpOH was only 1.93 mg/L. González et al.(2018) showed that TrpOH had a negative impact on the yeast growth, which might be the answer to the lower concentration of TrpOH produced by *Saccharomyces cerevisiae GIM2.188*. However, TrpOH could stimulate the growth of diploid pseudohypha of *S. cerevisiae* when nitrogen was deficiency, which greatly contributed to the cellular foraging reaction and biofilm formation (Reynolds and Fink, 2001; Hao and Gerald, 2006; Winters et al., 2019). In addition, TrpOH had a positive feedback regulation on the transcription of ARO genes which are vital to the production of aromatic alcohols(Deborah, 2006).

In the fermentation process, the signal molecules produced by *S. cerevisiae*

gradually accumulated to their peak. Simultaneously, the regulations of genes and the changes of the cell behaviors would occur which needed further investigation. Therefore, the peak concentrations of the QSMs determined in the sole fermentation of *S. cerevisiae* were selected as the exogenous addition dosages (120 mg/L 2-PE, 35 mg/L TyrOH, 2 mg/L TrpOH) to explore the effect of *S. cerevisiae* QSMs on bioethanol production.

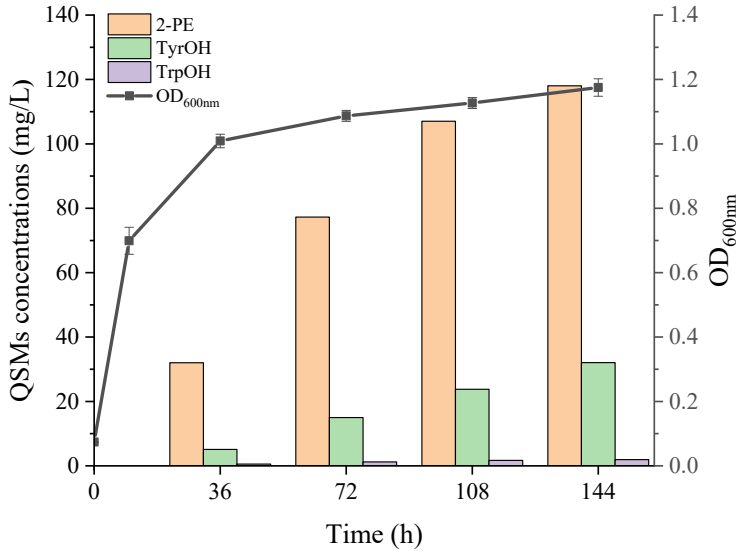


Fig. 1. QSMs concentrations and OD_{600nm} values in *S. cerevisiae* fermentation process

3.2 Effects of exogenous quorum sensing signal molecules in the sole fermentation process of *S. cerevisiae*

The effects of three exogenous QSMs on the cell growth and the ethanol yields in the sole fermentation of *S. cerevisiae* were studied. The results showed that three exogenous QSMs did not affect the growth of *S. cerevisiae* ($P > 0.05$) (Figure 2 (a-c)). This may be the reason that the dosage of the exogenous QSMs is not higher than that

S. cerevisiae itself can produce. Therefore, the growth of *S. cerevisiae* would not be promoted or inhibited. In addition, the growth curves of *S. cerevisiae* in the control group (SC) varied to a certain degree(Figure 2 (a-c)), and the glucose consumption patterns in SC are also not exactly the same (Figure 2 (d-f)). The reason was that the fermentation experiments added with three exogenous QSMs were carried out more than once, and the activities of inoculated *S. cerevisiae* for each experiment were not completely the same. Therefore, the control group (SC) was set up for each experimental trial, and the results of each trial was compared to their owns, avoiding the deviations caused by the activities of inoculated *S. cerevisiae*.

The curves of the ethanol concentrations were similar to that of OD_{600nm}, while the changes of the glucose were in the opposite (Figure 2 (d-f)). Being different from the results of Huang and Reardon(2021), the addition of TyrOH and TrpOH had no significant effect on the increase of the ethanol production ($P > 0.05$) (Figure 2 (e-f)), owing to that the concentrations of TyrOH and TrpOH added in this study did not inhibit the growth of *S. cerevisiae*, so the carbon flux in *S. cerevisiae* did not shift from cell growth to ethanol production. It is worth noting that the growth of *S. cerevisiae* was also not inhibited at 9 h by adding 120, 60 and 30 mg/L 2-PE, but the ethanol concentrations significantly increased by 58.7%, 54.7% and 44.8%, respectively, compared to the control group ($P < 0.01$). However, at 12 h, only the ethanol concentration of the experimental group added with 120 mg/L 2-PE was significantly increased by 8.9% compared to the control group($P=0.025 < 0.05$) (Figure 3).It has been reported that exogenous 2-PE can stimulate the biofilm

formation of *S. cerevisiae* at a low cell density, because the expression level of *FLO11* gene increased in *S. cerevisiae*, and *FLO11* encodes a flocculating protein, which plays a key role in the biofilm formation((Deli et al., 2021; Guo et al., 2000; Yang et al., 2018). The formation of biofilm promoted the yeast cell`s tolerance to ethanol, thereby increasing the ethanol production. However, 2-PE did not promote the ethanol production at 24~30h (P> 0.05), since the glucose in this period was depleted (Figure 2d). No sufficient glucose was available in the fermentation process for the yeast cells to produce ethanol, and the yeast cells growing mode changed from the ethanol fermentation to the cell respiration. These results showed that *S. cerevisiae* QSMs(2-PE, TyrOH and TrpOH) had no effect on the growth of *S. cerevisiae*, but 2-PE could promote the biofilm formation and then increase the ethanol production capacity.

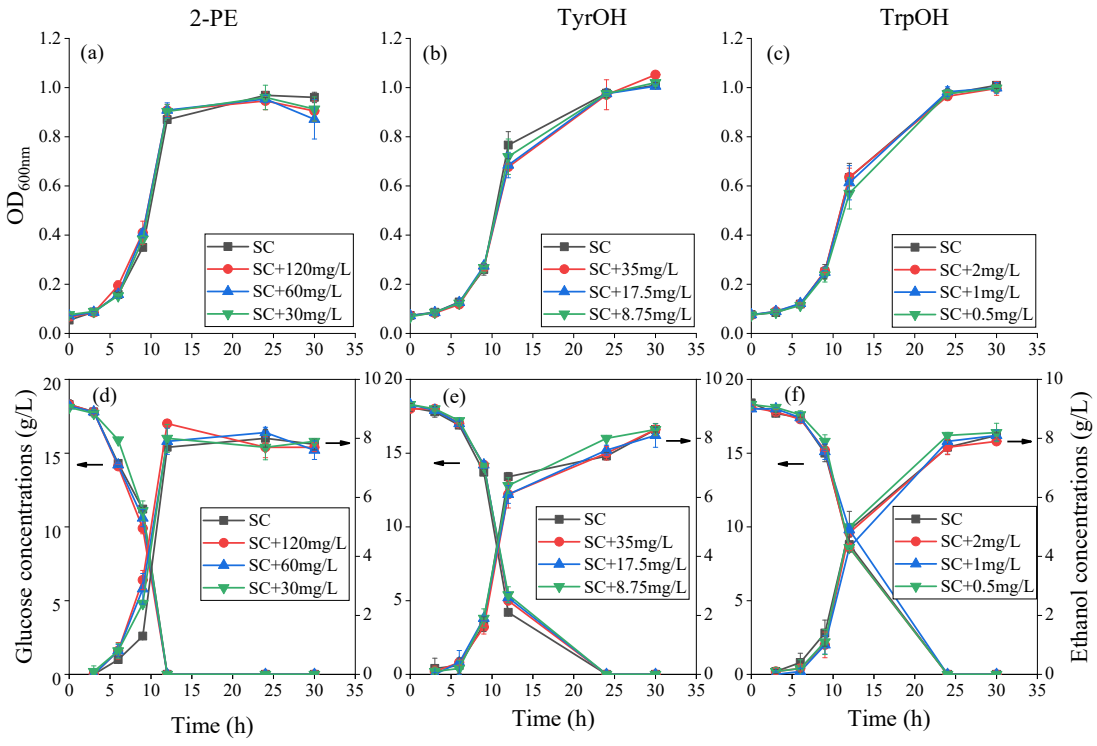


Fig. 2. Effects of *S. cerevisiae* QSMs on the growth, glucose consumption and ethanol production of *S. cerevisiae.* (Note: SC means *S. cerevisiae*)

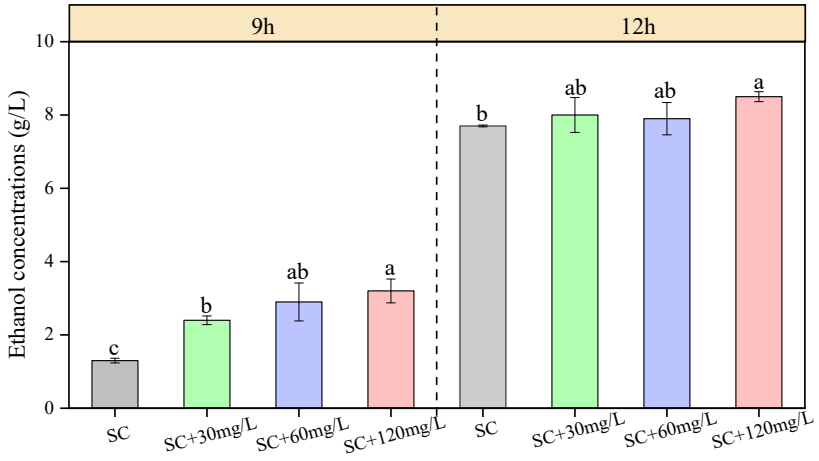


Fig. 3. Effects of 2-PE on the ethanol production of *S. cerevisiae* at 9 h and 12 h. (Note: SC means *S. cerevisiae*. Comparisons were done between groups at the same cultivation time. Non-overlap of letters indicates significant differences in ethanol production $P < 0.05$)

3.3 Effects of exogenous quorum sensing signal molecules on co-fermentation of *S. cerevisiae* and *L. plantarum*

It is well known that *L. plantarum* can inhibit the metabolism of *S. cerevisiae* by changing the embden-meyerhof-pamas (EMP) pathway, thereby reducing the ethanol production(Dong et al., 2015). In Figure4a, compared to the control group without LAB contamination, the ethanol concentrations in the contaminated group (SC+LP) were significantly decreased by 21.1% and 14.1%($P< 0.05$), respectively, within co-fermentation for 12~24h. When the co-fermentation lasted for 12 h, the rest glucose concentration in the contaminated group was 18% higher than that in the

358 control group (Figure4a), indicating that in the co-fermentation process, the presence
359 of *L. plantarum* reduced the carbon flux in the *S. cerevisiae* fermentation process for
360 ethanol production. This result was also confirmed by Dong et al.(2015). In addition,
361 the pH value in the co-fermentation reactor was 4.11 at 12h, which was similar to that
362 in the fermentation reactor with sole *L. plantarum*. While the pH value in the
363 co-fermentation reactor(SC+LP) was 11% lower than that in the sole fermentation
364 reactor (SC) at 24 h (Figure S1), which was caused by the growing of *L. plantarum*.
365 Furthermore, *S. cerevisiae* produces more acids and aldehydes in a lower pH medium,
366 which is detrimental to the ethanol production (Lastauskienė et al., 2014). Hence, *L.*
367 *plantarum* growing with *S. cerevisiae*in a fermenter reduced the ethanol production.
368 However, there were no significant differences in the growth curves of
369 microorganisms between contaminated and restored groups ($P > 0.05$, Figure S2). It
370 indicated that the exogenous QSMs didn`t affect the growth of *S. cerevisiae* and *L.*
371 *plantarum*. Regarding the ethanol yields, no significant improvement was noted in the
372 restored group with the addition of TyrOH and TrpOH, compared to the contaminated
373 group (SC+LP) ($P > 0.05$). While the ethanol concentrations in the contaminated
374 group (SC+LP) and the restored group added with 120 mg/L 2-PE were 27.6% and
375 39.7% higher than those in the control group (SC), respectively, at 9 h($P=0.030$ and
376 $P=0.002 < 0.05$).Besides, the ethanol concentration in the contaminated group was
377 21.1% lower than that in the control group at 12 h($P < 0.01$). The ethanol
378 concentration of the restored group added with 120 mg/L 2-PE were the same to the
379 control group at 12 h ($P=0.056 > 0.05$) (Figure 4b), which was 13.7% higher than that

of the contaminated group (P=0.011< 0.05).These results showed that 2-PE could promote the ethanol production in the co-fermentation process, and reduce the negative impact of *L. plantarum*. Interestingly, the ethanol concentration of the contaminated group was higher than that of the control group at 9 h. The reason may be that *S. cerevisiae* inhibited the growth of *L. plantarum* by producing more ethanol at the early stage of fermentation(Busti et al., 2010). Indeed, some scholars believe that the lactic acid produced by *L. plantarum* can become available carbon for *S. cerevisiae*, thereby promoting the ethanol yield (Sander et al., 2018).

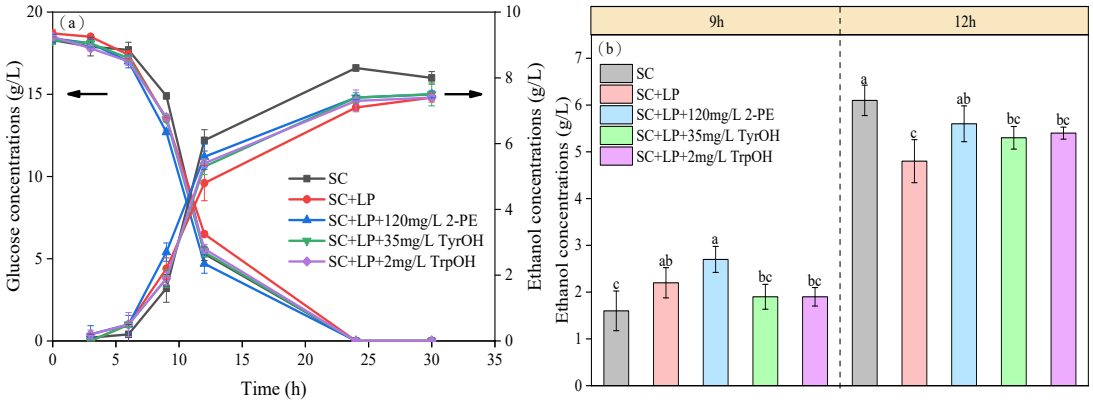


Fig. 4. (a)Effects of *S. cerevisiae*QSMs on the glucose consumption and the ethanol production in the co-fermentation process; (b) Ethanol production in the co-fermentation process at 9 h and 12 h.(Note: SC means *S. cerevisiae*. LP means *L. plantarum*. Comparisons were done between groups at the same cultivation time. Non-overlap of letters indicates significant differences in ethanol production P < 0.05)

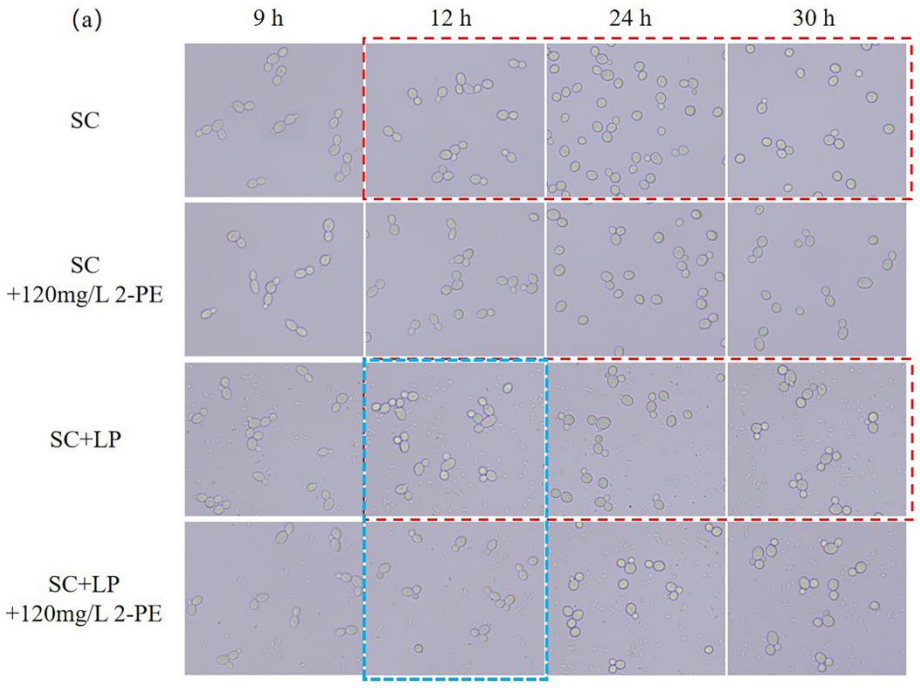
3.4 Effects of exogenous 2-PE on the morphology and budding rates of *S. cerevisiae*

Alcohols such as TyrOH and 2-PE can promote the conversion of dimorphic

yeast from the yeast stage to the mycelium stage (Khan and Murphy, 2020). In this work, it was found that the yeast cells in SC were mostly haploid which only have axial buddings, and the addition of 120 mg/L 2-PE did not stimulate the growth of filamentous yeast in both SC and SC+LP (Figure 5a). The reason may be that the nitrogen contents in the sole and co-fermentation processes were sufficient to the yeast. Hao and Gerald(2006) also showed that aromatic alcohols could not stimulate the formation of filaments in the growing of diploid cells of yeast which was cultured in the glucose medium being rich in ammonium sulfate. In addition, within fermentation of 12~30 h, the multi polar sprouting of the yeast cells in SC+LP was significantly increased compared to that in SC (Figure 5a, red dotted frame). It is also seen that the budding rates of SC+LP were over 40% within 12 h (Figure 5b), indicating that the presence of *L. plantarum* stimulated the proliferation of the yeast cells.

In order to further evaluate the effect of 2-PE on the cell morphology of the yeast, the budding rates of the yeast cells were counted by using an optical microscope, because the budding rate is an important indicator to reflect the fermentation ability of the yeast(Laverty et al., 2013). In general, the budding rates of the yeast cells are required to be in the range of 20~30% to present a strong reproduction capacity and a good fermentation ability in the industrial ethanol production process (Lian, 2015).If yeast budding occurs in large numbers, carbon flux will shift from ethanol fermentation to biomass production. In this test, the budding rates in all groups were less than30% at 9h. At 12 h, the budding ratesinSC+120 mg/L 2-PEreached 29.34%,

which were 5.82% higher than that in SC(P< 0.05). Simultaneously, the budding rates in SC+LP reached 43.19%, which was 19.67% higher than that in SC(P< 0.05). However, in the group of SC+LP+120 mg/L 2-PE, the budding rates reached 28.51%, which was 14.68% less than that in SC+LP (P< 0.05) (Figure 5b). Based on the blue dotted frame in Figure 5a, the multipolar budding of yeast cells was significantly reduced after adding 120mg/L 2-PE to the fermentation of *S. cerevisiae*and *L. plantarum*. This may be due to the addition of 2-PE which promotes the growth of the biofilm of *S. cerevisiae* and weakened the negative impact of *L. plantarum*(Deli et al., 2021; Yujing et al., 2021). Also, the ethanol yield in SC+LP+120 mg/L 2-PE was 5.6 g/L at 12 h, which was restored to the same level as that in SC (Figure 4b). This result further indicated that the addition of 2-PE could reduce the negative effect of *L. plantarum* on the ethanol production of *S. cerevisiae*.



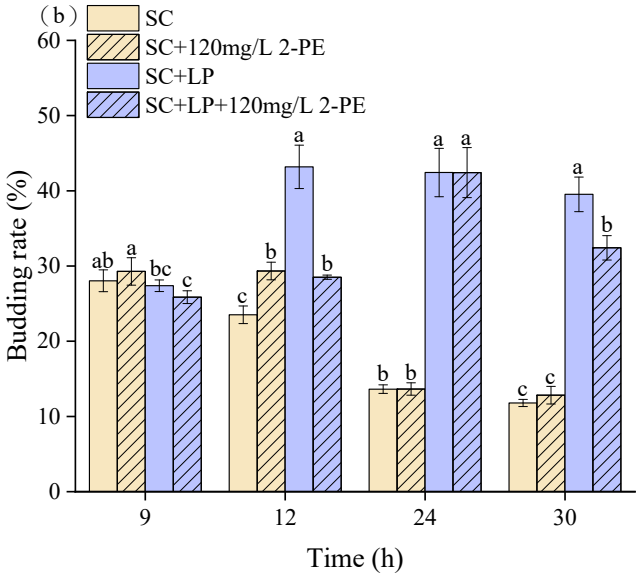


Fig. 5. (a)Microscopic analysis of *S. cerevisiae* cells; (b) Effects of 2-PE on the budding ratesof *S. cerevisiae*.(Note: SC means *S. cerevisiae*. LP means *L. plantarum*. Comparisons were done between groups at the same cultivation time. Non-overlap of letters indicates significant differences in ethanol production $P < 0.05$)

3.5 Effects of exogenous 2-PE on the ethanol production pathways and related gene expressions in *S. cerevisiae*

Compared to the sole fermentation of *S. cerevisiae*, the relative expression levels of key genes *HXK2, PFK1* and *PYK1* encoding the hexokinase 2, phosphofructokinase-1 and pyruvate kinaseon the EMP pathway did not change significantly ($P > 0.05$) in the sole fermentation of *S. cerevisiae* loaded with 120 mg/L 2-PE, and the relative expression level of *ZWF1* encoding theglucose-6-phosphate dehydrogenase on the hexose mono-phosphate pathway (HMP) did not change significantly ($P > 0.05$), either. It indicated that the exogenous addition of120mg/L2-PE had no effect on the EMP and HMP pathways (Figure6). Compared to SC, the relative expression of *ADH1* encoding the ethanol dehydrogenase was

significantly down-regulated by 25% in the group of SC+120 mg/L 2-PE($P < 0.05$).The ethanol dehydrogenase can catalyze the reduction of acetaldehyde to form ethanol, which is the last step on the glycolysis pathway(Mortimer and Schild, 1985). *S. cerevisiae* mainly synthesizes 2-PE through the Ehrlich pathway, in which the last step is the reduction of phenylacetal dehyde catalyzing by *ADH* genes(Hazelwood et al., 2008). Therefore, the exogenous addition of 2-PE may have led to the down-regulation of the expression of *ADH1*.

The relative expression levels of *MLS1* and *CIT2* genes involved in the glyoxylic acid cycle in SC+120 mg/L 2-PE were decreased by 26% and 30%, respectively, compared to those in SC(P=0.016 and P=0.005 $<$ 0.05). The down-regulation of *MLS1* and *CIT2*caused the less consumption of acetyl coenzyme A and the less utilization of non-fermentable carbon like glycerol and ethanol (Figure S3). In addition, the relative expression levels of *IDH1* and *CIT1* genes involved in the tricarboxylic acid cycle (TCA) decreased by 22% and 18% (P=0.006 and P=0.030 < 0.05), which reduced the consumption of the pyruvic acid on the EMP pathway (Figure S3). As an intermediate, the pyruvic acid generated acetaldehyde through the catalysis of pyruvic acid decarboxylase, and the acetaldehyde was further reduced to ethanol. The key gene *KGD1* responsible for the oxidative decarboxylation of α-ketoglutarate to the succinate coenzyme A did not change significantly (P> 0.05). These results showed that exogenous addition of 120 mg/L 2-PE inhibited the glyoxylic acid cycle and the TCA cycle of *S. cerevisiae*. Combined with the results of the sole fermentation test, the ethanol concentration in SC+ 120 mg/L 2-PE was the

highest at 9 h, reaching 3.2 g/L, while the glucose concentration was the lowest (9.9 g/L) (Figure 2d). It indicated that due to the inhibition of the glyoxylic acid cycle and the TCA cycle, more carbon flux switched from the glyoxylic acid cycle and the TCA cycle to the glycolysis pathway, and more glucose was consumed by the yeast, resulting in increased ethanol production. In addition, although exogenous addition of 2-PE led to the down-regulation of the expression level of *ADH1*, the existence of 2-PE had a positive effect on the formation of the biofilm of *S. cerevisiae* (Deli et al., 2021). The biofilm can protect the cells from being torn down by the various external stimuli, such as oxidative stress, osmotic pressure, heat shock, and improve the yeast`s tolerance toa higher concentration of ethanol (Jill and Aaron, 2006; Yang et al., 2018).Overall, the exogenous addition of 2-PE led to the down-regulation of the expression levels of *MLS1*, *CIT2*, and *IDH1*, *CIT1*, inhibiting the glyoxylic acid cycle and the TCA cycle. Carbon flux in *S. cerevisiae*cells were redistributed from respiratory to the glycolysis pathway, leading to a higher ethanol yield in SC+120 mg/L 2-PE.Besides, in the industrial bioethanol fermentation process, the glucose concentration is higher and the contaminating microorganisms are more diverse. Future research on increasing glucose concentrations gradiently and applying more contaminating microorganisms to explore the regulatory effects of exogenous 2-PE on *S. cerevisiae*is expected, which will provide more details to guide the industrial ethanol production.

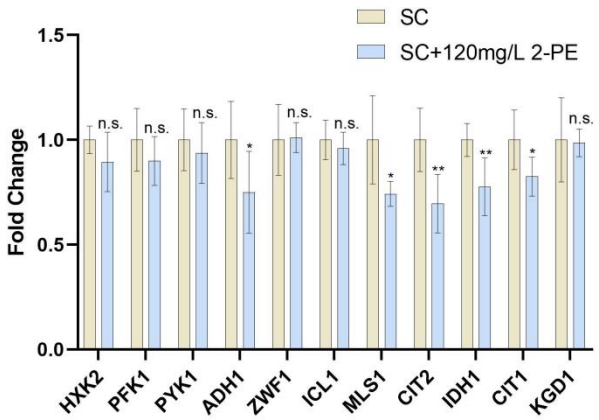


Fig. 6. Effects of 2-PE on the ethanol production pathways and the related genes expressions of *S. cerevisiae*.(Note: SC means *S. cerevisiae*, **P* value < 0.05; ***P* value < 0.01; n.s. indicated no statistically significance)

4. Conclusions

The effects of *S. cerevisiae* QSMs on the ethanol production were investigated in the sole fermentation of *S. cerevisiae* and co-fermentation of *S. cerevisiae* and *L. plantarum*, respectively. Among the three signaling molecules, the one with the addition of 120mg/L 2-PE showed the greatest improvement on the ethanol production and reduced most of the negative impacts of *L. plantarum* on the fermentation of *S. cerevisiae*. The qRT-PCR results showed that the addition of 120 mg/L 2-PE had no effect on the EMP and HMP pathways, but inhibited both the glyoxylic acid cycle and the TCA cycle. Resulting in more carbon flux shifting to the glycolysis pathway as well as the promotion of the yield of ethanol.

CRediT authorship contribution statement

Jun Tian: Investigation, Validation, Data curation, Writing - Original draft

Yunqin Lin: Project administration, Supervision, Writing - Review & Editing

Xiaoying Su: Investigation, Formal analysis, Data curation

Honghao Tan: Investigation, Validation,

Chaoyi Gan: Investigation, Validation,

Arthur J. Ragauskas: Writing - Review & Editing

Conflicts of interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

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Appendix A. Supplementary data

Supplementary data to this article can be found online at…

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