Acetic Acid Acting as a Signal Molecule in Quorum Sensing System Enhances Production of 2,3-Butanediol in *Saccharomyces Cerevisiae*

Chi Zhang

Heilongjiang University

Tianqi Tong

Heilongjiang University

Jingping Ge (✉ gejingping@126.com)

Heilongjiang University <https://orcid.org/0000-0003-1194-4325>

Research Article

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1 **Acetic acid acting as a signal molecule in quorum sensing system enhances production of 2,3-**
2 **butanediol in *Saccharomyces cerevisiae***

3 Chi Zhang^{1,2} Tianqi Tong^{1,2} Jingping Ge^{1,2,*}

4 ¹Engineering Research Center of Agricultural Microbiology Technology, Ministry of Education,
5 Heilongjiang University, Harbin 150500, China

6 ²Key Laboratory of Microbiology, College of Heilongjiang Province, School of Life Sciences,
7 Heilongjiang University, Harbin 150080, China

8 *Author for correspondence: Phone number: +86-0451-86609106; Fax: +86-0451-86608046; Email
9 address: gejingping@126.com

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Abstract:

Objectives: 2,3-butanediol (2,3-BD) has been extensively used in chemical syntheses. The traditional 2,3-BD production method has low yield and high cost. This study aimed to explore the use of acetic acid as a signal molecule to initiate a quorum sensing (QS) system in order to promote the production of 2,3-BD in *Saccharomyces cerevisiae* W141.

Results: We found that the yield of 2,3-BD from *S. cerevisiae* W141 is proportional to the cell density. *S. cerevisiae* W141 does not produce 2,3-BD when cell density was lower than the threshold concentration ($OD_{600\text{ nm}} = 10$ or cell density 4.4×10^8 CFU/mL). When 1.5 g/L acetic acid was added in the fermentation process, the yield of 2,3-BD was the highest reaching 3.01 ± 0.04 g/L (84 h). Subsequently, we found that *S. cerevisiae* W141 was co-cultured with *Acetobacter pasteurianus* Huniang 1.01 under the optimal conditions and the acetic acid production was increased by 76.7% and 30.6% compared with the original strain and the strain with 1.5 g/L acetic acid, respectively. In addition, the yield of 2,3-BD was respectively increased by 81.9% and 3.3%. The above results are attributable to the increased activity of acetolactate synthase (ILV2) and 2,3-BD dehydrogenase (BDH1) and the increase of the relative expression of *ilv2* and *bdh1* genes.

Conclusion: Our data showed that biosynthesis of 2,3-BD was regulated by acetic acid as a signaling molecule. Moreover the study provides a deeper understanding of the mechanisms underlying between acetic acid and 2,3-BD production.

Keywords: Signal molecule; Quorum sensing; Acetic acid; 2,3-butanediol; *Saccharomyces cerevisiae*; *Acetobacter pasteurianus*

Introduction:

The signal molecules are metabolites of bacterial growth, which can mediate quorum sensing (QS) system. The concentration of the signal molecules in the environment is directly proportional to the density of bacteria (Zhou et al. 2019). When the concentration reaches the threshold concentration, signal molecules will drive or inhibit the expression of biological characteristics genes in bacteria to regulate population behavior, further improving the ecological relationship of the population to adapt to environmental changes (Studer et al. 2008; Kareb and Aider 2019). Finally, it realizes physiological functions and regulatory mechanisms that cannot be accomplished by a single cell (Sun et al. 2016; Arena et al. 2017). When the signal molecules interact with the QS system, in addition to the information exchange between the cells, increasing attention has been paid to intracellular metabolism and cell growth state (Jia et al. 2018; Parente et al. 2010). Since the QS system can use the signal molecules as media to regulate the ecological relationship of different microorganisms to change the physiological state of microorganisms, QS system provides high research significance in bacteria. At the same time, large quantities of signal molecules have been identified, such as acetate and oligopeptides (Studer et al. 2008; Camilli and Bassler 2006).

At present, the gradual depletion of petroleum has reawakened people's strong interest in the production of bio-based chemicals including 2,3-butanediol (2,3-BD) from biofuels (Cho et al. 2012; Ge et al. 2020). However, 2,3-BD was originally separated from nature. Its yield is low and cannot be used in industrial production. Therefore, the current research concentrates on seeking a new strain and using metabolic engineering methods to increase the yield of 2,3-BD. In recent years, the production of 2,3-BD by microbial fermentation has become the focus of attention both at home and abroad (Celińska and Grajek 2009). The advantage of the proposed method is that it cannot only efficiently use glucose and other monosaccharides as carbon sources, but also use plant straws as raw materials to ferment to produce 2,3-BD. The method is cheap and safe (Gao et al. 2019; Kim et al. 2016; Kim et al. 2015). Although numerous microorganisms have been studied for the production of 2,3-BD, however *Saccharomyces cerevisiae* has become the main platform microorganism for many related researches due to its compliance with the principle of safe production (Turner et al. 2016). Although *S. cerevisiae* can naturally produce 2,3-BD, yet *S. cerevisiae* uses glucose to produce 2,3-BD by fermentation, which is usually accompanied by the formation of various metabolites and by-products (Fig. 1), such as ethanol and glycerol, competing for carbon sources in metabolic pathways. Ultimately, the lower yields of 2,3-

63 BD can be generated.

64 In the early stage of the current project, molecular biology was used to modify the metabolic pathway of
65 *S. cerevisiae*, aiming to increase the yields of 2,3-BD. The results demonstrated that although the yield
66 of 2,3-BD was improved in a short time, but the yield of 2,3-BD presented a slight downward trend due
67 to defects such as poor genetic stability of the engineered strain. Consequently, in order to solve the
68 present problem, this research attempts to depend on ecological principles and make attempts to enhance
69 the ecological relationship of strains from the perspective of QS to increase the yield of 2,3-BD. Studies
70 have demonstrated that for microorganisms, alkaline conditions are conducive to the formation of acids,
71 and acidic conditions contribute to the formation of alcohols (Guo et al. 2017; Priya et al. 2016). Similarly,
72 acetic acid can effectively activate the gene expression of pyruvate to 2,3-BD under low pH conditions
73 and promote the synthesis of 2,3-BD. Meanwhile, acetic acid serving as a fermentation product can
74 provide a powerful weapon for cell population competition and make microorganisms profit in ecological
75 competition. At the same time, acetic acid can also be used as a backup carbon source to regulate the
76 population behavior of cells (Anane et al. 2017). Therefore, in the metabolic process, the production of
77 2,3-BD may be regulated by QS, and acetic acid as a signal molecule can initiate QS system in order to
78 increase the production of 2,3-BD. In other words, it is beneficial to the production of 2,3-BD under
79 acidic conditions. Therefore acetic acid exerts an indispensable role in the 2,3-BD production process.
80 Meanwhile, the *Acetobacter pasteurianus* Huniang 1.01 in the present study can produce acetic acid
81 because the cells contain a large amount of alcohol dehydrogenase (ADH), aldehyde dehydrogenase
82 (ALDH) and the coenzyme pyrroloquinoline benzoquinone (PQQ). Alcohol is oxidized to aldehyde
83 under the combined action of ADH and PQQ, and aldehyde is further oxidized to acid under the action
84 of ALDH and PQQ (Fig. 1) (Mamlouk and Gullo 2013). We co-culture *S. cerevisiae* W141 and *A.*
85 *pasteurianus* HN 1.01 to explore the drive of acetic acid on QS under co-cultivation mode, further explore
86 the mechanism of acetic acid as a signal molecule, and clarify the relationship between the signal
87 molecule and 2,3-BD in the QS system. Simultaneously, it provides a theoretical basis for the production
88 of 2,3-BD by *S. cerevisiae*.

90 **Materials and Methods:**

91 **Bacterial strains and growth conditions**

The strains used were *S. cerevisiae* W141 and *A. pasteurianus* HN 1.01 in this study. Both bacterial strains were maintained in the Key Laboratory of Microbiology, Heilongjiang University, China. *S. cerevisiae* W141 was grown at 30 °C in Yeast Extract Peptone Dextrose (YEPD) liquid medium (2% peptone, 1% yeast extract, and 2% glucose; w/v). The initial pH was adjusted to 7.0. The loading volume was 125 mL/250 mL. Flask cultures were maintained at 140 rpm for 12-18 h. *A. pasteurianus* HN 1.01 was grown in seed medium containing 0.2% peptone, 1% yeast extract, 9% glucose, 0.1% KH₂PO₄, 0.02% MgSO₄·7H₂O, and 4% ethanol in 1 L H₂O. This was incubated at 37 °C for 12-18 h at a shaking speed of 150 rpm to grow the population.

Cell threshold concentration of *S. cerevisiae* W141

Overnight bacterial cultures of *S. cerevisiae* W141 that were grown in YEPD liquid medium for 12-18 h at 30 °C at a shaking speed of 140 rpm and were centrifuged (two times at 4500 r/min for 15 min) to recover the cells. The harvested cells were washed with sterile 0.9% saline. 9 different culture conditions were set to control cell density by adjusting the amount of carbon source in medium (100%, 50%, 20% carbon source) and inoculation amount (0.5%, 2%, 5%) and incubated for 96 h at 30 °C at a shaking speed of 140 rpm. The cell density (*OD*_{600nm} value) and the yield of 2,3-BD in different culture conditions were determined by spectrophotometry and high performance liquid chromatography (HPLC). Accordingly, in order to determine the minimum inhibitory threshold concentration, and the minimum cell density in the medium that would not inhibit microbes.

Effects of different concentrations of acetic acid on 2,3-BD

Firstly, *S. cerevisiae* W141 strains were precultured. Subsequently, *S. cerevisiae* W141 were inoculated into 150 mL/500 mL YEPD liquid medium at 5% inoculation volume. Inoculation volume of acetic acid were set to 1 g/L, 1.5 g/L and 2 g/L for 96 h at 30 °C at a shaking speed of 140 rpm. The original strain *S. cerevisiae* W141 was used as the control group. The changes in cell density (*OD*_{600nm} value) and 2,3-BD concentration were detected by spectrophotometry and HPLC. The effects of different acetic acid concentrations on 2,3-BD were observed. Thus, the optimal concentration of acetic acid can be determined to ensure the highest yield of 2,3-BD.

Orthogonal optimization experiment

According to the result of single factor, the four variables of inoculation ratio, temperature, rotation speed

and inoculation time are further optimized. This study uses four-factor $L_9(3^4)$ orthogonal design, each factor has three different levels (Table 1).

Establishment of co-culture system and index detection

The strains were individually precultured as described above. The strains *S. cerevisiae* W141 and *A. pasteurianus* HN 1.01, which were used in the co-culture experiment, were inoculated in their respective liquid medium and grew to the logarithm phase. Then these cultures were inoculated into fresh broth for continuous culture for 96 h at 30 °C at a shaking speed of 140 rpm. According to the physiological characteristics of *S. cerevisiae* and *A. pasteurianus*, the results of orthogonal optimization test were used as the optimal conditions for co-culture fermentation. Spectrophotometry and HPLC were used as detection methods, the yield of 2,3-BD and acetic acid was used as the detection standard, samples were taken every 12 h, single culture treatment of each strain was used as the control group. The cell density (OD_{600nm} value) was determined by spectrophotometry. The content of 2,3-BD, acetic acid, glucose and ethanol were determined by HPLC.

Real-time quantitative polymerase chain reaction to measure *ilv2* and *bdh1*

RNA was extracted from samples by using the RNA Prep Pure Bacteria Kit (Tiangen Biotech Co., Ltd., China), and both the purity and concentration of these RNA samples were determined using a NanoDrop 2000 spectrophotometer (Thermo Scientific Co., Ltd., USA), by measuring their absorbance ratio at 260/280 nm and 260/230 nm. Reverse transcription was performed using the BioRT cDNA First Strand Synthesis Kit (TransGen Biotech Co., Ltd., China). Real-time Quantitative Polymerase Chain Reaction (qRT-PCR) was performed with at least three biological replicates using SYBR Green-based detection in triplicate in a 7500 Real-Time PCR System (Applied Biosystems, Inc., USA). The 16S rRNA gene was chosen as an internal control to normalize RNA amounts. The following primers were used: *ilv2* (forward 5'-CGTCCAATTCCTCTTGCTTC-3', reverse 5'-ATGGCAATCCCTGTTCTACG-3'); *bdh1* (forward 5'-TTTGCTGAACAAGTCGTAGTC-3', reverse 5'-CCCAGTTTCTTGGCCATTTC-3'); 16S rRNA (forward 5'-TCACCAGGTCCAGACACAAT-3', reverse 5'-AGCAGACAAATCACTCCACC-3'). Diluted cDNA equivalent to 1 ng RNA starting material was used as the template for qRT-PCR amplification. The transcriptional levels of *ilv2* and *bdh1* were normalized to the transcriptional level of the 16S rRNA gene. The relative changes (*x*-fold) at the transcriptional level in different samples were calculated (Ge et al. 2020).

Enzyme activity detection

The 6 mL strain fermentation broth was centrifuged at 5000 rpm/min and 4 °C to collect the precipitate, washed twice with potassium phosphate buffer (100 mM, pH 7.4), and resuspended in the buffer. The cell supernatant after sonication is placed in an ice bath and used as a crude enzyme solution for measuring enzyme activity. The BDH1 enzyme unit is defined as the amount (μmol) of a unit of BDH activity per unit mass (mg) of total protein oxidized into NAD^+ in a unit of time (min). ILV2 enzyme unit is defined as the amount (μmol) of a unit of ILV2 activity per unit mass (mg) of enzyme protein to catalyze the synthesis of α -acetolactate in a unit of time (min) (Zhang et al. 2016). All enzyme activities are measured in triplicate.

Metabolite detection

Metabolites such as acetic acid and 2,3-BD were determined by HPLC. Shimadzu LC20A high-performance liquid chromatography analysis system: RID-10A differential detector, LC-10ATvp pump and HPX-87H column (300 $\times$ 7.8 mm, Aminex HPX-87H Ion Exclusion column). The fermentation broth sample was diluted 100 times and centrifuged and the supernatant was collected. After filtering with a 0.45 μm bacterial filter, the injection volume was 15 μL , 0.005 mol/L sulfuric acid was used as the mobile phase, the flow rate was 0.8 mL/min, and the column oven temperature was 65 °C, fermentation metabolite analysis for 18 min.

Statistical analysis

The data were statistically analyzed using Microsoft Office Excel 2016 (Microsoft, Redmond, WA) and SPSS 10.0 (SPSS Inc. Chicago, IL, USA) and are expressed the mean $\pm$ SD. In our work, the SD data were obtained from three independent experiments and averaged. OriginPro 2021 (OriginLab, Northampton, MA) was used for chart analysis. $p < 0.05$ (represented by *) was considered a significant difference, $p < 0.01$ (represented by **) was considered an extremely significant difference.

Results

Defining the threshold concentration of *S. cerevisiae* W141

The threshold concentration is defined as the culture condition that does not produce 2,3-BD when the cell density of *S. cerevisiae* W141 is lower than the threshold. It can be observed from Table 2 that

different inoculation amounts exert no obvious effect on the cell density, and the carbon source (glucose) in the medium is the main factor which can affect the bacterial count. When the carbon source is not changed (control group), the cell density ($OD_{600\text{ nm}}$ value) in the fermentation broth is the highest, the second is when the carbon source is 1/2, and the cell density of 1/5 is the lowest. In the meanwhile, the cup and dish method was employed to detect the relationship between the yield of 2,3-BD and cell density under 9 different culture conditions (Table 2). Under 1/5 carbon source culture conditions, the yield of 2,3-BD cannot be detected regardless of the inoculum amount of 5%, 2%, or 0.5%, and the $OD_{600\text{ nm}}$ values were respectively 9.683 ± 0.732 , 9.613 ± 0.611 and 9.014 ± 0.571 , all of which are less than 10. Therefore, it can be proved that the yield of 2,3-BD within the certain range is directly proportional to the cell density of the fermentation broth. The production process of 2,3-BD is dependent on cell density, and the threshold concentration to be reached is $OD_{600\text{ nm}}$ is 10 (cell density is 4.4×10^8 CFU/mL). In addition, it can also be determined that the threshold density culture condition is 1/5 carbon source medium and the inoculation amount is 5%. Therefore, this condition can be used to determine whether co-cultivation with other microorganisms can promote *S. cerevisiae* W141 in order to produce more 2,3-BD.

The effect of adding different concentrations of acetic acid on cell density and 2,3-BD

Studies have found that the addition of acetic acid can induce the synthesis of 2,3-BD, indicating that 2,3-BD as a neutral metabolite, can offset excessive acidification levels (Syu 2001). Therefore, the addition of acetic acid can increase 2,3-BD production by inducing enzymes involved in the 2,3-BD pathway (Celińska and Grajek 2009). However, a large amount of acetic acid will increase the carbon flux in the metabolic pathway. As a result, a large amount of acetic acid will be harmful to cell growth. In other words, when the concentration of acetic acid is too high, it can inhibit microorganism growth (Sandoval et al. 2011). Relevant studies have proved that in the addition of 1-10 g/L of acetic acid, high concentration of acetic acid is usually used as an inhibitor of bacterial growth (Martin et al. 2007; Garcia-Aparicio et al. 2006). At the same time, the growth of *Escherichia coli* was inhibited when the concentration of acetic acid was 0.5 g/L (Roe et al. 1998).

In the present study, we chose to add 1 g/L, 1.5 g/L and 2 g/L acetic acid to determine the changes in biomass and 2,3-BD yield in the fermentation process of *S. cerevisiae* W141 (Fig. 2). The results demonstrated that the $OD_{600\text{ nm}}$ value ended to first rise and then maintain stable, at the same time, with *S. cerevisiae* W141 as the control group, $OD_{600\text{ nm}}$ value reached the maximum of 15.29 ± 0.31 . After

adding 1g/L and 1.5 g/L acetic acid, the highest yields of 2,3-BD reached respectively 2.93 ± 0.02 g/L (84 h) and 3.01 ± 0.04 g/L (84 h), which were compared with the control group (1.71 ± 0.02 g/L). In addition, it was increased respectively by 71.4% and 76.0% ($p < 0.01$). At the same time, the $OD_{600\text{ nm}}$ value has no obvious effect after adding 1 g/L acetic acid, while the $OD_{600\text{ nm}}$ value dropped to 14.46 ± 0.93 (cell density is 6×10^8 CFU/mL) after adding 1.5 g/L. After adding 2 g/L acetic acid, the 2,3-BD yield decreased to 1.45 ± 0.02 g/L (84 h), which was 17.9% lower than the control group ($p < 0.01$). Meanwhile, the $OD_{600\text{ nm}}$ value dropped to 13.62 ± 0.48 . (cell density is 5.7×10^8 CFU/mL). Consequently, the relationship between acetic acid and 2,3-BD during the fermentation of *S. cerevisiae* W141 has a certain coupling, that is, when acetic acid reaches a concentration of 1.5 g/L, it can effectively promote the production of 2,3-BD, and excessive addition of acetic acid will lead to a decrease in the production and cell density of 2,3-BD and inhibit its growth.

The effect of acetic acid on microorganisms under co-culture conditions

According to the results of the previous single-factor experiment, the fermentation conditions for co-cultivation include the inoculation ratio is 4:2, the fermentation temperature is 30°C, the rotation speed is 140 r/min, and the inoculation time is 12 h. Besides, the output of 2,3-BD reached its maximum at that time. We further carry out orthogonal optimization test for four factors, and each factor has three different levels of orthogonal experimental design (Table 1). In accordance with the production of 2,3-BD determined by each experimental design and the influence of these factors on the production of acetic acid, according to the R value (Table 3), the influence of four factors on the concentration of 2,3-BD decreases in order: (A) inoculation amount, (B) temperature, (C) rotation speed and (D) inoculation time. Based on the order of K value (Table 3), the best combination is $A_2B_2C_3D_1$, corresponding to a fermentation temperature of 32 °C, a rotation speed of 160 r/min, an inoculation time of 0 h, and an inoculation amount of *S. cerevisiae* W141 and *A. pasteurianus* HN 1.01 are 4% and 2% respectively. Under the optimal technological conditions, the average output of 2,3-BD in the three parallel experiments was 2.612 ± 0.003 g/L.

Under optimal culture conditions, samples were taken from the culture medium in order to analyze changes in glucose concentration, biomass, ethanol, acetic acid, and 2,3-BD content. As shown in Fig. 3a, during the fermentation process, glucose was used as a carbon source and was quickly utilized by *S. cerevisiae* W141 in the first 24 h. At the same time, with the maximum amount being 40.02 ± 1.72 g/L (24 h), the content of ethanol increased significantly. Therefore, the first 24 h is the main synthesis period

of ethanol, and *A. pasteurianus* HN 1.01 passes a large amount of ethanol through ADH and ALDH oxidation produces acetic acid (Mamlouk and Gullo 2013). The output of acetic acid reached a maximum of 2.437 ± 0.056 g/L at 24 h, and then showed a slow decline. However, after 24 h, the output of acetic acid decreased. At this time, the content of 2,3-BD increased significantly. Through comparing the changes in the content of acetic acid and 2,3-BD, it can be observed that the output of 2,3-BD is inversely proportional to the content of acetic acid. It reveals that *S. cerevisiae* W141 is using acetic acid to produce 2,3-BD. As shown in Fig. 3b, when the biomass of *S. cerevisiae* W141 increased, the growth of *A. pasteurianus* HN 1.01 was inhibited, and the cell number decreased from 1.95×10^8 CFU/mL at 60 h to 1.41×10^8 CFU/mL at 48 h. However, the co-culture conditions exert no significant effect on the cell number of *S. cerevisiae* W141. In other words, the process of converting ethanol to acetic acid was masked by the inhibition of the activity of *A. pasteurianus* HN 1.01. Therefore the cell number of *A. pasteurianus* HN 1.01 decreased.

Comparison of the 2,3-BD yield changes under the optimal conditions of co-cultivation, yeast monoculture, and addition of 1.5 g/L acetic acid, can be seen from the results of Fig. 4a. Under the optimal culture conditions, the maximum yield of 2,3-BD tested was 3.11 ± 0.02 g/L (72 h), which was 81.9% higher than the original strain ($p < 0.01$). Simultaneously, compared with adding 1.5 g/L acetic acid, the yield increased by 3.3% ($p < 0.05$). Additionally, it could also be found that under the optimal culture conditions, the acetic acid produced was higher than that of *S. cerevisiae* W141 monoculture and 1.5 g/L acetic acid (Fig. 4b). Therefore, it can be proved that *S. cerevisiae* W141 will use the acetic acid produced by *A. pasteurianus* HN 1.01 to promote the production of 2,3-BD. The decrease of acetic acid content will lead to the production of 2,3-BD decline, also further explaining that acetic acid can be used as a signal molecule under co-culture conditions to affect the content of 2,3-BD produced by *S. cerevisiae* W141.

ILV2 and BDH1 enzyme activity detection and mRNA expression of related genes

In order to further explore that acetic acid acting as a signal molecule can promote the activity of key enzymes and gene expression in the metabolic pathway of *S. cerevisiae* W141, we will detect the enzyme activity of acetolactate synthase (ILV2) and 2,3-BD dehydrogenase (BDH1) in the metabolic pathway under the optimal conditions of co-cultivation. *S. cerevisiae* W141 was used as a control to detect the changes in the key enzyme activity of 2,3-BD under the optimal conditions of co-cultivation and after the addition of 1.5 g/L acetic acid. From the results of Fig. 5a, it is found that under the optimal culture

conditions for co-cultivation, the enzyme activity of ILV2 first increases, decreases and then increases with the extension of the fermentation time. Finally, it stabilizes, and the maximum enzyme activity reaches 1.519 ± 0.022 U/mg (24 h). Compared with the control group (0.774 ± 0.022 U/mg) and the 1.5 g/L acetic acid group (1.412 ± 0.022 U/mg), the increase was 96.3% and 7.6%, respectively. It can be seen from Fig. 5b that the BDH1 enzyme activity under the three groups presented a trend of increasing and then decreasing. In addition, the enzyme activity under the optimal conditions of co-cultivation reached a maximum of 0.1634 ± 0.0026 U/mg (60 h), respectively comparison with the control group (0.0976 ± 0.0015 U/mg) and the 1.5 g/L acetic acid group (0.1212 ± 0.0032 U/mg) increased by 67.4% and 34.8%. This proves that under the optimal conditions of co-cultivation, the key enzyme activities of the 2,3-BD metabolic pathway of *S. cerevisiae* W141 have been significantly increase. Consequently, the output of 2,3-BD has been greatly improved.

This study used qRT-PCR technology to detect the expression of *ilv2* and *bdh1* gene. The CT value of each sample was analyzed by employing the relative quantitative $2^{-\Delta\Delta CT}$ method, and the relative expression of the *ilv2* and *bdh1* genes was calculated (Fig. 6). According to the results, the relative expression levels of *ilv2* gene were higher than those in the control group after adding 1 g/L and 1.5 g/L acetic acid, which were 3.37 times and 5.56 times the gene expression levels respectively, and the relative expression level of *ilv2* gene under optimal co-culture condition was 4.52 times that of control group. The relative expression of *ilv2* gene was 4.52 times that of the control group ($p < 0.01$). After adding 2 g/L acetic acid, the relative expression of the *ilv2* gene was down-regulated, which was 20.5% lower than that of the control group ($p < 0.01$). At the same time, the relative expression of *bdh1* gene in *S. cerevisiae* W141 after the addition of 1 g/L and 1.5 g/L acetic acid was also higher than that of the control group, which was respectively 1.35 times and 1.12 times that of its gene expression. Under the optimal co-cultured conditions, the relative expression of *bdh1* gene was 4.52 times that of the control group ($p < 0.01$), while the relative expression of *bdh1* gene was down-regulated after adding 2 g/L acetic acid, which was 85.2% lower than that of the control group ($p < 0.01$). It shows that there is QS between *S. cerevisiae* and *A. pasteurianus* HN1.01, and the acetic acid produced by fermentation of *A. pasteurianus* HN1.01 can be used as a signal molecule to up-regulate the expression of key enzyme genes in the 2,3-BD synthesis pathway, and subsequently improve the activity of related enzymes to achieve the purpose of increasing 2,3-BD.

Discussion

2,3-BD acting as a platform chemical was extensively used in various fields such as food and medicine (Kim et al. 2019). In general, numerous bacteria had an endogenous metabolic pathway to produce 2,3-BD, including *S. cerevisiae* (Kim et al. 2016), *Enterobacter cloacae* (Li et al. 2015), *Klebsiella* (Rathnasingh et al. 2016; Ji et al. 2008) and *Bacillus licheniformis* (Qiu et al. 2016) and these bacteria have been proved to produce 2,3-BD. Lee et al. (2016) found that when *E. aerogenes* are cultured, the addition of external acetic acid could increase the production of 2,3-BD by 32.4%. Yu et al. (1982) discovered that the addition of less than 1 g/L of acetic acid can increase the production of 2,3-BD by 2-3 times when *K. pneumoniae* is a substrate containing nutrients such as glucose and xylose. For *S. cerevisiae*, excessive acetic acid will promote the production of acetolactate and decrease the intracellular pH. In order to avoid excessive acetic acid at this time, pyruvate tilts to the 2,3-BD pathway, and uses the means of converting acidic products to avoid the phenomenon of excessive acid in the cell. In other words, it is beneficial to the production of 2,3-BD under acidic conditions, consequently indicating that acetic acid exerts an indispensable role in the entire production process of 2,3-BD. At the same time, studies have been shown that acetic acid can be used as a signal molecule to affect cell membranes, bacteriocins and QS (Elmassry et al. 2020; Ge et al. 2019). Therefore, it can also be believed that in the *S. cerevisiae* quorum sensing system, acetic acid has the potential as a signal molecule.

Some studies have found that the production process of 2,3-BD is affected by a lot of factors, such as ventilation, pH and cell density (Tong et al. 2015; Kalia 2013). Based on our obtained data, the yield of 2,3-BD is affected by the cell density of *S. cerevisiae*. When the cell density of the fermentation broth is lower than the threshold concentration ($OD_{600\text{ nm}} = 10$ or cell density of 4.4×10^8 cells/mL), the production of 2,3-BD cannot be detected. Under the same conditions, when the cell density is higher than the threshold concentration, the production of 2,3-BD is found. Therefore, high cell density facilitates the production of high levels of 2,3-BD. However, it has been found that the signal molecules produced by some strains have a certain relationship with cell density. As a result, it shows that the production of 2,3-BD is affected and controlled by the cell density of *S. cerevisiae* W141 flora without being influenced by other substances. At the same time, in the metabolic process of 2,3-BD production by *S. cerevisiae*, acetic acid promotes the expression of the three key genes (*budA*, *budB* and *budC*) from pyruvate to 2,3-BD. Acetic acid can regulate the balance of acetoin to 2,3-BD and induce the enhancement of BDH enzyme activity. Additionally, it has been confirmed that under the premise of reasonable allocation of

carbon sources, the production of 2,3-BD can be increased by adding appropriate acetic acid (Xu et al. 2014). Moreover, the present study explored the effect of acetic acid as a signal molecule on the production of 2,3-BD by adding different concentrations of acetic acid from an external source. The results showed that when 1.5 g/L acetic acid was added to the fermentation broth of *S. cerevisiae* W141, the yield of 2,3-BD reached 3.01 ± 0.04 g/L (84 h), which was an increase of 76.02% compared with the control group. At this time, the strain $OD_{600\text{ nm}}$ value has fallen. It shows that adding a certain concentration of acetic acid from external sources can promote the production of 2,3-BD. At the same time, Lee et al. (2016) also reached a similar conclusion. When they explored the effect of acetic acid on the production of 2,3-BD by bacteria, they found that when a certain concentration of acetic acid was added to *E. aerogenes*, the pH of the medium was adjusted to 6.5 and the yield of 2,3-BD was increased, indicating that the addition of acetic acid exerted a certain stimulating effect on 2,3-BD.

Through conducting further co-cultivation experiments, we found that 2,3-BD production will be affected by the growth and metabolism of *A. pasteurianus* HN1.01 to produce acetic acid. Under the optimal co-cultivation conditions, the yield of 2,3-BD was higher than the original strain and the 1.5 g/L acetic acid group. There are reports showing that the metabolism of 2,3-BD is regulated by QS system (Jin et al. 2019). Suh et al. (2000) obtained similar results when exploring the mixed culture of *Monascus* J101 and *S. cerevisiae*, that is, the pigment yield of the co-cultivation was 30-40 times higher than that of *Monascus* alone. In the meanwhile, during the co-cultivation of *S. cerevisiae* and *A. pasteurianus*, Wang et al. (2013) found that the ethanol produced by *S. cerevisiae* directly as fermentation with carbon sources can achieve the purpose of saving costs, consequently shortening fermentation time and increasing yield. This result is consistent with the results of the present study. In addition, this study also found that under the optimal conditions of co-cultivation, the expression levels of *ilv2* and *bdh1* genes were significantly up-regulated, and were higher than that of the acetic acid group. This result is consistent with the results of Lou et al. (2016) Therefore, under co-cultivation conditions, the acetic acid produced by fermentation of *A. pasteurianus* HN1.01 can be used as a signal molecule to successfully increase the relative expression of the key enzyme genes *ilv2* and *bdh1* in the 2,3-BD metabolic pathway, and simultaneously lead to ILV2 and BDH1 activity is also significantly increased to achieve the purpose of increasing 2,3-BD.

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Compliance with ethical standards

Conflict of interest: The authors declare that there are no conflicts of interest.

Ethical statements: This article does not contain any studies with human participants or animals performed by any of the authors.

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466

467 **Tables**

468 **Table 1** Factors and their levels of orthogonal experiment $L_9(3^4)$

Constituent	Level 1	Level 2	Level 3
A: inoculation amount (% v/v)	3:3	4:2	2:4
B: fermentation temperature (°C)	30	32	34
C: rotate speed (r/min)	120	140	160
D: inoculation time (h)	0	12	24

469

470 **Table 2** The yield of 2,3-BD and cell density of *S. cerevisiae* W141 under 9 different culture conditions

Groups	Culture condition	Cell density ($OD_{600\text{ nm}}$)	2,3-BD yield (g/L)
1	100% carbon source, 5%	15.073 ± 0.627	1.71 ± 0.01
2	50% carbon source, 5%	10.664 ± 0.823	0.40 ± 0.02
3	20% carbon source, 5%	9.683 ± 0.732	0
4	100% carbon source, 2%	14.394 ± 0.599	1.37 ± 0.03
5	50% carbon source, 2%	10.553 ± 0.487	0.56 ± 0.03
6	20% carbon source, 2%	9.613 ± 0.611	0
7	100% carbon source, 0.5%	13.803 ± 0.806	0.81 ± 0.02
8	50% carbon source, 0.5%	10.494 ± 0.773	0.29 ± 0.01
9	20% carbon source, 0.5%	9.014 ± 0.571	0

471

472 **Table 3** Experimental design and results of the orthogonal experiment $L_9(3^4)$

Run	A ^a	B	C	D	2,3-BD yield (g/L)
1	1	1	1	1	0.372 ± 0.002
2	1	2	2	2	0.515 ± 0.001
3	1	3	3	3	0.577 ± 0.001
4	2	1	2	3	1.787 ± 0.003
5	2	2	3	1	2.612 ± 0.003
6	2	3	1	2	1.201 ± 0.002
7	3	1	3	2	1.133 ± 0.002
8	3	2	1	3	1.267 ± 0.002
9	3	3	2	1	1.058 ± 0.001
K_1	1.464 ± 0.002	3.292 ± 0.001	2.84 ± 0.003	4.042 ± 0.001	
K_2	5.600 ± 0.001	4.394 ± 0.002	3.360 ± 0.003	2.849 ± 0.004	
K_3	3.458 ± 0.004	2.836 ± 0.005	4.322 ± 0.001	3.631 ± 0.002	
R	2.068	0.779	0.741	0.5965	
Optimal level	2	2	3	1	

473 a The symbols were the same as those in Table 1

474

Figure captions

Fig. 1 The interaction between *A. pasteurianus* HN 1.01 and *S. cerevisiae* W141 in co-culture system. The red area represents the acetic acid metabolism system from *A. pasteurianus* HN 1.01 and the blue area represents the 2,3-BD metabolic system from *S. cerevisiae* W141

Fig. 2 The effects of adding different concentrations of acetic acid on the production of 2,3-BD and cell density ($OD_{600\text{ nm}}$ value). The symbols indicate the different concentrations of acetic acid. 1 g/L, 1.5 g/L and 2 g/L signify respectively the concentration of acetic acid under different treatment conditions. The bar chart shows the yield of 2,3-BD during the fermentation of *S. cerevisiae* W141, corresponding to the left Y-axis; The point plot shows the change of cell concentration ($OD_{600\text{ nm}}$ value) of *S. cerevisiae* W141, corresponding to the right Y-axis. (■) and (—■) represent isolated culture of *S. cerevisiae* W141; (□) and (·-□-·) represent the addition of 1 g/L acetic acid during the fermentation of *S. cerevisiae* W141; (■) and (—●) denote the addition of 1.5 g/L acetic acid during the fermentation of *S. cerevisiae* W141; (▨) and (·-○-·) represents the addition of 2 g/L acetic acid during the fermentation of *S. cerevisiae* W141

Fig. 3 The change of metabolites and cell density ($OD_{600\text{ nm}}$ value) of *S. cerevisiae* W141 and *A. pasteurianus* HN 1.01 under optimal co-cultivation conditions. (a) The change of the yields glucose, ethanol, acetic acid and 2,3-BD under optimal co-culture conditions. The black square (—■), the black circle (—●), the open square (·-□-·) and the open circle (·-○-·) indicate respectively glucose, ethanol, acetic acid and 2,3-BD. (b) The change of cell density ($OD_{600\text{ nm}}$ value) of *S. cerevisiae* W141 and *A. pasteurianus* HN 1.01 under the conditions of individual culture and optimal co-culture, respectively. The black square (—■) represents the cell density in co-culture of *S. cerevisiae* W141. The black circle (—●) signifies the cell density in co-culture of *A. pasteurianus* HN 1.01. The open square (·-□-·) represents the cell density of *S. cerevisiae* W141 when isolated culture and the open circle (·-○-·) represent the cell density of *A. pasteurianus* HN 1.01 when being solely cultured

Fig. 4 When *S. cerevisiae* W141 and *A. pasteurianus* HN 1.01 were optimally co-cultured, the original strain *S. cerevisiae* W141 was solely cultured and *S. cerevisiae* W141 was added with 1.5 g/L acetic acid in the production of 2,3-BD and acetic acid. (a) Metabolism of 2,3-BD under three treatment conditions;

(b) Metabolism of acetic acid under three treatment conditions. (■) and (—■—) represent *S. cerevisiae* W141 and *A. pasteurianus* HN 1.01 were co-cultured under optimal conditions; (■) and (·-○-·) stand for the original strain *S. cerevisiae* W141 was solely cultured; (▨) and (—▲—) represent that *S. cerevisiae* W141 was added with 1.5 g/L acetic acid. Significance levels are presented as follows: * $p < 0.05$, ** $p < 0.01$

Fig. 5 The enzyme activity of ILV2 and BDH1 of *S. cerevisiae* W141 and *A. pasteurianus* HN 1.01 were optimally co-cultured, the original strain *S. cerevisiae* W141 was solely cultured and *S. cerevisiae* W141 was added with 1.5 g/L acetic acid. (a) The change of enzyme activity of ILV2 under three treatment conditions; (b) The change of enzyme activity of BDH1 under three treatment conditions. The red area (■) represents *S. cerevisiae* W141 and *A. pasteurianus* HN 1.01 were co-cultured under optimal conditions; the blue area (■) denotes the original strain *S. cerevisiae* W141 was solely cultured and the green area (■) represents *S. cerevisiae* W141 was added with 1.5 g/L acetic acid

Fig. 6 The gene expression levels of *S. cerevisiae* W141 and *A. pasteurianus* HN 1.01 were optimally co-cultured, the original strain *S. cerevisiae* W141 was solely cultured and *S. cerevisiae* W141 was added with 1.5 g/L acetic acid. (□) represents the *ilv2* gene expression level at 24 h; (■) denotes the *bdh1* gene expression level at 60 h. Significance levels are as follows: * $p < 0.05$

Figures

Figure 1

The interaction between *A. pasteurianus* HN 1.01 and *S. cerevisiae* W141 in co-culture system. The red area represents the acetic acid metabolism system from *A. pasteurianus* HN 1.01 and the blue area represents the 2,3-BD metabolic system from *S. cerevisiae* W141

Figure 2

The effects of adding different concentrations of acetic acid on the production of 2,3-BD and cell density (OD_{600 nm} value). The symbols indicate the different concentrations of acetic acid. 1 g/L, 1.5 g/L and 2 g/L signify respectively the concentration of acetic acid under different treatment conditions. The bar chart shows the yield of 2,3-BD during the fermentation of *S. cerevisiae* W141, corresponding to the left Y-axis; The point plot shows the change of cell concentration (OD_{600 nm} value) of *S. cerevisiae* W141, corresponding to the right Y-axis. (■) and (—■) represent isolated culture of *S. cerevisiae* W141; (□) and (—□) represent the addition of 1 g/L acetic acid during the fermentation of *S. cerevisiae* W141; (■) and (—■) denote the addition of 1.5 g/L acetic acid during the fermentation of *S. cerevisiae* W141; (■) and (—■) represents the addition of 2 g/L acetic acid during the fermentation of *S. cerevisiae* W141

Figure 3

The change of metabolites and cell density (OD600nm value) of *S. cerevisiae* W141 and *A. pasteurianus* HN 1.01 under optimal co-cultivation conditions. (a) The change of the yields glucose, ethanol, acetic acid and 2,3-BD under optimal co-culture conditions. The black square ($\blacksquare$), the black circle ($\bullet$), the open square ($\square$) and the open circle ($\circ$) indicate respectively glucose, ethanol, acetic acid and 2,3-BD. (b) The change of cell density (OD600 nm value) of *S. cerevisiae* W141 and *A. pasteurianus* HN 1.01 under the conditions of individual culture and optimal co-culture, respectively. The black square ($\blacksquare$) represents the cell density in co-culture of *S. cerevisiae* W141. The black circle ($\bullet$) signifies the cell density in co-culture of *A. pasteurianus* HN 1.01. The open square ($\square$) represents the cell density of *S. cerevisiae* W141 when isolated culture and the open circle ($\circ$) represent the cell density of *A. pasteurianus* HN 1.01 when being solely cultured

Figure 4

When *S. cerevisiae* W141 and *A. pasteurianus* HN 1.01 were optimally co-cultured, the original strain *S. cerevisiae* W141 was solely cultured and *S. cerevisiae* W141 was added with 1.5 g/L acetic acid in the production of 2,3-BD and acetic acid. (a) Metabolism of 2,3-BD under three treatment conditions; (b) Metabolism of acetic acid under three treatment conditions. (⊠) and (⊞) represent *S. cerevisiae* W141 and *A. pasteurianus* HN 1.01 were co-cultured under optimal conditions; (⊠) and (⊞) stand for the original strain *S. cerevisiae* W141 was solely cultured; (⊠) and (⊠) represent that *S. cerevisiae* W141 was added with 1.5 g/L acetic acid. Significance levels are presented as follows: * $p < 0.05$, ** $p < 0.01$

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The enzyme activity of ILV2 and BDH1 of *S. cerevisiae* W141 and *A. pasteurianus* HN 1.01 were optimally co-cultured, the original strain *S. cerevisiae* W141 was solely cultured and *S. cerevisiae* W141 was added with 1.5 g/L acetic acid. (a) The change of enzyme activity of ILV2 under three treatment conditions; (b) The change of enzyme activity of BDH1 under three treatment conditions. The red area (⊠) represents *S. cerevisiae* W141 and *A. pasteurianus* HN 1.01 were co-cultured under optimal conditions; the blue area (⊠) denotes the original strain *S. cerevisiae* W141 was solely cultured and the green area (⊠) represents *S. cerevisiae* W141 was added with 1.5 g/L acetic acid

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