Effect of Dissolved Oxygen and Acid on the Yield of 2,3-butanediol by Saccharomyces Cerevisiae W141

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Research

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Abstract

Background: The yeast *Saccharomyces cerevisiae* is a promising host cell to produce 2,3-butanediol (2,3-BDO). However, the fermentation environment restricts 2,3-BDO yield, productivity, and titre from engineered yeast. In the present study, we propose a strategy in which a suitable dissolved oxygen content and acid stress level can improve the 23-BDO yield of *S. cerevisiae* W141. Five different concentrations of short-chain fatty acids were evaluated and noxE overexpression was performed to disrupt the intracellular redox balance and alter the NADH content associated with 2,3-BDO synthesis, which can significantly increase or inhibit 2,3-BDO yield.

Results: The five assayed short-chain fatty acids have different effects on the fermentation characteristics of yeast, were formic, butyric and valeric acids can inhibit the synthesis of 2,3-BDO. Only low concentrations of acetic and propionic acids could significantly increase the yield of 2,3-BDO, especially when 1 g/L acetic acid was added, which stimulated the expression of acid stress-related genes in *S. cerevisiae* W141 (*haa1p* and *hog1p*) and increase the 2,3-BDO yield by 29.74%. To further verify that acid stress primarily disrupts the intracellular redox balance by altering the NADH content, we constructed a *S. cerevisiae* strain, W141-E, which overexpresses the *noxE* gene of Lactobacillus. After adding 1 g/L acetic acid, the 2,3-BDO yield from in *S. cerevisiae* W141-E increased by 43.64%, confirming the validity of our strategy. When the optimized fermentation oxygen content was 0.6vvm, the 2,3-BDO yield from *S. cerevisiae* was greatly improved after the addition of acetic acid.

Conclusions: In the present study, we demonstrated that a suitable dissolved oxygen and acid stress are highly effective for increasing the 2,3-BDO yield from *S. cerevisiae* W141. 2,3-BDO biosynthesis was heavily dependent on the intracellular NADH content, which is closely associated with glycolysis and the TCA cycle and is likely important for the production of 2,3-BDO by *S. cerevisiae*.

Background

2,3-Butanediol (2,3-BDO) is a natural metabolite and an important potential platform chemical. There are three optical isomers of 2,3-BDO that are widely used in solvents, fuels, polymers, cosmetics, pharmaceuticals and other fields [1–3]. 2,3-BDO can be produced by different microorganisms, such as *Klebsiella pneumoniae*, *Enterobacter cloacae* and *Bacillus thuringiensis* [2, 4, 5]. Compared to prokaryotes, the eukaryotic model organism *Saccharomyces cerevisiae* primarily produces (2R, 3R)-2,3-BDO, which is an important precursor material for the synthesis of chiral reagents and ligands and has extremely high industrial value [6, 7]. However, the 2,3-BDO output obtained using *S. cerevisiae* is extremely low, which has become a bottleneck limiting large-scale industrial yields. At present, the primary strategies to increase 2,3-BDO yields from *S. cerevisiae* can be divided into four categories: (1) reducing the formation of other by-products in *S. cerevisiae* [8]; (2) modifying the levels of relevant cofactors in the 2,3-BDO metabolic pathway [9]; (3) enhancing glycolysis and the 2,3-BDO biosynthetic pathway [10]; and (4) exogenously expressing the 2,3-BDO synthesis pathway [11]. Among these
approaches, the elimination of ethanol and glycerol yields and rebalancing cellular redox are the key factors for increase the yield of 2,3-BDO from yeast [8, 9].

The process by which *S. cerevisiae* produces 2,3-BDO is the same as that observed in prokaryotes, which is heavily dependent on the consumption of NADH (Fig. 1). Interestingly, disrupting the NADH/NAD⁺ balance, which affects the intracellular redox balance, has been shown to promote the synthesis of 2,3-BDO in *S. cerevisiae* [9, 12, 14, 15]. However, *S. cerevisiae* commonly encounters acid stress in industrial settings, which will greatly affect the redox balance in the cell and metabolite yields [16–18]. The acid stress response mechanism of yeast cells includes the following four aspects: (1) the cell membrane maintains the stability of intracellular pH by restricting the penetration of high levels of acid; (2) the cell membrane channel proteins that regulates transcription factors and acid transport maintains the intracellular stabilization of pH; (3) adenosine triphosphatase H⁺-ATPase (encoded by the *PMA1* gene) on the cell membrane can hydrolyse ATP to produce energy, pumping protons out of the cell to maintain a normal neutral pH environment in the cell; and (4) maintaining the integrity and fluidity of cell membranes by modulating fatty acid composition [13, 19, 20]. Among these processes, maintaining the stability of intracellular pH through the massive consumption of ATP in the cell is the most important, but the results obtained using this method will lead to intracellular acidification, thereby inhibiting cell growth and metabolism, especially for the TCA cycle, with the Crabtree effect having the most notable influence [12, 19, 21]. In addition, the induction of acid stress mechanisms will also lead to the consumption of intracellular reactive oxygen species and alter metal ion metabolism, which will affect the synthesis of intracellular DNA/RNA, amino acids, and proteins and alter the intracellular redox balance [22–25]. Previous studies have shown that the mechanisms used to cope with acid stress are not conducive to yeast ethanol fermentation, but there are few reports on its effects on other metabolic by-products [18, 26].

In the present study, under laboratory conditions, we exogenously added 5 types of short-chain fatty acids and constructed a strain (W141-E) that overexpresses the NADH oxidase gene (*noxE*) of *Lactococcus lactis*, which further demonstrated that acid stress and dissolved oxygen affect the internal redox balance that controls 2,3-BDO biosynthesis in yeast. The primary goals of the of the present study were as follows: (1) compare the effects of different short-chain fatty acids on 2,3-BDO biosynthesis by the evaluated strain; (2) assess the effects of acid stress and dissolved oxygen contents on 2,3-BDO biosynthesis in yeast; and (3) optimize the dissolved oxygen conditions during the fermentation of W141 to produce 2,3-BDO. The results of the present study provides new ideas for improving the industrial yields of 2,3-BDO by *S. cerevisiae*.

**Methods**

2.1 Strains, plasmids, and media

The bacterial strains *S. cerevisiae* W141 and *Escherichia coli* DH5α were used in the present study. *L. lactis* NZ9000, which was primarily used to construct the mutant strain W141-E, was a gift from
Professor Meng of Northeast Agricultural University. *E. coli* DH5α was cultivated in Luria-Bertani medium (10 g/L tryptone, 5 g/L yeast extract, and 5 g/L sodium chloride) supplemented with 100 µg/mL ampicillin. Yeast cells were activated at 30°C in yeast-peptone-dextrose-adenine (YPD) medium (10 g/L yeast extract, 20 g/L Bacto peptone and 20 g/L glucose) and cultivated in fermentation medium (SD; 80 g/L glucose, 20 g/L Bacto peptone, 3.4 g/L YNB, 10 g/L (NH₄)₂SO₄, 11.8 g/L KH₂PO₄, and 3 g/L K₂HPO₄). Transformed yeast cells were screened on YPD medium supplemented with 175 µg/mL of hygromycin B. *L. lactis* NZ9000 was cultured in MRS medium [27]. The vector pMD18-T (Takara Bio Inc., Dalian, China) and the plasmid pSH69 (gifted by Professor Du from Guangxi University) were used to clone and express the *noxE* gene, respectively. All chemical reagents used in the present study were obtained from Tianjin Kemiu Chemical Reagent Co., Ltd., China. A Micro Pyruvate Decarboxylase (PDC) Assay Kit and a Micro Alcohol Dehydrogenase (ADH) Assay Kit were purchased from Solarbio Science&Technology Co., Ltd., China. A 2,3-butanediol dehydrogenase (2,3-BDODS) ELISA Kit was purchased from Mlbio Bioengineering Co., Ltd., China.

2.2 Plasmid construction and quantitative PCR

The *noxE* gene was PCR amplified from *L. lactis* NZ9000 genomic DNA using the following forward and reverse primers: 5'-CGCAAGCTTAAAATGAAAATCGTAGTTATCGGTA-3' (HindIII site is underlined) and 5'-ACGCGTCGACTTTATTTGGCATTCAAAGCT-3' (SalI site is underlined). The amplified fragment was digested with HindIII and SalI and then inserted into the HindIII/SalI sites of the vector pMD18-T to construct pSH69-noxE. The *bdh1*, *hog1p*, *haa1p* and *noxE* gene expression was analysed by RT-PCR using a PrimeScript™ RT Reagent Kit (Takara Bio Inc., Shiga, Japan). RT-qPCR was performed using a Nanodrop 2000 spectrophotometer (Thermo Scientific Co., Ltd., USA) and an Applied Biosystems™ 7500 (Applied Biosystems, Inc., USA). The forward and reverse primers used for *bdh1*, *hog1p*, *haa1p*, *noxE* and 18S rRNA gene amplification are shown in Additional file 1 (Table S1). The primers were designed using Primer5 software. The CT values of each sample were analysed using the relative quantitative 2^-ΔΔCT method and were corrected using the reference gene 18S rRNA.

2.3 Validation of acid stress mechanism

*S. cerevisiae* W141 was inoculated into SD medium with different concentrations (0, 0.5, 1, 1.5, 2, and 2.5 g/L) of short-chain fatty acids (formic, acetic, propionic, butyric, and valeric acids). The 2,3-BDO yield was determined after 60 h of fermentation (refer to the maximum concentration of 2,3-BDO produced by W141), and RT-PCR was performed to determine gene expression levels, and the activities of key enzymes were determined using an enzyme activity kit following the manufacturer's instructions. In addition, an experimental group was set up to adjust the pH (3, 4, 5, 6, 7, 8) of the initial fermentation of SD medium by adding inorganic acid (sulfuric acid) to verify the yield conditions when evaluating the acid stress mechanism.

2.4 Construction of *S. cerevisiae* W141-E
The plasmid pSH69 (7566 bp) was digested with HindIII/SalI, and the resulting 6582-bp fragment was recovered and purified using a TIANgel Midi Purification Kit (Tiangen Biotech Co., Ltd. Beijing, China). Subsequently, it was ligated to the cloned target noxE gene fragment using DNA ligase at 15°C overnight to obtain the pSH69-noxE (HindIII/SalI) plasmid. A Yeast Transformation Kit (Maokang Bio Inc., Shiga, Japan) was used to obtain S. cerevisiae mutant strains that were cultivated on solid YPD medium supplemented with 175 μg/mL hygromycin B.

2.5 Dissolved oxygen control

The dissolved oxygen content of the SD medium was controlled by changing the number of gauze (3, 8, and 12 layers) during fermentation, the rotation speed of the shaker (100, 140, and 180 rpm) and the volume of liquid in a 250-mL triangular bottle (50-, 75-, and 100-mL), using a Mettler Toledo Inpro 6800 probe connected to a CE O₂ 4050 transmitter for dissolved oxygen detection. The conditions were optimized through orthogonal experiments.

2.6 HPLC analysis of intermediate metabolites

Full details of the LC-MS/MS analysis of the intermediate metabolites are described in Additional file 1: Method S1. Metabolite analysis was performed using a previously described method.

2.7 Statistical analysis

Statistical analysis of the test data was performed using Statistical Product and Service Solutions (SPSS), with the results presented as X ± SD. Probability values of less than 0.05 (p<0.05) were considered to indicate a significant difference. Origin 2020 was used for data statistical analysis and to generate charts.

Results

3.1 Effect of Exogenous Short-chain Fatty Acids on the Fermentation Performance of S. cerevisiae W141

The results of 25 groups of experiments (Fig. 2A-F) showed that the addition of formic, butyric and valeric acids severely inhibited the 2,3-BDO and ethanol yields of W141. Among them, the inhibitory effect of formic acid was the most notable. When 0.5 g/L formic acid was used, the 2,3-BDO yield from W141 was 1.25 ± 0.09 g/L after 60 h, which was 35.90% lower than that of the control group (1.95±0.12 g/L, 60 h), and the maximum ethanol output was 22.65 ± 0.11 g/L after 48 h, which was 24.40% lower than that of the control group (29.96 ± 0.15 g/L, 48 h), and as the formic acid concentration increased, the ability of W141 to synthesize 2,3-BDO was lost, and the ethanol fermentation activity also gradually decreased. When 2.5 g/L formic acid was used, W141 could barely grow (details are provided in Additional file 1: Results S1). Although the inhibitory effect of butyric and valeric acids on W141 ethanol synthesis was relatively low at the same concentration, the inhibitory effect of valeric acid on 2,3-BDO synthesis by W141 was stronger than that of butyric acid. Upon the addition of 1.5 g/L valeric acid, the ability of W141
to synthesize 2,3-BDO was lost, while butyric acid needed to be added at 2.5 g/L to achieve this result. Among the 5 groups of acids, propionic acid had the smallest effect on the ethanol fermentation of W141. When 2.5 g/L propionic acid was used, the maximum ethanol yield of W141 was 28.12 ± 0.16 g/L after 48 h, which was only 6.14% lower than that of the control group. At this time, a trace amount of 2,3-BDO was still being synthesized, which is not observed for the other four acids. The addition of 0.5 g/L propionic acid also increased the 2,3-BDO yield by 12.31% (2.19 ± 0.14 g/L, 60 h). Based on the results of the 25 experiments, the addition of 1 g/L acetic acid was considered to be the most effective condition to increase the yield of 2,3-BDO. Under these conditions, the 2,3-BDO yield from strain W141 increased by 29.74%, reaching 2.53 ± 0.05 g/L after 60 h, but compared to propionic acid, when the concentration of acetic acid was greater than or equal to 2.0 g/L, 2,3-BDO synthesis by W141 abruptly stopped. The effect of acetic acid on the ethanol fermentation of W141 was second only to propionic acid. After adding 2.5 g/L acetic acid, the maximum ethanol yield was 26.48 ± 0.06 g/L after 48 h, which was 11.62% lower than that of the group without acetic acid. The above results indicate that formic, butyric and valeric acids inhibit the ethanol and 2,3-BDO fermentation properties of W141, with the toxicity of formic acid being the most lethal. However, low concentrations of propionic and acetic acids promoted 2,3-BDO synthesis by strain W141, and the two acids had little effect on ethanol fermentation.

In addition, after adding 1 g/L acetic acid, the enzymatic activity BDH1, which is involved in 2,3-BDO synthesis, increased by 32.26% in strain W141, and the relative expression of the bdh1 gene increased by 1.55-fold compared to the control. In addition, for the genes haa1p and hog1p, which are involved in the acid stress response, the relative gene expression increased by 1.45- and 1.37-fold, respectively. These results further show that the exogenous addition of 1 g/L acetic acid stimulates the acid stress response mechanism of W141, which promotes the biosynthesis of 2,3-BDO.

The results of the exogenous addition of inorganic acid (H$_2$SO$_4$) (Fig. 3A, B) shows that when the pH value of the initial fermentation broth was neutral or alkaline, the growth of W141 and the synthesis of 2,3-BDO was inhibited, but at a specific pH range (pH = 3~5), the amount of H$^+$ had little effect on the growth of W141 and the synthesis of 2,3-BDO. Combined with the fermentation results for short-chain fatty acids, this result further shows that different acid radical ions play a crucial role in 2,3-BDO synthesis, and acid stress is primarily caused by acid radical ions.

**3.2 Effect of intracellular redox balance on the 2,3-BDO yield from S. cerevisiae W141**

Acid stress will cause strains to consume a great deal of energy. Compared to W141, the engineered strain *S. cerevisiae* W141-E, which was constructed by exogenously overexpressing the noxE gene of *L. lactis* NZ9000, the maximum 2,3-BDO yield decreased by 15.38% (1.65 ± 0.14 g/L, 60 h) (Fig. 4A), while the maximum ethanol output occurred 12 h earlier than W141 and increased by 10.08%, (32.98 ± 0.16 g/L, 36 h). The maximum enzymatic activity of PDC, which plays a key role in the ethanol metabolism pathway, was 0.704 ± 0.013 U/mg at 36 h and increased by 29.54% compared to that observed for W141 (0.494 ± 0.006 U/mg, 36 h). However, the maximum activity of the ADH enzyme was 0.203 ± 0.04 U/mg at 36 h, which was 25.91% lower than that observed for W141 (0.274 ± 0.011 U/mg, 36 h). In addition, the
maximum activity of the BDH1 enzyme was $0.032 \pm 0.004$ U/mg at 48 h, which was 23.81% lower than that of W141 ($0.042 \pm 0.002$ U/mg, 48 h). These results show that overexpression of the \textit{noxE} gene inhibits the 2,3-BDO metabolic pathway in W141. In the presence of oxygen, NADH oxidase will consume a large amount of intracellular NADH, causing an imbalance of intracellular NADH/NAD$^+$, which in turn affects the W141 carbon metabolism pathway.

After the addition of 1 g/L acetic acid, the fermentation performance for ethanol and 2,3-BDO production by W141-E changed significantly. The 2,3-BDO yield increased by 43.64% (Fig. 4B), from 1.65 $\pm$ 0.14 g/L to 2.37 $\pm$ 0.14 g/L at 60 h, but the maximum ethanol yield dropped by 9.10% to 29.98 $\pm$ 0.07 g/L at 48 h. This result shows that the addition of 1 g/L acetic acid can effectively alleviate the inhibitory effect of overexpressing the \textit{noxE} gene on W141 2,3-BDO synthesis. The above results further indicate that acid stress is associated with the intracellular NADH content.

### 3.3 Influence of dissolved oxygen on the 2,3-butanediol yield

NADH is consumed intracellularly and requires the activity of NOXE enzymes as well as the presence of intracellular dissolved oxygen. Under laboratory conditions, through the orthogonal test optimization of the number of gauze layers, the speed of the shaker and the amount of liquid in the triangle bottle during fermentation (Fig. 5A), the dissolved oxygen content in the fermentation broth can be effectively controlled (the orthogonal table is provided in Additional file 1: Table S3). The above results show that a dissolved oxygen concentration of 0.6 vvm (A2B1C3) was the best fermentation conditions for 2,3-BDO production by W141 (Fig. 5B).

When the dissolved oxygen content was 0.6 vvm, the 2,3-BDO yield from W141 reached 2.56 $\pm$ 0.13 g/L at 60 h, which was 31.28% higher than that observed in the control group, and the expression of \textit{bdh1} gene increased by 1.31-fold. In addition, the 2,3-BDO yield from W141-E increased by 36.36% compared to the control group, reaching 2.25 $\pm$ 0.16 g/L at 60 h. Under the above conditions, after adding 1 g/L acetic acid, the 2,3-BDO yields from W141 and W141-E increased by 21.33 and 20.31%, respectively, compared to the group without acetic acid. The above experimental results show that the yield of 2,3-BDO from W141 is closely related to the amount of dissolved oxygen in the cell. Conditions with a small amount of dissolved oxygen were more suitable for 2,3-BDO synthesis. In addition, under in the presence of 0.6 vvm of dissolved oxygen, the maximum ethanol yields from W141 and W141-E were increased by 4.17 and 9.84% compared to the control group, reaching 31.21 $\pm$ 0.13 and 33.23 $\pm$ 0.07 g/L at 48 h, respectively. The maximum ethanol yields of W141 and W141-E in the presence of 1 g/L acetic acid were almost unchanged compared to the group without acid, reaching 31.48 $\pm$ 0.10 and 33.67 $\pm$ 0.10 g/L at 48 h, respectively. Under optimal external dissolved oxygen conditions for 2,3-BDO production, the external oxygen is strictly controlled, and \textit{noxE} gene overexpression led to the consumption of intracellular NADH being inhibited, causing 2,3-BDO synthesis in the strains to return to their original levels or even higher. When 1 g/L acetic acid was added at this time, the 2,3-BDO yield could be increased by 21.33%, indicating that the acetic acid stress on the strains was related to the intracellular NADH content.
Discussion

In the present study, we assessed the effects of the dissolved oxygen content and the presence of the noxE genes, which led to significant increases and decreases in 2,3-BDO and ethanol production by W141, respectively (Figs. 4, 5). Dissolved oxygen plays a crucial role in the production and metabolism of industrial fermentation microorganisms. Based on the genome-scale metabolic model (GSM) of S. cerevisiae, Mesquita et al developed a control system for ethanol production by manipulating feed and dissolved oxygen levels, which increased the maximum ethanol yield of the assayed strain by 15% [14]. Chopda et al developed a platform using a built-in electronic system of a reactor using with the TCP/IP protocol in LabVIEW to realize the decoupled input and output linearization controller (DIOLC). Compared to that observed using the PID controller, the biomass yield of yeast cells increased by 23% using the DIOLC [28]. Kirk et al developed a membrane-aerated microbial reactor driven by an oscillating jet without any moving parts that could accurately control the dissolved oxygen concentration and measure the oxygen absorption rate in real time, and they verified the performance of the device with using S. cerevisiae [29]. Oxygen utilization is a key factor for glucose catabolism in S. cerevisiae, and regulating the oxygen supply threshold is key to the regulation of yeast metabolic pathways. If the dissolved oxygen threshold is too low, yeast metabolism will shift to produce glycerol and other metabolic by-products, thereby reducing ethanol yield. In contrast, when the dissolved oxygen content is too high, although the maintenance of cell viability and the formation of yeast biomass is promoted, these conditions will also reduce ethanol biosynthesis [14]. In the present study, we also observed that when the amount of dissolved oxygen in the fermentation environment gradually decreased, the ethanol metabolic capacity of the yeast gradually decreased, and the production of 2,3-BDO and glycerol first increased and then decreased. When the dissolved oxygen content was 0.6 vvm, the largest yield of 2,3-BDO was obtained, reaching 2.56 ± 0.13 g/L. These results were similar to the optimal dissolved oxygen amount for metabolically engineered Klebsiella oxytoca KMS005 to synthesize 2,3-BDO (0.8 vvm), whereas the optimal amount of dissolved oxygen for meso-2,3-BDO produced by Bacillus subtilis was 0.02 vvm [30, 31]. These results show that different microorganisms that produce 2,3-BDO have their own optimal dissolved oxygen conditions.

In addition, in the present study, although the ethanol yields from the W141-E strain increased by 10.08%, the 2,3-BDO and glycerol yields decreased by 15.38 and 19.23%, respectively. Microbial 2,3-BDO biosynthesis is heavily dependent on the intracellular NADH content [9, 15]. Exogenous expression of the noxE gene of L. lactis in yeast will disrupt the intracellular NADH/NAD⁺ dynamic balance, which has often been used to alleviate the intracellular redox imbalance caused by the knockout of key genes in the yeast ethanol and glycerol metabolic pathways [12]. The strains engineered from S. cerevisiae ΔPDC are the primary strains used for the industrial production of 2,3-BDO. For example, 2,3-BDO production by S. cerevisiae (BD5_G1CtPDC1) was increased 2.3-fold compared to the control group to reach 121.8 g/L (fed-batch fermentation), and after the exogenous expression of the noxE gene from L. lactis, its 2,3-BDO yield increased by 80.7%, reaching 154.3 g/L (fed-batch fermentation) [27]. However, because the degree of reduction (γ = 5.5) of 2,3-BDO was less than ethanol (γ = 6), the accumulation of NADH occurs, leading
to the biosynthesis of glycerol during industrial production. Kim et al in 2019 knocked out the genes GPD1 and GPD2, encoding glycerol-3-phosphate dehydrogenases, from S. cerevisiae (BD5_G1CtPDC1) to further solve the problem of interference with glycerol metabolism during 2,3-BDO synthesis [8].

Studies have shown that in bacteria, acid can promote the production of 2,3-BDO. The addition of acetic acid, propionic acid, and pyruvate during the fermentation Paenibacillus polymyxa was shown to increase the production of 2,3-BDO, where 1.0 g/L acetic acid increased the yield of 2,3-BDO by 2-3-fold [32]. After adding a certain amount of acetic acid to an Enterobacter aerogenes ATCC 29007 culture, the 2,3-BDO yield significantly increased [33]. In the present study, after the addition of butyric and valeric acids, the 2,3-BDO and ethanol yields from W141 decreased, and its growth was also significantly inhibited. These results occur because under anaerobic and micro-aerobic conditions, the tricarboxylic acid cycle (TCA) in S. cerevisiae is inhibited, causing it to be unable to produce sufficient ATP to synthesize medium and short-chain fatty acids (MCFAs, C4:0-C12:0), thereby inhibiting cell growth [34]. In addition, after adding formic acid, the growth and metabolism of W141 were significantly inhibited, and the synthesis of 2,3-BDO and ethanol was reduced. The results of a transcriptome study by Li et al showed that when formic acid is present in the fermentation environment, it will accelerate energy consumption by the cell to activate biotransformation, the stress response and transmembrane transport in the cell to resist formic acid [35]. However, formic acid can inhibit mitochondrial cytochrome oxidase at the end of the respiratory chain, and low concentrations of formic acid can cause a rapid burst of reactive oxygen species in the mitochondria that can kill yeast cells [36, 37]. Therefore, adding formic, butyric or valeric acids is not conducive to the growth of yeast cells and is even more unfavourable for synthesis of 2,3-BDO or other metabolites. However, the fermentation results obtained after adding acetic and propionic acids were different, and they were beneficial for 2,3-BDO synthesis by W141 at a specific concentration. Propionic acid had a relatively small stimulatory effect on the ability of W141 to produce ethanol and 2,3-BDO. A low concentration of propionic acid (0.5 g/L) could increase 2,3-BDO production by W141 by 12.31%. Furthermore, when the propionic acid concentration was 2.5 g/L, W141 could still produce ethanol and 2,3-BDO, which was the biggest difference compared to the other four acids. When S. cerevisiae uses cassava mash as a substrate for fermentation, researchers also observed that low concentrations of propionic acid have no obvious side effects on ethanol fermentation by yeast, but higher concentrations of propionic acid have an inhibitory effect [38]. Subsequently, the same research team verified that a low concentration of propionic acid had no obvious side effects on ethanol production during the coupling fermentation of propionic acid to ethanol-methane, and it could even increase ethanol production by 7.6% [39]. The promoting effect of propionic acid on ethanol fermentation can be explained from two aspects. 1) Propionic acid is converted into propionyl-CoA, which is used in the 2-methylcitric acid pathway to make part of pyruvate flow towards the ethanol fermentation pathway [40]. 2) The presence of propionic acid will stimulate the cells to increase energy production (such as ATP) to maintain the steady state of cytoplasmic pH [41], thereby increasing the ethanol output. Thus, a low concentration of propionic acid can promote 2,3-BDO metabolism in yeast, and the concentration of propionic acid has no obvious inhibitory effect on the ethanol metabolism of the strain. Although the fermentation results obtained after adding acetic and propionic acids were similar, the inhibitory effect of
acetic acid on 2,3-BDO production was stronger. In the presence of 2 g/L acetic acid, W141 halted the biosynthesis of 2,3-BDO, whereas the addition of an appropriate amount of acetic acid increased the 2,3-BDO production of W141. When 1 g/L acetic acid was added, 2,3-BDO production by W141 increased by 29.74% compared to the control group, and the relative expression of the bdh1 gene increased by 1.55-fold, while the BDH1 enzyme activity increased by 32.26%. These results occurred because in yeast mitochondria, acetic acid, like pyruvate, will be converted into acetyl-CoA in the TCA cycle, but too high a concentration of acetic acid will lead to an imbalance of intracellular acetylation, which will be detrimental to cell survival [19]. In addition, we observed that the relative expression of the haa1p and hog1p genes in W141 cells increased by 1.45- and 1.37-fold, respectively, indicating the cells it responded to the stress of acetic acid. The transcription factor encoded by haa1p regulates 80% of the genes related to acetic acid tolerance [42], such as the acid-tolerance gene hog1p. Hog1p can bind to and phosphorylate the channel protein Fps1p, causing it to undergo endocytosis and reducing the entry of acetic acid into the cell [43]. Increasing evidence shows that determinants of acetic acid tolerance allow glycolysis and the TCA cycle to provide sufficient nicotinamide adenine dinucleotide (NADH) [16]. The experimental results described above further indicate that acid stress and the amount of dissolved oxygen regulate 2,3-BDO biosynthesis in yeast by altering the intracellular redox balance.

**Conclusion**

In the present study, the strain *S. cerevisiae* W141-E was constructed and analysed, the results of which further verified that changes in the 2,3-BDO fermentation performance of *S. cerevisiae* under acid stress conditions are primarily caused by changes in intracellular redox. Overexpression of the noxE gene or the addition of formic, butyric and valeric acids was not conducive to the biosynthesis of 2,3-BDO in yeast cells, but a specific amount of acetic and propionic acids promoted increased 2,3-BDO yields from W141. In addition, after adding 1 g/L acetic acid, the 2,3-BDO yield from W141 and W141-nxoE increased by 20.31 and 21.33%, and the relative expression of the haa1p and hog1p genes in W141 cells was increased by 1.45- and 1.37-fold, respectively. The results of the present study indicate that an appropriate level of acetic acid stress may improve the 2,3-BDO fermentation performance of yeast cells.

**Abbreviations**

2,3-BDO: 2,3-butanediol; BDH: butanediol dehydrogenase; RT-qPCR: quantitative real time PCR; noxE: NADH oxidase gene; OD: optical density; PDC: pyruvate decarboxylase; ΔPDC: PDC-deficient yeast; TCA: tricarboxylic acid; YPD: yeast–peptone–dextrose–adenine; NADH: nicotinamide adenine dinucleotide; NAD⁺: nicotinamide adenine dinucleotide; HPLC: high pressure liquid chromatography.

**Declarations**

**Authors’ contributions**

ZLY, DAL, JPG and WXP wrote the manuscript. All authors read and approved the final manuscript.
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Competing interests

The authors declare that they have no competing interests.

Availability of data and materials

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Consent for publication

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Ethics approval and consent to participate

Not applicable.

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**Figures**

Figure 1

Acid stress and dissolved oxygen regulate 2,3-BDO biosynthesis by altering the redox balance in yeast cells. The red dashed circle shows the strategy of using the *S. cerevisiae* pyruvate carbon pathway to increase 2,3-BDO yields, and the blue dashed arrow shows the influence of the intracellular redox balance on 2,3-BDO yields [12, 13].
Figure 2

Fermentation results obtained by the exogenous addition of 5 types of short-chain fatty acids at different concentrations. A: The heat map shows the change in the 2,3-BDO yield from W141 when the fermentation progressed to 60 h. The ordinate shows different types of acids, while the abscissa shows the acid concentration. B-F: The broken dotted line graph shows the influence of 5 different types of acids (formic, acetic, propionic, butyric and valeric acids) on ethanol fermentation by W141. The hollow dots
represent the changes in glucose concentration in the fermentation medium, and the solid dots represent the changes in ethanol yield. The fermentation period was 72 h. The data are presented as the means ± standard deviation of three independent experiments (n = 3 each).

Figure 3

The effect of adding mineral acid (H2SO4) on the fermentation performance of W141. A: The bar chart shows the 2,3-BDO fermentation results for W141, where mineral acid (H2SO4) was added to adjust the...
initial pH value of the fermentation medium (pH 3, 4, 5, 6, 7, or 8). B: The solid scatter graph shows the change in the OD600nm value of W141 under different initial pH conditions, and the broken dotted line graph shows the change in the pH value. The data are presented as the means ± standard deviation of three independent experiments (n = 3 each).
Fermentation results for W141 and W141-E in the presence of 0 and 1 g/L acetic acid. A: The broken dotted line graph shows the fermentation results for ethanol and 2.3-BDO produced by W141 and W141-E after the addition of 0 g/L acetic acid. B: The broken dotted line graph shows the fermentation results for ethanol and 2.3-BDO produced by W141 and W141-E after the addition of 1 g/L acetic acid (AA). The data are presented as the means ± standard deviation of three independent experiments (n = 3 each).
The number of gauze (3, 8, and 12 layers), the speed of the shaker (100, 140, and 180 rpm) and the amount of liquid in the 250-mL Erlenmeyer flask (50-, 75-, and 100-mL) was changed to influence the production of 2,3-BDO by W141. A: The bar chart shows the effect of changing a single factor on the 2,3-BDO yield of W141. The number of gauze layers is represented by A (1, 2, and 3), the speed of the shaker is represented by B (1, 2, and 3), and the amount of liquid in the 250-mL triangular bottle is represented by C (1, 2, and 3) means. B: The scatter diagram shows the fermentation results for 2,3-BDO production by W141 after optimization by orthogonal experiments. The data are presented as the means ± standard deviation of three independent experiments (n = 3 each).

Figure 6

The fermentation results for different treatment groups of W141 and W141-E, where the dissolved oxygen content was 0.6 vvm. The broken dotted shows line graph shows the ethanol fermentation results for the strains, and the bar graph shows the 2,3-BDO fermentation result for the strains.

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