Ergosterol Supplementation Improves Furfural Tolerance of *Saccharomyces cerevisiae* to Produce Ethanol and Its Underlying Mechanism

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Furfural produced during lignocellulose pretreatment to reduce the recalcitrance inhibits the growth of *Saccharomyces cerevisiae* and reduces ethanol yield. To reduce the adverse effect of furfural on *S. cerevisiae*, exogenous ergosterol was supplemented and the impact on *S. cerevisiae* under furfural stress was studied. The lag phage was shortened by 50%, and the maximum ethanol yield was increased by 158% with 50 mg/L ergosterol supplementation under 4 g/L furfural stress. Flow cytometry analysis results showed that permeable cells and intracellular reactive oxygen species were decreased by 45 and 53%, respectively with the addition of ergosterol under furfural stress. The fatty acid composition of *S. cerevisiae* was changed; the intracellular glycerol and ergosterol content was increased after ergosterol supplementation. The saturation of fatty acid was increased. Addition of ergosterol promoted cell growth by decreasing oxidative stress. Under 4 g/L furfural stress, the lag phage of *S. cerevisiae* BY4741 (*erg3⊿*) and *S. cerevisiae* BY4741 (*erg5⊿*) was longer than that of *S. cerevisiae* BY4741, and the maximum ethanol concentration was decreased.

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INTRODUCTION

The process of biofuel production from lignocellulose includes pretreatment, enzymatic hydrolysis, fermentation, and distillation (Yao *et al.* 2022). During biomass pretreatment, various inhibitors such as furfural are generated, and these suppress the growth of *Saccharomyces cerevisiae* and consequently limit the ethanol production (Huang *et al.* 2019). Furfural is one of the main toxic chemicals formed from carbohydrates *via* pentose dehydration during acid-catalyzed biomass pretreatments. It prevents the growth of yeast and interferes with the critical fermentation step, thus affecting the production of bioethanol (Liu *et al.* 2021).

Earlier studies showed that furfural of 3 g/L reduced the production of ethanol by blocking cell growth (Gonçalves *et al.* 2015). *S. cerevisiae* oxidizes furfural to furoic acid with oxygen or reduces it to furfural alcohol without oxygen to achieve the purpose of detoxification (Wang *et al.* 2016). Therefore, it has been widely used in ethanol

fermentation. Under stress conditions, such as ethanol at high concentration and lignocellulose hydrolysate, reactive oxygen species (ROS) accumulate in cells, which hinders their growth and fermentation (Jayakody and Jin 2021). Previously, the addition of 3.5% biochar (as an additive) was shown to improve the ethanol production of *Zymomonas mobilis* with furfural at high concentration (Wang *et al.* 2020). The addition of proline and inositol to the medium has a protective effect on the growth of *S. cerevisiae* under furfural stress, and the intracellular ROS is decreased with the increase of proline content (Wang *et al.* 2015). Furthermore, the yeast's tolerance to acetic acid is improved by adding zinc sulfate to the culture medium (Wan *et al.* 2015).

Under furfural stress, cell integrity plays a vital role in maintaining cell viability and normal metabolic function. Furfural stress conditions affect the lipid content of the cell membrane (Lopes *et al.* 2015). Supplementation with oleic acid and ergosterol relieves the oxidative stress of *S. cerevisiae* (Landolfo *et al.* 2010).

As a structural analogue to cholesterol, ergosterol plays vital role in fungal plasma membrane (Beni *et al.* 2014). It regulates the permeability of the plasma membrane and the activity of proteins for membrane-binding (Wollam and Antebi 2011). It is widely applied in medicine, and its derivatives have notable antitumor activities (Kitchawalit *et al.* 2014). Although ergosterol biosynthesis process in yeast has been clarified, the role of ergosterol in yeast under furfural stress is unclear.

The effects of ergosterol on furfural tolerance of *S. cerevisiae* were assessed in the present study. To examine the underlying mechanism, the response of the cell to furfural stress was observed. The effects of ergosterol on furfural tolerance of *S. cerevisiae* were demonstrated by the determination and comparison of the content of glycerol, hydrogen peroxide, and catalase activity. Finally, the key genes *erg3⊿* and *erg5⊿* knockout strains of ergosterol synthesis were used to validate the effect of ergosterol on furfural tolerance of *S. cerevisiae*.

EXPERIMENTAL

Microorganism

Saccharomyces cerevisiae (isolated and given by Angel Yeast Co., Ltd), *S. cerevisiae* BY4741 (American type culture collection 18824), *S. cerevisiae* BY4741 (*erg3⊿*) (engineered and stored in our own lab), and *S. cerevisiae* BY4741 (*erg5⊿*) (engineered and stored in our own lab) were maintained in slants (glucose 20 g/L, agar 20 g/L, peptone 10 g/L, yeast extract 5 g/L) at 4 °C.

Different concentrations of furfural (0.0, 1.0, 2.0, 4.0 g/L) were added to the yeast extract peptone dextrose (YEPD) medium to evaluate the potential impact of furfural on *S. cerevisiae*. Ergosterol supplementation under furfural stress (4.0 g/L) was evaluated with different concentrations (0 mg/L, 5 mg/L, 10 mg/L, 20 mg/L, 50 mg/L).

Precultures were grown overnight at 30 ℃ in 250 mL shake flasks containing 50 mL YEPD medium without furfural and ergosterol. The initial OD600 of 0.5 was cultivated in an orbital shaker at 30 ℃ and 200 rpm with different addition of furfural with or without ergosterol supplementation.

Methods

Determination of biomass, glucose, and ethanol

The concentration of yeast biomass was determined by the measurement of absorbance at 600 nm. Glucose content was measured by the DNS method (Dai *et al.* 2020). Ethanol was determined with biological sensor analyzer (SBA-40C, Biological Institute of Shandong Academy of Science, Shandong province, China).

Determination of furfural

Furfural was analyzed by high performance liquid chromatography (Shimadzu LC-20A, Kyoto, Japan) with a UV detector at a wavelength of 277 nm. Furfural was separated on a C18 column (SinoChrom ODS-AP 300A 5μm), 0.1% (v/v) acetic acid was applied as eluent, at a flow rate of 1.0 mL/min.

Flow cytometry analysis

A flow cytometer (FACS, Becton-Dickinson, Franklin Lakes, NJ, USA) was used to monitor the physiological stress response of *S. cerevisiae* under furfural stress and ergosterol supplementation. Propidium iodide (PI, P-1304) was applied to monitor the membrane integrity, and 2',7'-dichlorofluorescein diacetate (DCFDA) was used to determine the accumulation of ROS.

One milliliter of fermentation broth with OD_{600} 0.25 was centrifuged at 2000 rpm for 5 min to collect the cells. The cells were washed three times with PBS. Pre-cooled 70% (v/v) ethanol was added to fix the cell at 4 ℃ for 1 h, and then 1 mL of PBS was added to resuspend the cells. After filtering with a 400-mesh screen, the cells were mixed with 10 μL of corresponding dye solution. The cells were protected from light at 4 ℃ for 30 min and ultrasoniced for 1 min before flow cytometry analysis.

Extraction and determination of ergosterol

The precipitate was separated from 10 mL of fermentation broth by centrifugation followed by washing with distilled water (5 mL×2). Approximately 20 mL of 20% (v/v) sodium hydroxide solution and 10 mL of 95% (v/v) ethanol were added. After reflux saponification in a water bath for 1.5 h at 85 to 90 ℃, another 4 mL of 95% (v/v) ethanol was added, and saponification was continued for 1 h. After adding 25 mL of acetone, the mixture was shaken violently for 15 min and then left to incubate without shaking for 2 h. The upper extract was detected by a UV spectrophotometer at 282 nm (Pastinen *et al.* 2017).

Determination of fatty acids

The methods used for harvesting, saponification, methylation, and extraction of cellular fatty acids followed published protocols (Dai *et al.* 2014). The fatty acid composition analysis of the extracted yeast cells was carried out using an Agilent gas chromatograph (6890 N, Palo Alto, CA, USA) and the MIDI Sherlock YEAST6 program. The temperature of the hydrogen ion flame detector (FID) was 300 ℃. A Hp-ultra2 capillary column was used, and the temperature of the vaporization chamber was 250 ℃. The carrier gas was 30 mL/min (H_2). The furnace temperature rose from 170 °C to 260 °C at a rate of 5 °C/min.

Determination of glycerol, hydrogen peroxide, and catalase

The concentration of H_2O_2 was quantified using a hydrogen peroxide detection kit (BC3590, Beijing Solarbio Technology Co., Ltd, Beijing, China). Catalase was determined by a catalase detection kit (A007-1-1, Nanjing Jiancheng Bioengineering Institute, Nanjing, China). The concentration of glycerol was detected by a glycerol detection kit (Beijing pulley Gene Technology Co., Ltd., EL002, Beijing, China).

Statistical analysis

All experiments were performed in triplicate, and the mean ± standard deviation values were determined. The cells were collected at mid-exponential phase, unless stated otherwise. The diagrams were drawn by OriginPro 2019b software

(Northampton, MA, USA) and FlowJo_V10. Statistica 23.0 software (SPSS Inc., Chicago, IL, USA) was applied for significant difference analysis.

RESULTS AND DISCUSSION

Effect of Ergosterol Supplementation on Furfural Tolerance of *S. cerevisiae*

Influence of furfural on growth and ethanol production of S. cerevisiae

To investigate the impact of furfural on the growth of *S. cerevisiae*, different concentrations of furfural were added to the YEPD medium. The addition of 1 g/L furfural did not show obvious influence on the growth of *S. cerevisiae*. When the furfural concentration was increased to 2 and 4 g/L, the lag phase was extended from 4 h (1 g/L furfural) to 12 (2 g/L furfural) and 48 h (4 g/L furfural), respectively, as shown in Fig. 1A. The X_{max} was decreased under higher furfural stress, as illustrated in Table 1. Notably, *S. cerevisiae* began to grow when the furfural concentration was decreased to 0.1 g/L regardless of the initial concentration. Figure 1C shows that the concentration of furfural decreased sharply during the first 10 h. The rate of decrease slowed until all furfurals were converted.

The glucose consumption and ethanol production profile are presented in Fig. 1B and 1D, respectively. When *S. cerevisiae* began to grow, glucose concentration decreased sharply, and ethanol production increased. Furfural in a low concentration (1 g/L) did not exhibit significant impact on glucose consumption and ethanol production of *S. cerevisiae*. At higher concentrations, furfural showed a great impact on the ethanol production of *S. cerevisiae*. The $EtOH_{max}$ was 9 g/L (control), 8.75 g/L (1 g/L), 7.00 g/L (2 g/L), and 5.50 g/L (4 g/L), respectively. The time corresponding to $EtOH_{max}$ was delayed from 8 h (control) to 12 h (1 g/L), 24 h (2 g/L), and 48 h (4 g/L). The higher concentration of furfural had a larger impact on ethanol production from *S. cerevisiae*.

Previous studies have shown that when furfural is less than 2.5 g/L, it has no inhibitory effect on ethanol fermentation of highly active dry yeast. The increasing furfural concentration slows furfural metabolism, reduces ethanol yield, and prolongs the fermentation cycle. A previous study showed that ethanol yield is reduced to 87% at 32 h after addition of 5 g/L of furfural (Fu *et al.* 2014).

Table 1. Kinetic Parameters for *S. cerevisiae* in YEPD Medium with Increasing Initial Furfural Concentrations

Furfural (g/L)	Lag phase (h)	μ (/h)	X_{max} (g/L)	$Y_{X/S}$ (mg/g)	$EtOH_{max}$ (g/L)	$Y_{P/S}$ (g/g)
0	2	0.041±0.002	45.2±0.43	3.1±0.82	9±0.42	0.62±0.01
1	4	0.042±0.005	42.3**±0.48	2.9±0.20	8.8±0.48	0.57*±0.02
2	12	0.028**±0.001	42.8*±0.97	2.4±0.29	7.0**±0.35	0.39***±0.01
4	48	0.017***±0.001	39.4*±2.38	1.9±0.04	5.5***±0.41	0.27***±0.01

Note: μ refers to specific rate of growth, X_{max} is the maximum biomass obtained, $Y_{X/S}$ represents the biomass yield, $EtOH_{max}$ is the maximum ethanol obtained, $Y_{P/S}$ refers to the ethanol yield. The significance was analyzed by comparing with 0 g/L furfural, "*" ($0.01 \le P < 0.05$), "**" ($0.001 \le P < 0.01$), "***" ($P < 0.001$).

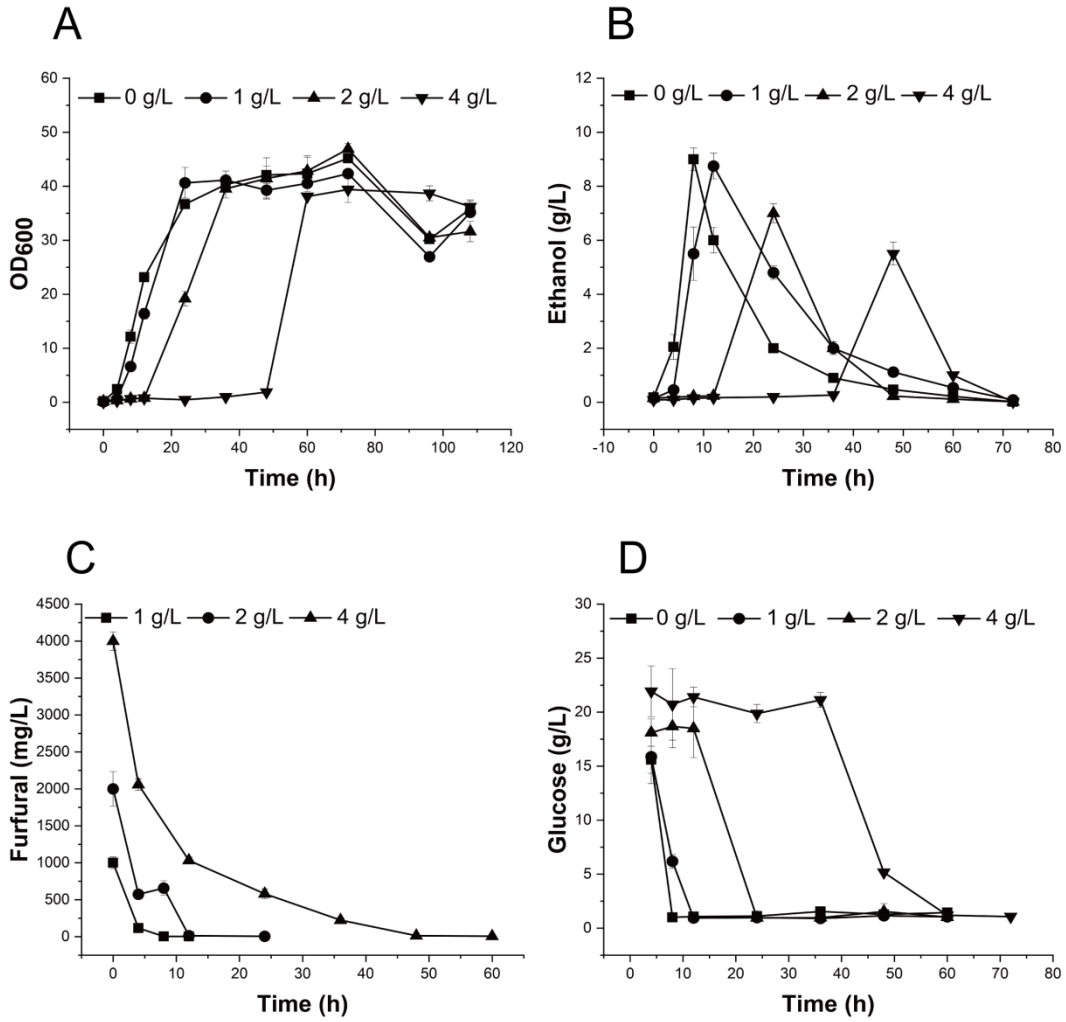

Fig. 1. Cultures profiles of *S. cerevisiae* in YEPD medium with increasing initial furfural concentrations. (A, OD_{600}; B, ethanol; C, furfural concentration; D, glucose)

Ergosterol supplementation increased furfural tolerance of S. cerevisiae

At 4 g/L furfural loading, different concentrations of initial ergosterol were added into the medium to analyze the influence of ergosterol supplementation on furfural tolerance of *S. cerevisiae*. As shown in Fig. 2A and Table 2, the lag phase was shortened with the increased ergosterol supplementation, which was 48 h (0 mg/L), 36 h (5 mg/L, 10 mg/L), and 24 h (20 mg/L, 50 mg/L), respectively. In addition, the X_{max} was also increased with increased ergosterol supplementation. *S. cerevisiae* began to grow when the furfural concentration was decreased to 0.1 g/L. The results implied that ergosterol supplementation might help speed up furfural detoxification (Fig. 2C).

The glucose consumption and ethanol production profile with ergosterol supplementation at 4 g/L furfural loading are presented in Figs. 2B and 2D. With the growth of *S. cerevisiae*, glucose decreased, and ethanol was increased accordingly. Ergosterol supplementation shortened the lag phase and increased the ethanol yield. The $EtOH_{max}$ was 6.0, 6.2, 6.9, 9.7, and 15.5 g/L, for control, 5 mg/L, 10 mg/L, 20 mg/L, and 50 mg/L ergosterol supplementation, respectively. The results indicated that ergosterol supplementation increased ethanol production of *S. cerevisiae* under furfural stress (4 g/L).

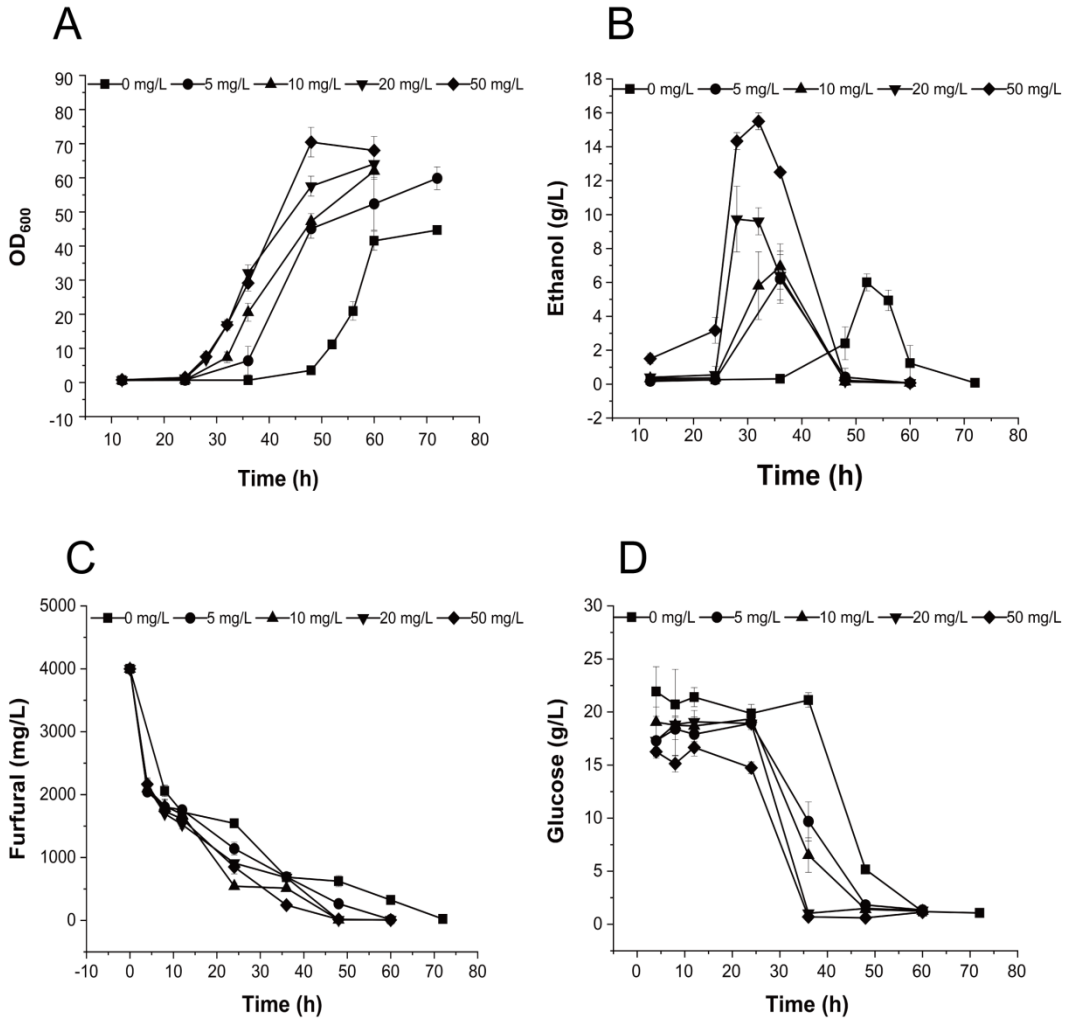

Fig. 2. Cultures profiles of *S. cerevisiae* in YEPD media containing 4 g/L furfural and different ergosterol supplementation. (A, OD_{600}; B, ethanol; C, furfural concentration; D, glucose)

Table 2. Kinetic Parameters for *S. cerevisiae* in YEPD Media with Increasing Initial Ergosterol Concentrations under 4g/L Furfural Stress

Ergosterol (mg/L)	Lag phase (h)	μ (/h)	X_{max} (g/L)	$Y_{X/S}$ (mg/g)	$EtOH_{max}$ (g/L)	$Y_{P/S}$ (g/g)
0	48	0.016±0.003	44.7±1.1	2.1±0.69	6.0±0.5	0.28±0.01
5	36	0.021±0.004	59.9**±3.3	3.4±0.58	6.2±1.44	0.35**±0.02
10	36	0.028**±0.002	62.0***±1.1	3.4*±0.29	6.9±1.33	0.38**±0.02
20	24	0.042***±0.003	64.1**±4.6	3.5±0.78	9.7*±1.94	0.53***±0.01
50	24	0.042***±0.002	70.4**±4.3	4.3*±0.81	15.5***±0.5	0.96***±0.04

Note: μ refers to specific rate of growth, X_{max} is the maximum biomass obtained, $Y_{X/S}$ represents the biomass yield, $EtOH_{max}$ is the maximum ethanol obtained, $Y_{P/S}$ refers to the ethanol yield. The significance was analyzed by comparing with 0 g/L ergosterol, "*" ($0.01 \le P < 0.05$), "**" ($0.001 \le P < 0.01$), "***" ($P < 0.001$).

Supplementing components in the medium (lauric acid, palmitoleic acid, stearic acid, linolenic acid, and ergosterol) can change the lipid composition of cells, and different lipid composition is closely related to the performance of *S. cerevisiae* at low

temperatures (Marian *et al.* 2009). After supplementing oil, the fermentation cycle is reduced, which are consistent with the present results. Landolfo *et al.* (2010) found that supplementing lipid nutrients under adverse conditions reduces the oxidative stress and oxidative damage to *S. cerevisiae*.

To determine whether the addition of ergosterol promotes the production of ethanol without furfural, only ergosterol was added to the YEPD medium. As shown in Fig. 3A, compared with the control, adding ergosterol of different concentrations increased the biomass (OD_{600}) of *S. cerevisiae*. The maximum biomass increased with the increase of ergosterol concentration. Figure 3B shows that when ergosterol was added, compared with control, the yield of ethanol was not increased. When 50 mg/L ergosterol was added, glucose was exhausted within 6 h, two hours earlier than the other groups (Fig. 3C). The results confirmed that the addition of ergosterol improved the furfural tolerance of yeast. More importantly, the ethanol yield of *S. cerevisiae* was improved under high furfural stress.

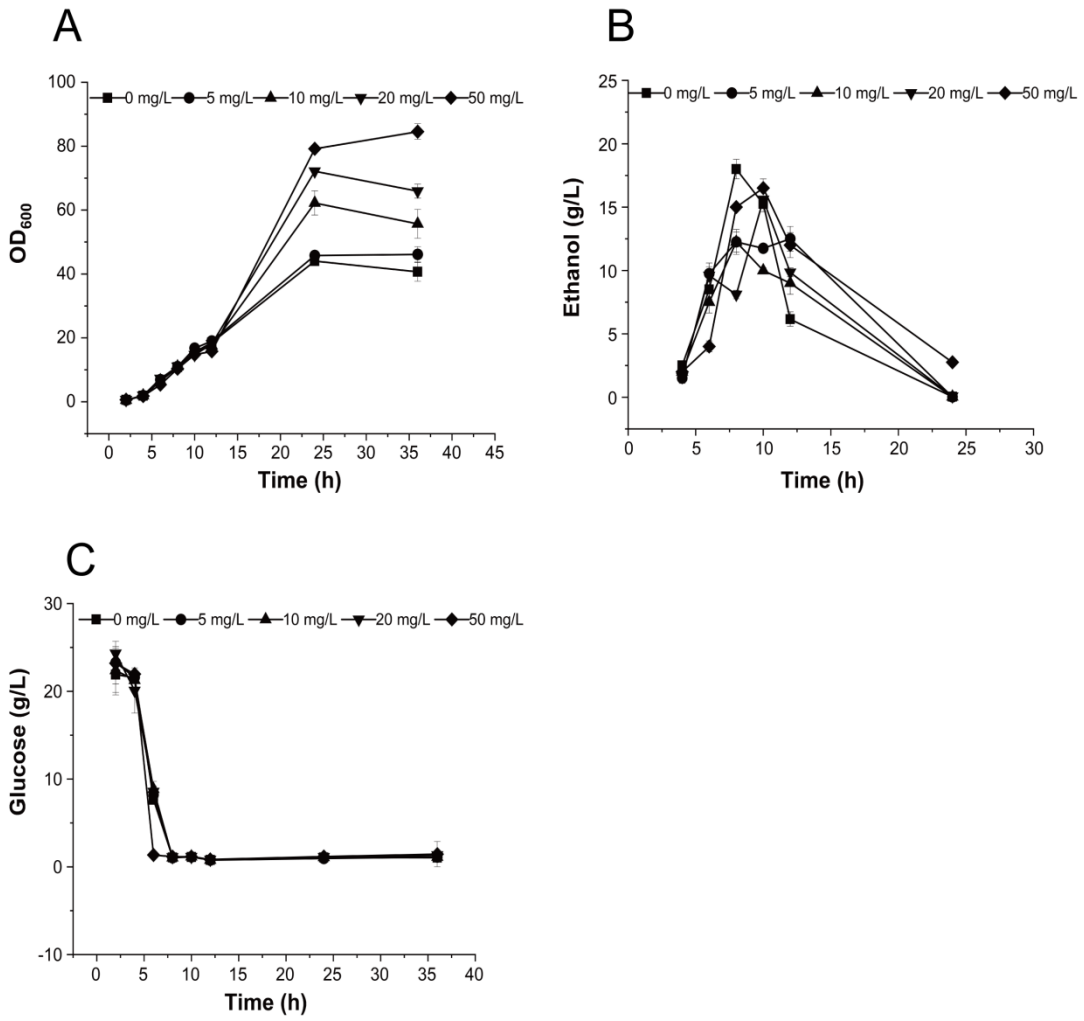

Fig. 3. *S. cerevisiae* batch cultures profiles in YEPD media containing increasing initial ergosterol concentrations. (A, OD_{600}; B, ethanol; C, glucose)

Analysis of cell membrane porosity of S. cerevisiae under furfural stress with ergosterol supplementation

To elucidate the mechanism of ergosterol enhancing furfural tolerance of *S. cerevisiae*, cell membrane porosity was determined and compared with furfural at 4 g/L with 50 mg/L ergosterol. As shown in Fig. 4, propidium iodide (PI) was applied to

evaluate the porosity of the membrane. The membrane permeability of control was 0.13%, which was increased to 15% with 4 g/L furfural, indicating that cells with permeable cell membrane were increased under furfural stress. The proportion of cells with incomplete cell membrane decreased to 8.5% with ergosterol supplementation, which was 45% lower than that of the furfural group.

The results indicated that the membrane integrity of yeast cells was affected by the addition of furfural. Ergosterol supplementation improved cell membrane integrity under furfural stress. In previous studies, under 4 g/L furfural stress, the proportion of permeabilized cells of *S. carlsbergensis* increased to 57%, and the proportion of healthy cells in the highest concentration furfural group was the lowest at the end of culture. The integrity of the cell membrane was influenced by the presence of furfural (Lopes *et al.* 2015). Additionally, furfural had a serious impact on the membrane potential. The membrane potential of *S. cerevisiae* was low in the lag phase. The decrease of membrane potential was the greatest when furfural, vanillin, and acetic acid were present (López *et al*. 2021). In this study, similar results were obtained.

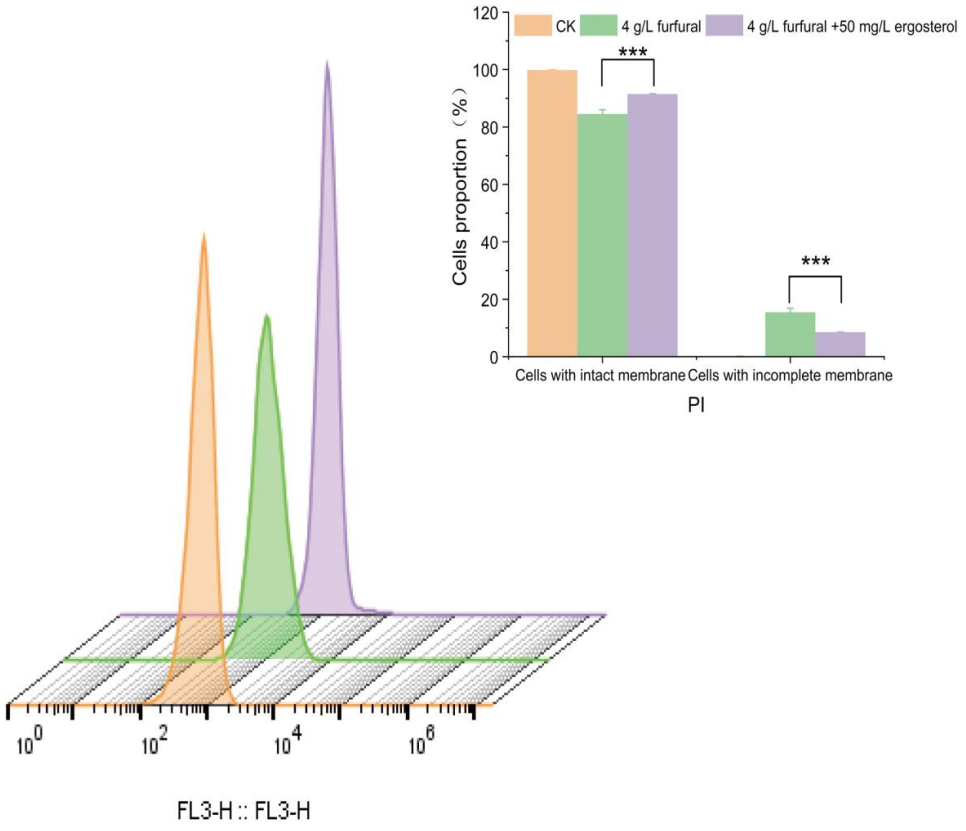

Fig. 4. *S. cerevisiae* subpopulations under 4 g/L furfural stress and 50 mg/L ergosterol. (PI), "***" ($P < 0.001$)

Analysis of ROS of S. cerevisiae under furfural stress with ergosterol supplementation

ROS accumulation leads to DNA and protein damage, and reduced viability of cultured cells (Moradas *et al.* 1996). As shown in Fig. 5, the proportion of cells with ROS under furfural stress was 28%, and this was decreased to 13% with ergosterol addition, indicating that the ROS level was decreased with ergosterol supplementation. The proportion of cells with ROS decreased by 53% after ergosterol addition. Earlier studies indicated that lipid nutrients could reduce cell-to-cell variations under oxidative stress, and intracellular ROS content was lower (Landolfo *et al* 2010).

ROS accumulated gradually in the lag phase and growth phase with acetic acid, furfural, vanillin, and other inhibitors. Compared with furfural and vanillin, greater ROS accumulation was observed with acetic acid (López *et al.* 2021). Furfural-induced ROS accumulation in *S. cerevisiae* was studied by other researchers (Allen *et al* 2010). Compared with 50 mM furfural, the damage induced by furfural was not very serious with 25 mM furfural.

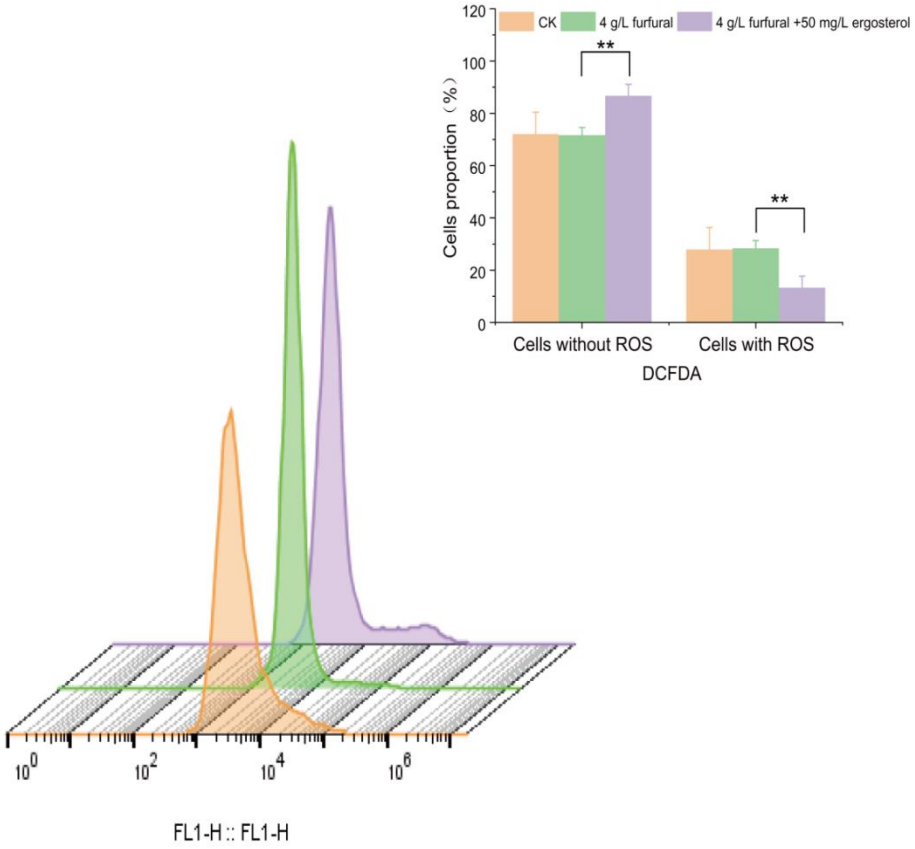

Fig. 5. Effect of ergosterol supplementation on intracellular ROS content in *S. cerevisiae* under furfural stress, "**" ($0.001 \leq P < 0.01$)

Regulation of total fatty acid composition of S. cerevisiae under furfural stress with ergosterol supplementation

The results showed that supplementation with lipid nutrients (ergosterol) reduced the oxidative stress of *S. cerevisiae*. Fatty acid composition under furfural stress should be studied to explore the mechanism of ergosterol supplementation.

The fatty acids in *S. cerevisiae* mainly include palmitoleic acid (C16:1), palmitic acid (C16:0), oleic acid (C18:1), myristic acid (C14:0), lauric acid (C12:0) and myristic acid (C14:1), as shown in Table 3. The content of main unsaturated fatty acids in *S. cerevisiae* cells was 88%. Under 4 g/L furfural stress, the content of main unsaturated fatty acids in *S. cerevisiae* increased to 93%. After supplementation with 50 mg/L ergosterol, the content of main unsaturated fatty acids was 85%.

Table 3. Effect of Ergosterol Supplementation on Total Fatty Acid Composition of *S. cerevisiae* under Furfural Stress

Group	Fatty Acid Composition (%)								Saturated Fatty Acids (%)	Unsaturated Fatty Acids (%)	Unsaturated /Saturated Fatty Acids
	$C_{16:1}$	$C_{18:1}$	$C_{16:0}$	$C_{14:0}$	$C_{12:0}$	$C_{14:1}$	$C_{18:0}$	$C_{15:0}$			
Control	67.1	18.5	7.5	2.6	1.5	1.2	-	-	11.7	88.3	7.5
4 g/L Furfural	63.1	27.5	5.7	-	1.3	-	-	-	7.2	92.8	12.9
4 g/L Furfural +50 mg/L Ergosterol	47.4	31.5	7.5	1.1	1.4	-	2.5	1.7	15.3	84.8	5.6

Compared with the control, exogenous addition of furfural and ergosterol changed the composition of fatty acids of *S. cerevisiae*. When furfural was added, the ratio of unsaturated fatty acids increased, resulting in an increase of 71% of unsaturated/saturated fatty acids and a decrease in cell membrane fluidity. When ergosterol was added, the proportion of unsaturated fatty acids decreased by 26%. The proportion of saturation of fatty acids increased, resulting in increased stability of cell membrane.

The increase of lipid nutrients (ergosterol) can maintain the stability of the membrane. The ergosterol content of yeast cells was determined and compared, as shown in Fig. 6A. In the control group, the ergosterol content of cells in logarithmic phase and stationary phase was 0.27% and 0.43% respectively, which was comparable under furfural stress. The ergosterol contents in cells of logarithmic phase and stationary phase after supplementation with ergosterol were increased to 0.49% and 0.91% respectively. This result indicated that exogenous addition of ergosterol increased the ergosterol content, thus changing the fatty acid composition. It has been reported that the proportion of ergosterol in cell membrane of *S. cerevisiae* increases under vanillin stress (Ayako *et al*. 2009). The increase of ergosterol content in cell membrane could improve the thermal stress resistance of *S. cerevisiae* (Caspeta *et al*. 2014). Ergosterol and unsaturated lipids may cooperate with each other to maintain the optimal membrane thickness, which can protect membrane proteins from ethanol (Vanegas *et al*. 2014).

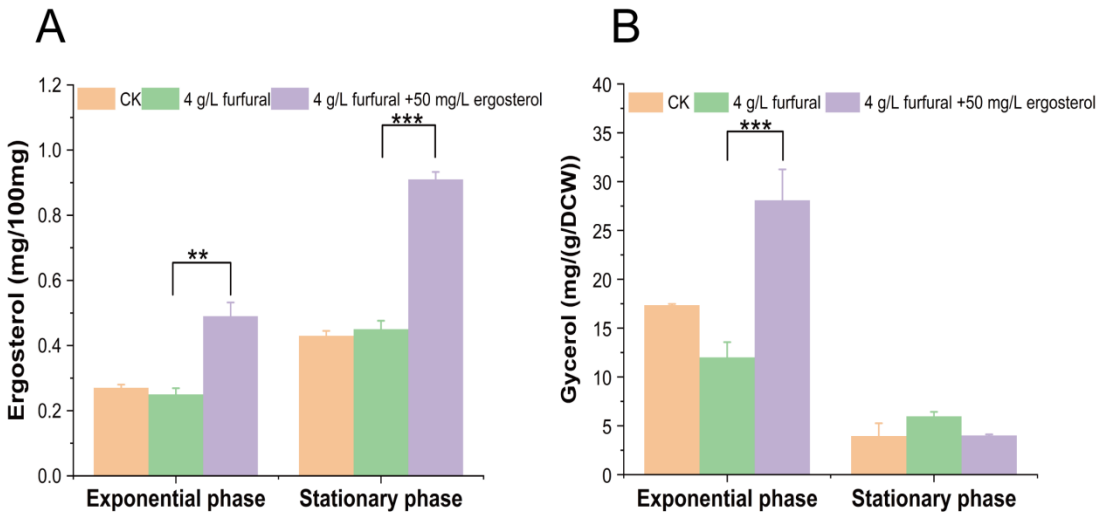

Fig. 6. Effect of ergosterol supplementation on A, ergosterol content; B, glycerol content of *S. cerevisiae* under furfural stress, "**" ($0.001 \leq P < 0.01$), "***" ($P < 0.001$). To be specific, the exponential phase was evaluated at the beginning of this phase.

Glycerol is another important component in the cell membrane of *S. cerevisiae* under stress. As shown in Fig. 6B, normally, the accumulation of glycerol in the logarithmic phase (12 h) was 18.9 mg/L. The cells grow rapidly in the logarithmic phase, and the intracellular glycerol begins to be consumed as a carbon source to support the growth of cells, such as the synthesis of lipids or other protective substances (Ansell *et al.* 1997). With 4 g/L furfural, the intracellular glycerol content of *S. cerevisiae* was lower in logarithmic phase. Excessive NADH is re-oxidized during furfural detoxification, resulting in reduced glycerol content (Liu *et al. 2020*). With 50 mg/L ergosterol supplementation, the intracellular glycerol content in logarithmic growth stage was nearly 5 times higher

than that under furfural stress. Metabolomics research shows that yeast respond to furfural stress by regulating glycerol synthesis, improving glycolytic activity (Jung *et al. 2017*).

An earlier study found that *S. cerevisiae* 1200 was more tolerant to ethanol than *S. cerevisiae* AJL2155 (Chi and Arneborg 1999). The proportion of unsaturated fatty acids of *S. cerevisiae* 1200 and the ratio of ergosterol to phospholipid was much higher than that of *S. cerevisiae* AJL 2155. The fermentation ability of *S. cerevisiae* was improved after cultured with supplementation of different lipids (Marian *et al.* 2009). The fermentation time of cells added with palmitoleic acid (C16:1) was reduced, and the viability of cells was higher. *S. cerevisiae* was cultured with different fatty acids and ergosterol to change the lipid composition of the membrane. The supplementation of ergosterol did not show impact on the total content of fatty acids, but it increased unsaturated fatty acids. Another study showed that in order to resist salt stress, the ergosterol content of *Zygosaccharomyces rouxii* was increased to maintain the stability of cell membrane, reduce the fluidity of cell membrane and increase the proportion of unsaturated fatty acids, resulting in the increase of the proportion of unsaturated / saturated fatty acids (Wang *et al.* 2020), which was found in the present study.

Changes of CAT and hydrogen peroxide under furfural stress with ergosterol supplementation

The antioxidant defense system of *S. cerevisiae* is composed of super oxide dismutase and catalase (CAT), which catalyze superoxide anion in cytoplasm and mitochondria to produce hydrogen peroxide and oxygen, and then decompose hydrogen peroxide into water and oxygen (Li *et al.* 2020). When cells are damaged by ROS, *S. cerevisiae* forms a defense by inducing catalase (Eleutherio *et al. 2017*). As shown in Fig. 7, CAT with 4 g/L furfural was higher than that with 4 g/L furfural and 50 mg/L ergosterol.

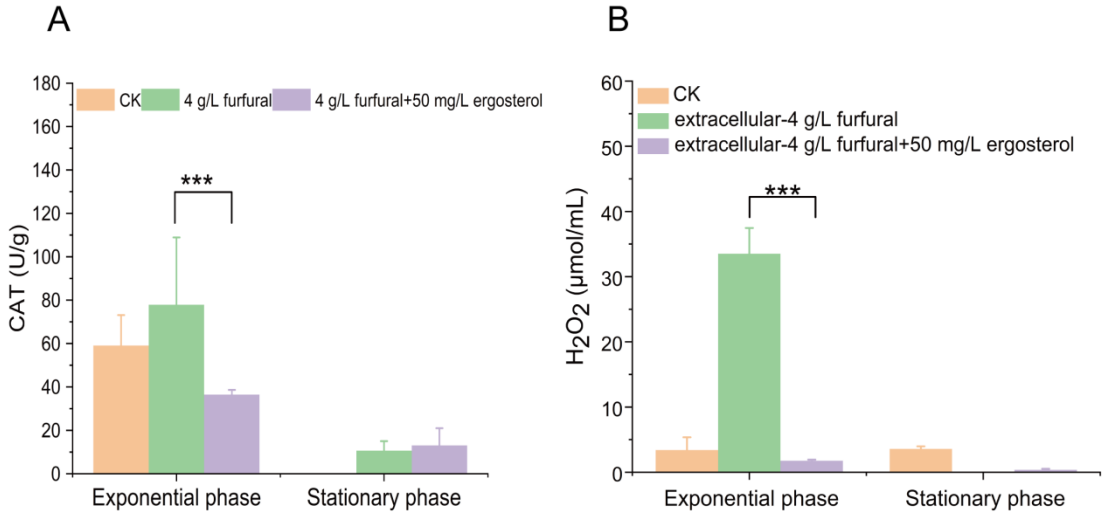

Fig. 7. Effect of ergosterol supplementation on CAT and hydrogen peroxide content in *S. cerevisiae* under furfural stress, "***" ($P < 0.001$). To be specific, the exponential phase was evaluated at the beginning of this phase.

The content of extracellular hydrogen peroxide was much higher without ergosterol under furfural stress in lag phase, indicating that the addition of ergosterol alleviated the oxidative stress of yeast cells. That might be one of the reasons that ergosterol

supplementation could shorten the lag phase of *S. cerevisiae* under furfural stress. The ability of TEMPOL (4-hydroxy-2,2,6,6-tetramethylpiperidin-l-oxyl) to scavenge intracellular ROS in *S. cerevisiae* was studied by Li *et al.* (2020). The addition of TEMPOL enhances cell growth and increased the CAT activity, resulting in higher H_2O_2 tolerance. The overexpression of YCR102C promotes the growth and ethanol production of *S. cerevisiae* under acetic acid stress. Through the expression of YCR102C, the activity of CAT was increased by 47% under 5 g/L acetic acid stress (Chen *et al.* 2019).

erg⊿mutants exhibit varying furfural sensitivity

Ergosterol plays an important role in stress adaption. Ergosterol levels are closely related to the ability of yeast to tolerate in low temperature, low sugar, high alcohol, and other stress (Hu *et al.* 2017; Kamthan *et al.* 2017). Ergosterol biosynthesis is complex, involving many enzymes and energy. The last steps of ergosterol biosynthesis are catalyzed by ERG3/ERG4/ERG5. Although mutations of these genes do not affect yeast growth, they do have impact on the composition of the cell membrane, thus influencing stress tolerance (Kodedová *et al.* 2015).

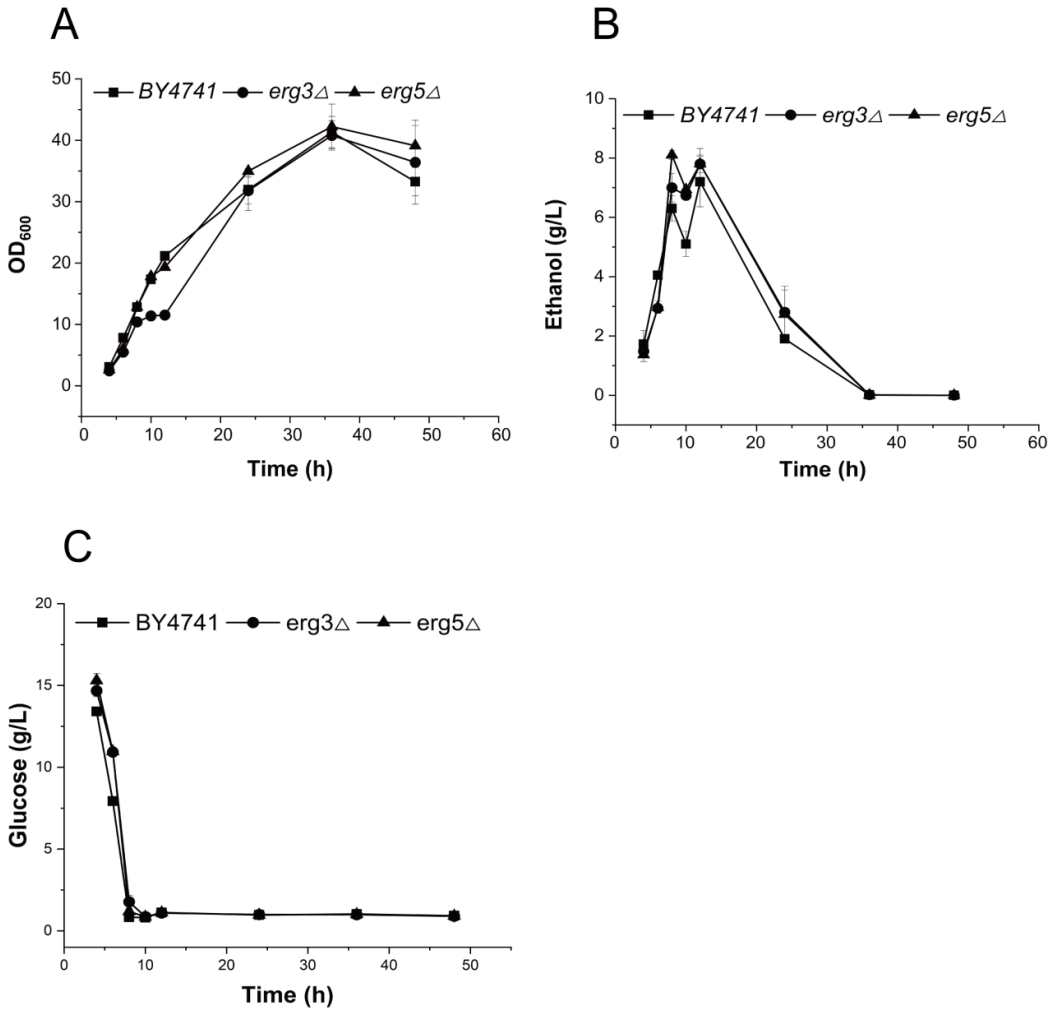

Fig. 8. Batch cultures profiles of different *S. cerevisiae* in YEPD media, curve with square refers to *S. cerevisiae* BY4741, curve with round represents *S. cerevisiae* BY4741 *erg3⊿*, curve with triangle is *S. cerevisiae* BY4741 *erg5⊿* (A, OD_{600}; B, ethanol; C, furfural concentration; D, glucose)

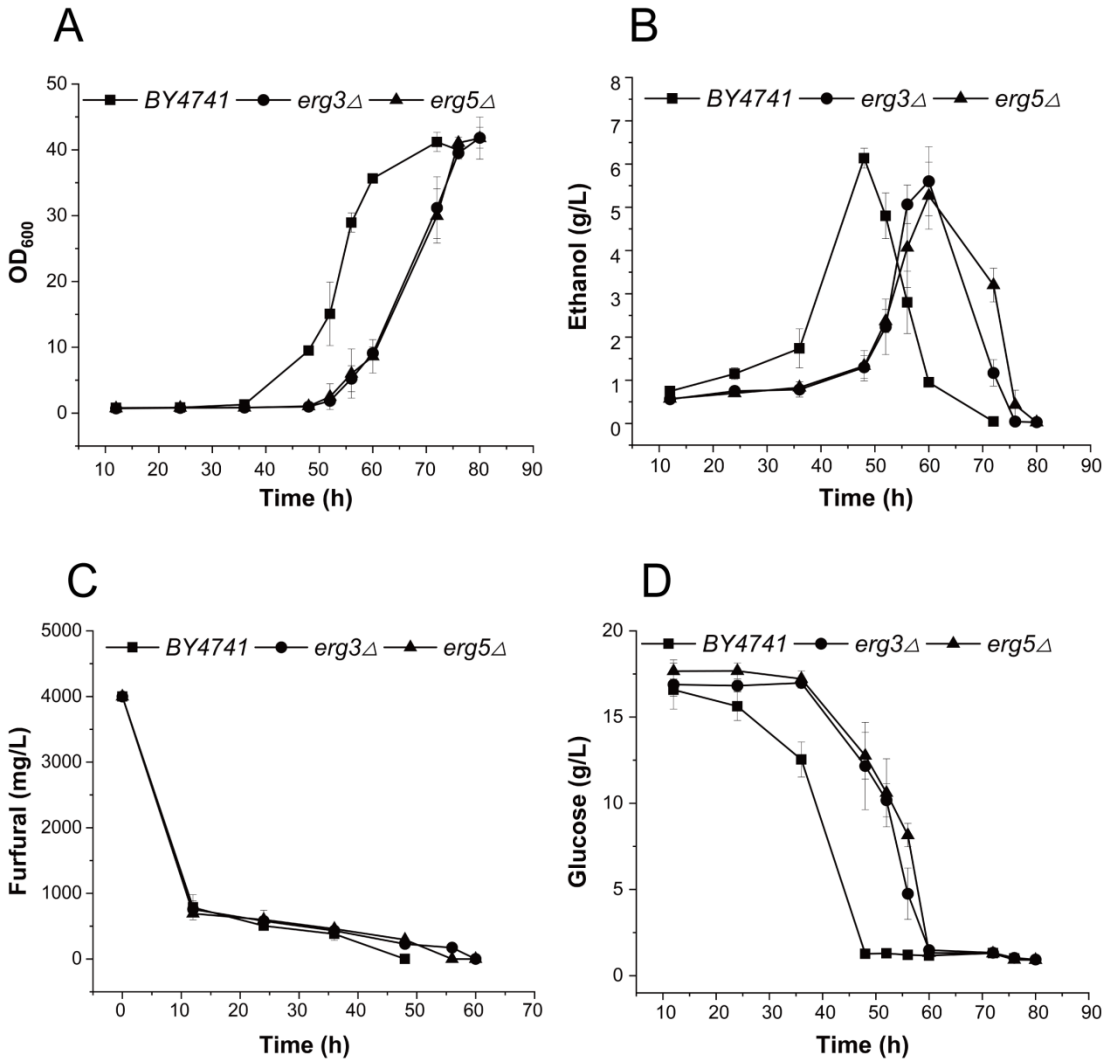

Fig. 9. Batch cultures profiles of different *S. cerevisiae* in YEPD medium under 4g/L furfural concentrations, curve with square refers to *S. cerevisiae* BY4741, curve with round represents *S. cerevisiae* BY4741 *erg3⊿*, curve with triangle is *S. cerevisiae* BY4741 *erg5⊿* (A, OD_{600}; B, ethanol; C, furfural concentration; D, glucose)

To further verify the effect of ergosterol in *S. cerevisiae* under furfural stress, ergosterol synthase knockout strains (*S. cerevisiae* BY4741 (*erg3⊿*) and *S. cerevisiae* BY4741 (*erg5⊿*)) were applied. The results in Fig. 8, Fig. 9, and Table 4 showed that without furfural stress, the kinetic parameters of the three strains were comparable. The value of μ was 0.042 h^{-1}, X_{max} was around 41.00 g/L, and $Y_{P/S}$ was about 0.56 g/g.

The lag phase of *S. cerevisiae* BY4741 was 36 h under 4 g/L furfural stress. The best ethanol performance was 6.1 g/L at 48 h. Furfural was converted, and glucose was consumed completely within 48 h. *S. cerevisiae* BY4741 (*erg3⊿*) began to grow after 52 h. The best ethanol performance was 5.6 g/L at 60 h.

Table 4. Kinetic Parameters for Different *S. cerevisiae* in YEPD Media

Strain	Furfural (g/L)	Lag phase (h)	μ (/h)	X_{max} (g/L)	$Y_{X/S}$ (mg/g)	$EtOH_{max}$ (g/L)	$Y_{P/S}$ (g/g)
S. cerevisiae BY4741	0	2	0.039±0.008	41.3±2.5	3.04±0.05	7.2±0.85	0.57±0.01
	4	36	0.016*±0.003	41.2±1.5	2.6±0.38	6.1±0.23	0.39***±0.01
S. cerevisiae BY4741 (*erg3*⊿)	0	2	0.039±0.006	40.8±2.4	2.8±0.36	7.8±0.52	0.56±0.03
	4	52	0.014**±0.001	41.8±1.6	2.6±0.31	5.6*±0.80	0.35***±0.01
S. cerevisiae BY4741 (*erg5*⊿)	0	2	0.039±0.004	42.2±3.7	2.8±0.42	8.1±0.15	0.56±0.02
	4	52	0.014**±0.004	41.8±3.2	2.4±0.46	5.3**±0.77	0.31***±0.01

μ refers to specific rate of growth, X_{max} is the maximum biomass obtained, $Y_{X/S}$ represents the biomass yield, $EtOH_{max}$ is the maximum ethanol obtained, $Y_{P/S}$ refers to the ethanol yield. The significance was analyzed by comparing with 0 g/L furfural of the same strain, "*" ($0.01 \le P < 0.05$), "**" ($0.001 \le P < 0.01$), "***" ($P < 0.001$).

Furfural was converted and glucose was consumed completely within 60 h. Compared with *S. cerevisiae* BY4741, the time required for furfural degradation was significantly longer. The corresponding time of the maximum ethanol concentration of *S. cerevisiae* BY4741 (*erg3⊿*) was 12 h later than that of *S. cerevisiae* BY4741. The maximum ethanol concentration was 8.6% lower than that of the original strain.

With 4 g/L furfural, *S. cerevisiae* BY4741 (*erg5⊿*) began to grow after 52 h. The best ethanol performance was 5.30 g/L at 60 h. Furfural was completely converted within 58 h, which was significantly longer than that of the original strain. The corresponding time of the maximum ethanol concentration was 12 h longer than that of the original strain, and the maximum ethanol concentration was 14% lower than that of the original strain.

The deletion of genes (*ERG6*, *ERG 2*, *ERG 3*, *ERG 5*, and *ERG 4*) in the final step of ergosterol biosynthesis had an impact on the physiological function of *S. cerevisiae* plasma membrane. Most *ERG* mutants are more sensitive to various stress than wild-type ones. The sensitivity was positively correlated with the hyperpolarization of plasma membrane. Under NaCl stress, *erg2⊿, erg3⊿* and *erg6⊿* mutants are the most sensitive to acid stress (Kodedová *et al.* 2015). E*rg3⊿* knockout strain BS-1, a key gene for ergosterol synthesis, was constructed. Compared with the original strain, BS-1 showed lower tolerance to salt stress. It was found that mutants of *S. cerevisiae* (*erg2⊿, erg3⊿, erg4⊿, erg5⊿, erg6⊿, erg24⊿, erg28⊿*) were sensitive to acid (Kawahata *et al.* 2006).

CONCLUSIONS

1. The addition of ergosterol can shorten the lag period and increase the content of ethanol in the presence of high concentration furfural (4 g/L) of *S. cerevisiae*.
2. Ergosterol supplementation could decrease membrane permeability. When ergosterol was added under furfural stress, the proportion of unsaturated fatty acids was decreased, the proportion of unsaturated/saturated fatty acids was decreased, which increased the stability of cell membrane and ensured the fluidity of cell membrane.
3. 3. Under 4 g/L furfural stress, the lag period of *S. cerevisiae* BY4741 (*erg3⊿*) and *S. cerevisiae* BY4741 (*erg5⊿*) was longer than that of the original strain, and the ethanol content decreased. It can be confirmed that ergosterol has a great effect on the furfural tolerance of *S. cerevisiae*.

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