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# Role of phosphatidylinositol (PI) in ethanol production and ethanol tolerance by a high ethanol producing yeast

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**The effect of inositol addition on phospholipids, cell growth, ethanol production and ethanol tolerance in a high ethanol producing *Saccharomyces* sp were studied. Addition of inositol greatly influenced major phospholipid synthesis. With inositol in the fermentation medium, phosphatidylinositol (PI) content was increased, while phosphatidylcholine (PC) and phosphatidylethanolamine (PE) were decreased. However, without inositol in the fermentation medium, PI content dropped down within 24 h, then increased, but was lower than in the presence of inositol. When yeast cells had a higher content of PI, they produced ethanol much more rapidly and tolerated higher concentrations of ethanol. During ethanol shock treatment at 18% (v/v) ethanol, yeast cells with a higher concentration of PI lost their viability much more slowly than those with a lower concentration of PI, indicating that the PI content in these yeast cells can play an important role in ethanol production and ethanol tolerance. Fatty acids and ergosterol were not responsible for high ethanol tolerance and high ethanol production in this yeast strain.**

**Keywords:** inositol; phosphatidylinositol (PI); high ethanol production; high ethanol tolerance; *Saccharomyces*

## Introduction

In recent years, phosphatidylinositol (PI) has received increasing attention among phospholipids in yeasts, as it plays important roles in a variety of cellular processes, such as cell growth, metabolism of proteins, nucleic acids and carbohydrates, as well as lipids themselves and signal transduction [25].

PI is synthesized from CDP-diacylglycerol (CDP-DAG) and inositol by the action of PI synthase (PIS). Inositol is one of the key regulatory factors in PI biosynthesis [24]. Hanson *et al* [12] reported that upon withdrawal of inositol, PI, cell wall, protein and RNA synthesis and cell division were affected adversely in *Saccharomyces*. The ultimate result of the changes was loss of cell viability, known as inositol-less death. Because of cell membrane damage, inositol deficiency caused loss of fermentation, respiration and sugar transport activity [31]. The synthesis of phosphatidylcholine (PC) from phosphatidylethanolamine (PE) by the methylation pathway was also regulated by inositol in *Saccharomyces* [11]. With inositol addition, PE methyltransferase was low. The presence of both inositol and choline or ethanolamine in the growth medium resulted in reduction of CDP-diacylglycerol synthase in *S. cerevisiae* [16]. Kelley *et al* [17] found that addition of inositol to the growth medium of *S. cerevisiae* resulted in rapid increase in the rate of PI synthesis at the expense of phosphatidylserine (PS), PE and PC. When wild-type cells entered the stationary phase of growth, CDP-DAG, PS, PC and PE synthesis decreased, but PI content remained constant.

The mechanisms of ethanol tolerance in the yeast strains commonly used in the fermentation industries were extensively studied [20,21]. The mechanisms are very complex. Yeast cells have different mechanisms to respond to ethanol toxicity based on the different strains and different cultivation conditions and many genes are involved [8]. The exact mechanism(s) of ethanol tolerance in commonly used yeast strains are still unknown.

Ethanol is thought to be an active agent which can damage the cell membrane by altering membrane organization and permeability. Thus, the plasma membrane lipids in yeast cells are regarded as the main targets of ethanol toxicity, although other cell components may also be related to ethanol tolerance [1,26,28]. An increase in the unsaturation index is correlated with increased ethanol tolerance [1,21]. However, according to enhanced saturation of fatty acids in immobilized yeast cells with improved alcoholic fermentation rates compared with freely suspended cells, Hilge-Rotmann *et al* [15] assumed that higher saturation of fatty acids favors faster removal of ethanol from the cells.

Sterols, especially ergosterol promote cell growth and alcohol tolerance, because they can provide rigidity to the cell membrane and have barrier-forming ability against the entry of ethanol into cells [13,30]. Del Castillo Agudo [9] also observed that in the case of *Saccharomyces* strain LA1, ergosterol markedly increased when the ethanol concentration in the medium was increased from 6 to 12% (v/v). However, Novotny *et al* [22] found that ethanol tolerance did not correlate with yeast strains with different sterol levels.

Phospholipids are main components in the yeast plasma membrane. Therefore, phospholipids were thought to be an important factor in ethanol tolerance in yeast cells. So far, only PS synthesized by yeast cells and added PC have been suggested to play some role in ethanol tolerance [13,21,27].

Although PI is connected to many cellular functions in yeasts, so far no report about the role of PI in high ethanol production and high ethanol tolerance has appeared. There-

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fore, this study is aimed at understanding the relationship between PI content and high ethanol production and high ethanol tolerance in *Saccharomyces* sp W4, a high ethanol producing yeast strain.

## Materials and methods

### *Yeast strains*

*Saccharomyces* sp $W_4$, a high ethanol producing yeast strain, was obtained previously [6].

### *Fermentation tests*

Yeast cells were grown aerobically at 30°C in a synthetic medium with 2% (w/v) glucose [7] for 18 h; 36 ml of the culture (equal to about 0.135 g of cell dry weight) were transferred to a 500-ml bottle with 264 ml of the synthetic medium containing 20% sucrose and 1% ammonium sulfate. The bottles were fitted with rubber bungs perforated by a needle and incubated statically at 30°C. The loss of weight by $CO_2$ liberation during fermentation was monitored daily until fermentation ceased. As fermentation proceeded, additional sucrose was complemented to keep a suitable concentration of sucrose in the medium. In order to investigate the effects of added inositol, the fermentation medium with 100 $\mu$g ml$^{-1}$ inositol and the fermentation medium without inositol were used.

### *Lipid extraction*

Yeast cells were harvested by centrifugation and washed three times with deionized water. The washed cells were resuspended in 10 ml of deionized water. The cell suspension was mixed with 19 ml of glass beads (0.25–0.30 mm diameter). The mixture was shaken in a Merckenschlager cell homogenizer (Braun-Melsungen, Germany) with $CO_2$ cooling for 7 min. The disrupted cells were mixed with 80 ml of chloroform/methanol (2 : 1) and transferred to a 500-ml flask and stirred with a magnetic bar for 2 h at room temperature in a hood. The mixture was then filtered and washed with another 40 ml of chloroform/methanol (2 : 1). The filtrate was mixed with 20 ml of 0.034% $MgCl_2 \cdot H_2O$ and the mixture was left to separate overnight at 4°C. The lower organic phase was mixed with 3 ml of benzene/ethanol (3 : 2) and taken to dryness on a rotary evaporator at 35–40°C. The residue was immediately dissolved in 2 ml of chloroform/methanol (2 : 1). The lipid sample was stored at −20°C [32].

### *Preparation and analysis of fatty acid methyl esters*

Lipid extract (0.5 ml) was dried under a stream of nitrogen. Then, 0.5 ml of benzene (GR) and 2 ml of boron trifluoride (20%) in methanol were added to the lipid trace and the mixture was heated at 100°C for 45 min. After cooling, 1 ml of water and 3 ml of petroleumbenzine (GR) were added and fatty acid methyl esters were extracted into petroleumbenzine. The fatty acid methyl esters were analyzed by gas chromatography (Hewlett Packard 5890) under the following conditions: injection temperature 140°C, initial time 5 min, rate 10 deg min$^{-1}$, rate A 10 deg min$^{-1}$, final temperature 200°C, final temperature A 250°C, final time 25 min, final time A 5 min, injection B temperature 250°C, detector A temperature 300°C, carrier ($N_2$) 370 ml min$^{-1}$, air 280 ml min$^{-1}$, $H_2$ 150 ml min$^{-1}$ and column crosslinked 5% phlle silica (25 m $\times$ 0.2 mm $\times$ 0.33 $\mu$m film thickness H.P port No. 190913).

### *Phospholipid analysis*

Individual phospholipids in the lipid extract were separated by two-dimensional thin-layer chromatography (TLC), in the first direction with chloroform-methanol-25% ammonia (65 : 35 : 5 by volume) and in the second direction with hloroform-acetone-methanol-acetic acid-water (50 : 20 : 10 : 10 : 5 by volume). Spots of the different phospholipids were located by exposing the plates to iodine vapor, marked with a pin and scraped off the plates. Phospholipids were extracted from the spots with chloroform/methanol (1 : 4) and dried under a stream of nitrogen.

Phospholipid content was determined by assaying the amount of phosphorus in the phospholipid extract. The phospholipid was digested using 0.4 ml of a mixture of concentrated sulfuric acid/perchloric acid (9 : 1 by volume) at 180°C until the digest was clear and colorless. After cooling, 9.6 ml of the reagent containing 5 ml of 0.26% $NH_4(MoO_7)_7 \cdot 4H_2O$ and 0.22 ml of ANSA (1-amino-2-hydroxy-4-naphthalenesulfonic acid) suspension was mixed with the digest and incubated at 90°C for 20 min. Absorbency of the solution was read at 830 nm in a spectrophotometer (Hitachi UV-1100). $KH_2PO_4$ (0.1 mg $PO_4^{3-}$ ml$^{-1}$) was employed as standard throughout.

### *Ethanol assay*

Ethanol concentration in fermented media was measured using the enzymatic method described by the manufacturer (Boehringer Mannhein Gmbh, Mannheim, Germany).

### *Measurement of cell dry weight*

Yeast cells from 20 ml of the culture were collected and washed by filtration using membrane filters with pore size 1.2 $\mu$m. Then they were dried to constant weight at 100°C.

### *Ethanol shock treatment*

Cells from the culture ($1 \times 10^8$) were harvested by centrifugation and washed three times with distilled water. The washed cells were resuspended in 8.2 ml of sterile water and 1.8 ml of absolute ethanol was added to the cell suspension. Then, the cells were mildly shaken at 50 rpm in the water bath (30°C). During the high ethanol shock treatment, samples were taken periodically from the cell suspension and their viabilities were determined following appropriate dilutions and plating on YPD plates. The colonies were counted after 48 or 72 h incubation at 30°C. All plates were run in duplicate, with the results averaged for each duplication. Ethanol tolerance expressed as percentage survivors was determined by comparing the colony count of the ethanol-shocked cells with that of a non-shocked control.

### *Analysis of neutral lipid*

Neutral lipids were determined according to methods described by Leber *et al* [18].

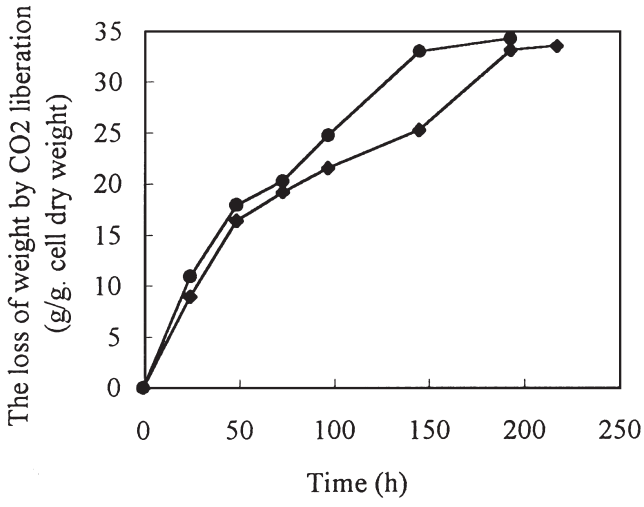


**Figure 1** Ethanol production with the synthetic medium containing 20% (w/v) sucrose. In the presence of inositol (—●—), the final ethanol concentration in the fermented medium was 16.3% (v/v). In the absence of inositol (—◆—), the final ethanol concentration in the fermented medium was 15.5% (v/v). The values are means of two independent experiments.

## Results

### *Effects of inositol on ethanol production and cell growth*

Figure1 shows time courses of $CO_2$ output during fermentation in the synthetic medium. In the presence of inositol, the yeast produced more ethanol at a higher fermentation rate than in the absence of inositol. At the end of fermentation, *Saccharomyces* W4 yielded 16.3% (v/v) ethanol in the fermentation medium with inositol, but only 15.5% (v/v) without added inositol.

The results shown in Figure 2 indicate that in the presence of inositol cells grew more quickly than in the absence of inositol. Within 72 h of fermentation, cell dry weight per 100 ml of fermentation media both containing inositol and lacking inositol was increased continuously. After that, autolysis of cells occurred so that cell dry weight decreased. Cells from both the culture with added inositol and that without inositol were in mid-log phase after 24 h.

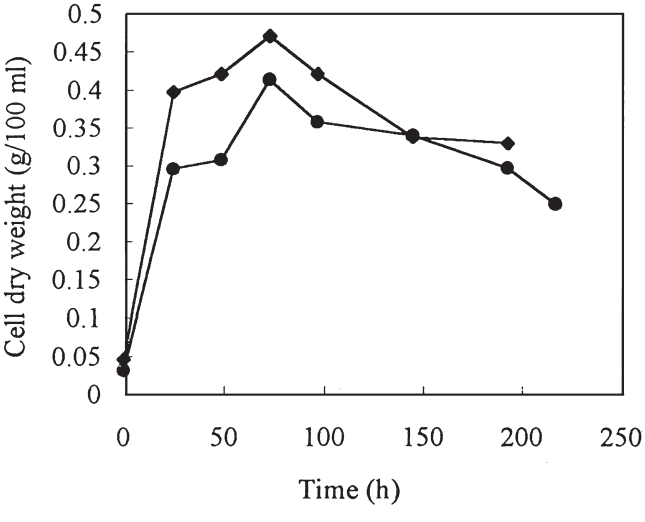


**Figure 2** Cell growth (cell dry weight per 100 ml of the fermentation medium) during high ethanol production with the synthetic medium containing 20% (w/v) sucrose. In the presence of inositol (—◆—); in the absence of inositol (—●—). The values are means of two independent experiments.

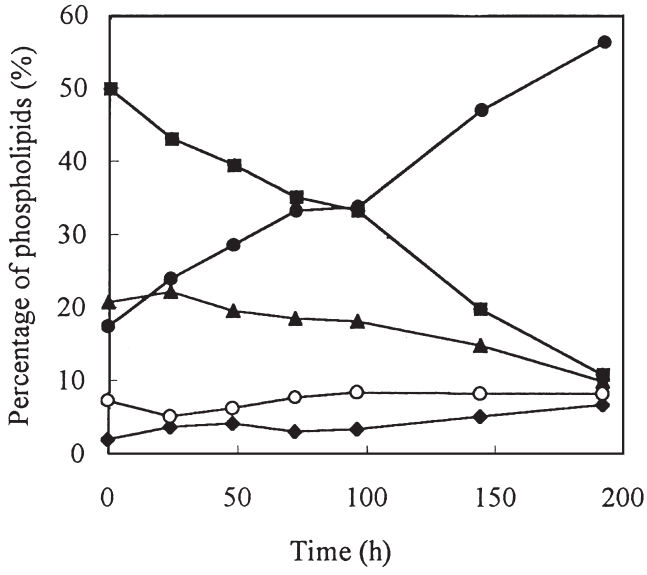


**Figure 3** The changes in percentage of phospholipids in the presence of inositol during high ethanol production. PA (—○—); PE (—▲—); PC (—■—); PI (—●—); PS (—◆—). The values are means of two independent experiments.

### *Effects of inositol addition on phospholipid synthesis*

The effects of inositol addition on phospholipid synthesis are shown in Figures 3 and 4. In the presence of inositol, cells could maintain a relatively high concentration of PI, while other phospholipids, especially PC and PE decreased rapidly. After 96 h of fermentation, PI became the major phospholipid (Figure 3). It is strange that the PI content in the media without inositol dropped down rapidly within 24 h of fermentation, then increased (Figure 4). But cells

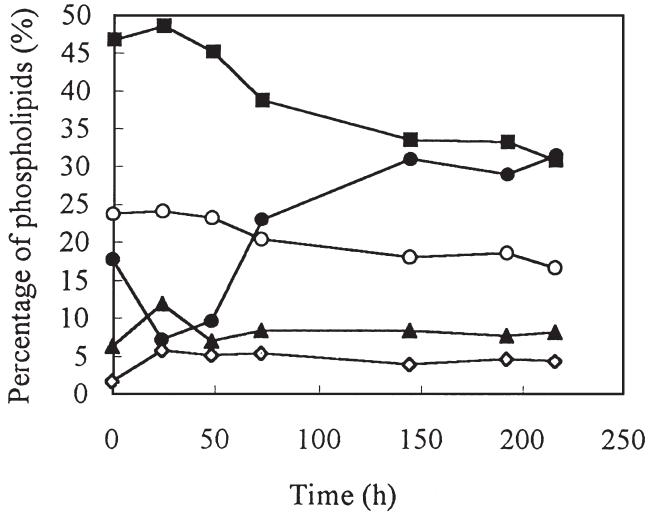


**Figure 4** The changes in percentage of the major phospholipids in the absence of inositol during high ethanol production. PA (—▲—); PC (—■—); PE (—○—); PI (—●—); PS (—◇—). The values are means of two independent experiments.

had a lower PI content in medium without inositol than in medium with inositol during the fermentation. For example, PI content decreased from 17.54% to 7.18% in cells grown in medium without inositol within 24 h, while the PI content was increased from 16.44% to 23.92% in cells grown in medium with inositol within the same period. The PC and PE contents also decreased with fermentation in the media without inositol. But PC and PE contents of cells grown in medium minus inositol were higher than those of the same yeast cells grown in medium plus inositol throughout the fermentation. In both cases of presence of inositol and the absence of inositol, PS and phosphatidic acid (PA) contents in the yeast cells remained low and constant.

### *Cell survival after ethanol shock*

The PI content of yeast cells cultivated in medium without inositol dropped down very quickly within 24 h, while cells grown in medium with inositol maintained a high concentration of PI (Figures 3 and 4). Therefore, the yeast cells from the fermentation broth at this stage were collected and washed with sterile water three times. Then the washed cells were treated by ethanol shock at 18% (v/v). During this period, the cell suspension was mildly shaken on a rotary shaker (50 rpm) at 30°C and their viabilities were determined by plate count.

Yeast cells with a lower content of PI were more sensitive to high ethanol shock than those with a higher content of PI (Figure 5). For example, cells from the fermentation broth with inositol maintained 74.27% cell viability within 1 h of the treatment, while the cells from the fermentation broth without inositol addition showed only 47.36% cell viability within 1 h of the treatment.

### *Fatty acid composition*

Tables 1 and 2 present changes in fatty acid composition during fermentation. The percentage of saturated fatty acids increased, especially $C_{16:0}$, while the percentage of unsaturated fatty acids decreased. But cells grown in the presence of inositol contained more saturated fatty acids and less unsaturated fatty acids than those gorwn in the absence of inositol. $C_{10:0}$ and $C_{12:0}$ fatty acids also increased. For example, $C_{10:0}$ fatty acid in cells grown in the presence of inositol increased from 0.20% to 7.7% within 24 h, while $C_{10:0}$ fatty acid in the cells grown in the absence of inositol increased from 0.2% to 2.0% within the same period.

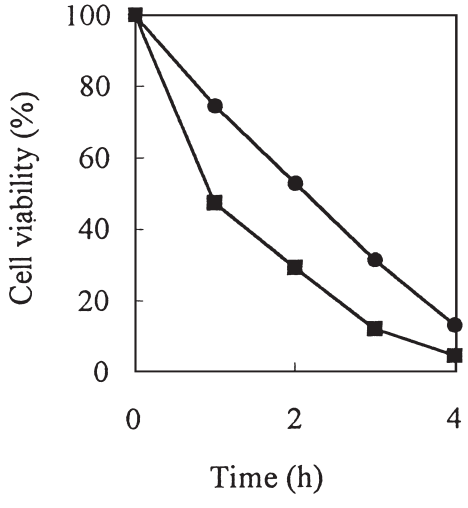


**Figure 5** Cell viability during high ethanol shock treatment (18.0% v/v ethanol). (—●—) Cells from 24-h fermentation cultures plus inositol. (—■—) Cells from 24-h fermentation cultures without added inositol. The values are means of two independent experiments.

**Table 1** Changes in fatty acid composition during high ethanol production in the presence of inositol

| Time (h) | Fatty acids (%) | | | | | | |
|---|---|---|---|---|---|---|---|
| | $C_{10:0}$ | $C_{12:0}$ | $C_{14:0}$ | $C_{16:1}$ | $C_{16:0}$ | $C_{18:1}$ | $C_{18:0}$ |
| 0 | 0.2 | 0.6 | 0.8 | 40.0 | 7.6 | 43.3 | 5.4 |
| 24 | 7.7 | 5.1 | 1.4 | 18.9 | 32.4 | 20.4 | 11.7 |
| 48 | 6.0 | 5.0 | 0.9 | 17.0 | 34.9 | 19.0 | 15.9 |
| 72 | 5.8 | 4.9 | 1.0 | 16.4 | 34.4 | 17.4 | 17.7 |
| 96 | 6.0 | 5.2 | 0.9 | 15.9 | 34.3 | 16.2 | 19.6 |
| 144 | 5.0 | 5.5 | 0.8 | 16.3 | 33.3 | 17.4 | 19.8 |
| 240 | 4.5 | 5.6 | 0.7 | 15.0 | 35.0 | 16.8 | 20.9 |

The results are the average of two experiments.

**Table 2** Changes in fatty acid composition during high ethanol production in the absence of inositol

| Time (h) | Fatty acids (%) | | | | | | |
|---|---|---|---|---|---|---|---|
| | $C_{10:0}$ | $C_{12:0}$ | $C_{14:0}$ | $C_{16:1}$ | $C_{16:0}$ | $C_{18:1}$ | $C_{18:0}$ |
| 0 | 0.2 | 0.6 | 0.8 | 40.0 | 7.6 | 43.3 | 5.4 |
| 24 | 2.0 | 4.3 | 2.2 | 24.3 | 30.0 | 26.0 | 9.2 |
| 48 | 3.1 | 5.2 | 1.2 | 23.5 | 31.8 | 23.1 | 10.3 |
| 72 | 3.8 | 5.5 | 0.8 | 19.3 | 34.2 | 20.2 | 14.0 |
| 120 | 3.6 | 6.3 | 0.9 | 17.3 | 35.8 | 16.1 | 17.8 |
| 192 | 2.7 | 4.4 | 0.6 | 20.7 | 30.1 | 22.2 | 17.5 |
| 216 | 3.2 | 6.1 | 0.9 | 19.6 | 32.8 | 18.1 | 17.0 |

The results are the average of two experiments.

### *Changes of ergosterol*

Ergosterol in the yeast cells decreased rapidly within 24 h (Figure 6). However, in the presence of inositol cells contained less ergosterol than those in the absence of inositol during the whole period of fermentation.

## Discussion

In recent years, attention has been given to the mechanisms of ethanol tolerance in yeasts. Understanding the mechanisms will be helpful to further improve the performance of large-scale ethanol production [8]. The cell membranes in the commonly used yeast strains play an important role in ethanol tolerance.

Several yeast strains which could produce over 17.5% (v/v) ethanol within a short period were isolated and some processes for high concentration ethanol production from molasses and starch were developed [3–6,10,14,29]. However, the mechanisms of high ethanol tolerance and production in these yeast strains are still obscure.

Phospholipids are the most important components in cell

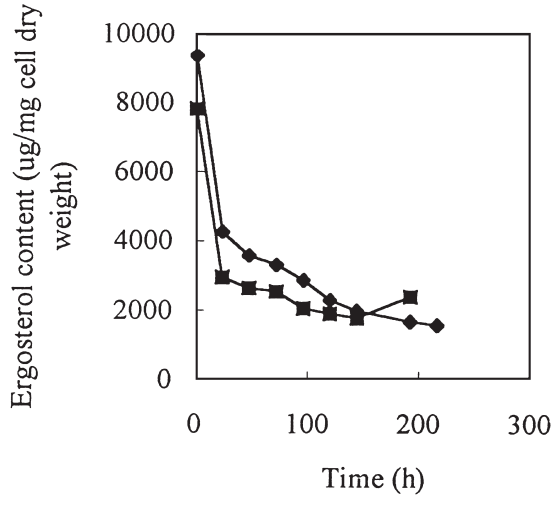


**Figure 6** The changes in ergosterol content during high ethanol production. In the presence of inositol (—■—); in the absence of inositol (—◆—). The values are means of two independent experiments.

membranes. So far only PC and PS were found to be in connection with fermentative activity and ethanol endurability in *Saccharomyces sake* [13,21,27]. Our results (Figures 3–4) indicate that whether there was added inositol or not in the fermentation medium, PC and PE contents in *Saccharomyces sp* $W_4$ decreased rapidly during fermentation. In the presence of inositol, cells could keep a relatively high concentration of PI (Figure 3). It is strange that PI contents in cells grown in medium without inositol addition decreased rapidly within 24 h, then increased (Figure 4). But cells maintained a lower PI content in medium without inositol than in medium with inositol. When the yeast contained a higher content of PI, it produced ethanol more quickly and accumulated a higher concentration of it (Figure 1). During high ethanol shock treatment, yeast with a higher content of PI died more slowly than that with a lower content of PI (Figure 5). These results support our conclusion that PI plays an important role in high ethanol production and high ethanol tolerance. In yeast, PI has many functions, such as regulation of proteins, cell wall, nucleic acids and synthesis of other lipids. PI is also involved in cell morphology and in the second messenger [25]. The present study is the first to obtain evidence that PI is related to high ethanol production and high ethanol tolerance in yeast. Recently, Arneborg *et al* [2] observed that *S. cerevisiae* cells adapt to increased concentrations of produced ethanol by increasing the proportion of PI at the expense of PC and keeping the composition of the other phospholipids constant. They suggested that PI may play a major role in the adaptive response of yeast cells to ethanol tolerance. However, it is still unknown how PI enhances ethanol production and ethanol tolerance in yeast and how PI protects cells from damage by ethanol at high concentration. One possibility is that membrane proteins, especially ATPase, and membrane integrity could be protected from damage by ethanol when they contain a higher content of PI, because the conformation of membrane proteins depends on the lipid structure [19]. These problems may be solved by understanding the relationship between PI content and plasma membrane ATPase activity, PI content and substrate transport across plasma membrane as well as PI content and ethanol-induced leakage in this yeast strain.

PA and PS were not involved in high ethanol tolerance and high ethanol production because the cells from both the culture with inositol and that without inositol contained almost the same percentage of PS and PA.

Unsaturated fatty acids in the yeast cell membrane are crucial for enhanced ethanol tolerance and production [21]. Unsaturated fatty acids may protect yeast cells from the inhibitory effects of ethanol shock [23]. Unsaturated fatty acids also play an important role in keeping cell membrane fluidity and functions. However, during alcohol fermentation, the concentration of oxygen in the medium is very limited. In this case, desaturation of saturated fatty acids in yeast cells will be inhibited, resulting in both a decrease in unsaturated fatty acids and an increase in saturated fatty acids [24]. It is of interest that the yeast strain could synthesize a considerable amount of short-chain fatty acids, such as $C_{10:0}$ and $C_{12:0}$ fatty acids after fermentation began (Tables 1 and 2).

Sterols, especially ergosterol are able to promote cell growth and alcohol tolerance because they can provide rigidity to the cell membrane and have barrier-forming ability against the entry of ethanol into cells [13,30]. Del Castillo Agudo [9] also observed that in the case of strain LA1, ergosterol markedly increased when ethanol increased from 6 to 12% (v/v). However, this study has shown that although the yeast cells grown in the presence of inositol could produce more ethanol during the fermentation (Figure 1) and tolerate higher added ethanol during high ethanol shock treatment (Figure 5) than those grown in the absence of inositol, they contained less ergosterol (Figure 6). Thus ergosterol did not play any role in high ethanol tolerance and high ethanol production in this strain.

## Acknowledgements

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