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Indentation with atomic force microscope, *Saccharomyces cerevisiae* cell gains elasticity under ethanol stress

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ABSTRACT

During bioethanol fermentation process, *Saccharomyces cerevisiae* cell membrane is the first target to be attacked by the accumulated ethanol. In such a prominent position, *S. cerevisiae* cell membrane could reversely provide protection through changing fluidity or elasticity secondary to remodeled membrane components or structure during the fermentation process. However, there is yet to be a direct observation of the real effect of the membrane compositional change. In this study, atomic force microscope-based strategy was performed to determine Young's modulus of *S. cerevisiae* to directly clarify ethanol stress-associated changes and roles of *S. cerevisiae* cell membrane fluidity and elasticity. Cell survival rate decreased while the cell swelling rate and membrane permeability increased as ethanol concentration increased from 0% to 20% v/v. Young's modulus decreased continuously from 3.76 MPa to 1.53 MPa while ethanol stress increased from 0% to 20% v/v, indicating that ethanol stress induced the *S. cerevisiae* membrane fluidity and elasticity changes. Combined with the fact that membrane composition varies under ethanol stress, to some extent, this could be considered as a forced defensive act to the ethanol stress by *S. cerevisiae* cells. On the other hand, the ethanol stress induced loosening of cell membrane also caused *S. cerevisiae* cell to proactively remodel membrane to make cell membrane more agreeable to the increase of environmental threat. Increased ethanol stress made *S. cerevisiae* cell membrane more fluidized and elastic, and eventually further facilitated yeast cell's survival.

1. Introduction

As an ideal biofuel with high renewability and little pollution, bioethanol can be a good alternative to the traditional fossil energy to ease off energy crisis and contribute to reduction of environmental pollution (Farrell et al., 2006; Goldemberg, 2007; Balat and Balat, 2009). Due to slight pollution, fast fermentation speed and high bioethanol productivity, biological fermentation has become the major method for bioethanol production. *Saccharomyces cerevisiae* is the most used industrial ethanologenic strain (Bai et al., 2008; Wood et al., 2014). However, bioethanol fermentation by *S. cerevisiae* is usually stressed by the accumulated ethanol which is considered to be the major hindrance to the complete bioethanol fermentation (Aguilera et al., 2006; Mira et al., 2010). Even *S. cerevisiae* can tolerate a certain degree of ethanol, accumulated ethanol could also inhibit the *S. cerevisiae* cell growth which would limitethe bioethanol yield and finally lead to economic loss, leaving industrial process crying for better-performing strains with higher ethanol tolerance (Baeyens et al., 2015; Steensels et al., 2014; Khattak et al., 2014).

Under ethanol stress, *S. cerevisiae* cell membrane is the first target to be attacked (Izawa, 2010). Previous studies indicated that content changes of membrane components such as unsaturated fatty acids could be determined to deduce the membrane fluidity under ethanol stress and thereby affect cell's tolerance abilities (Kajiwara et al., 2000; Aguilera et al., 2006; Li et al., 2012; Caspeta et al., 2014). And membrane fluidity is largely associated with ion transportance and thereby, viability (Lam et al., 2014). The function of monounsaturated fatty acids (MUFAs) in the ethanol tolerance of *S. cerevisiae* was controversial (Kajiwara et al., 2000; Li et al., 2012). Increased concentrations of MUFAs might lead to more membrane fluidity (Kajiwara et al., 2000). But others found that the dienoic fatty acids were more valuable for the ethanol tolerance of *S. cerevisiae* cells (Kajiwara et al., 2000). As previous views or conclusions about membrane fluidity were mainly deduced from ethanol

Abbreviations: AFM, atomic force microscopy; MUFAs, monounsaturated fatty acids; PFQNM, peak force quantitative nanomechanical mapping; SEM, standard error of the mean; YPD, yeast extract peptone dextrose.

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stress-associated changes of membrane compositions, changes and roles of *S. cerevisiae* cell membrane fluidity under ethanol stress were also controversial (Li et al., 2012). One view suggested that the plasma membrane should retain structural integrity as much as possible to counteract the ethanol stress (Li et al., 2012). On the basis of such hypothesis, factors that make the plasma membrane more rigid might be important for cells to maintain membrane stability and cell survival under ethanol stress. However, another view indicated that maintaining membrane fluidity is essential to maintain the *S. cerevisiae* cell viability (Kajiwara et al., 2000). Therefore, direct evidence of ethanol stress-associated change of fluidity or other mechanical properties (i.e., elasticity)of *S. cerevisiae* cell membrane was needed to clarify the exact roles of membrane playing in the ethanol tolerance of *S. cerevisiae*.

Cells can change their mechanical properties such as elasticity, viscoelasticity, etc. to respond to the environmental physical stimuli (Radmacher et al., 1996; Pelling et al., 2004). As physical damage to cells could be minimized as much as possible, atomic force microscopy (AFM) has become a powerful tool to study the mechanical properties of cells by detecting interaction force (i.e., atomic force and Van der Waals forces) between the probe tip and cell with nanoscale resolution (Green et al., 2002; Kunda et al., 2008; Lekka and Laidler, 2009). Within the elastic limit of the object texture, the ratio of external stress to the response of the object is named as Young's modulus which depends only on the physical property of the detected object itself according to Hooke's law ($F = -k \cdot x$) (Murnaghan, 1944; Calahorra et al., 2015). Young's modulus is widely used to characterize the rigidity of the cell surface; and the greater Young's modulus, the more difficult the cell is deformed. For example, the cell stiffness of hepatic metastatic cancer cells that taken from the body (pleural) fluids of patients with suspected lung, breast or pancreas cancer was about 70% softer than that of benign cells, indicating that mechanical analysis performed by AFM can distinguish tumor cells from normal ones even while they show similar shapes (Cross et al., 2007). Therefore, AFM can not only observe the cell morphology, but also determine the environmental physical stimuli-associated mechanical property changes through force-distance curve at pinewton force sensitivity level to unravel abnormal cell function (Pelling et al., 2005; Gaboriaud and Dufrêne, 2007; Liu et al., 2014).

In this study, *S. cerevisiae* S288c cells were exposed to various concentrations of ethanol stress to determine the effect of ethanol stress on cell viability and membrane integrity (indicated by membrane permeability and cell swelling). Moreover, AFM strategy was employed to evaluate ethanol stress-associated changes of *S. cerevisiae* cell membrane fluidity and elasticity (indicated by Young's modulus value), thereby contributing to a better understanding of the roles of membrane functioned in the ethanol tolerance or adaptation of *S. cerevisiae*.

2. Materials and methods

2.1. Strain, media and culture conditions

S. cerevisiae S288c used in this study was cultured in yeast extract peptone dextrose (YPD) broth (2% glucose, 1% yeast extract, 2% peptone) at 30°C and shaken at 150 rpm in 250 ml cotton-plugged flasks.

2.2. Grouping design

While ethanol concentration is higher than 100 g/L, yeast growth was obviously inhibited and cell survival rate also decreased (Devantier et al., 2005). Moreover, our previous study also indicated that *S. cerevisiae* almost did not grow under 15% v/v ethanol stress (Li et al., 2012). Therefore, five ethanol concentrations (i.e., 0%, 5%, 10%, 15%, and 20% v/v) were selected to stress *S. cerevisiae* cells for evaluating cell viability, membrane integrity and ethanol stress associated-changes of membrane mechanical properties (i.e., fluidity, elasticity). *S. cerevisiae* cell without exposure to ethanol stress (i.e., 0% v/v) was considered as control group.

2.3. Methylene blue staining for measurement of survival rate

Methylene blue staining was performed to distinguish living and dead cells according to previous reports (Teparić et al., 2004). *S. cerevisiae* cells at exponential phase were harvested by centrifugation (4000 × *g*, 5 min). To evaluate the degree of cell viability under ethanol stress, the collected *S. cerevisiae* cells were rinsed in water twice before being exposed to a challenge with 0%, 5%, 10%, 15% or 20% v/v ethanol for 4 h. The cell suspensions were mixed with an equal volume of methylene blue stain and incubated at room temperature for 5 min. The number of living cells that can render the methylene blue stain colorless was recorded as N_1, while the number of dead cells that would be blue colored was recorded as N_2. Then the survival rate was calculated according to the percentage of living cells (colorless) according to the following equation:

$$\text{Cell survival rate} = \frac{N_1}{N_1 + N_2} \times 100\%$$

The experiment was performed in triplicate.

2.4. Determination of cell membrane permeability

Cell membrane electric conductivity was determined to represent the cell membrane permeability. *S. cerevisiae* cells at exponential phase were harvested by centrifugation (4000 × *g*, 5 min). The collected *S. cerevisiae* cells were rinsed in water twice and respectively exposed to a challenge with 0%, 5%, 10%, 15% or 20% v/v ethanol for 4 h before measurement of the membrane electric conductivity with conductometer (DDS-307, Leici Co., China). The mean value of sample electric conductivity was recorded as C_1, and that of water was recorded as C_0. After being boiled for 10 min to make cell membrane completely broken and transparent, the electric conductivities of the cooled sample solution and water were measured and recorded as $C_{1'}$ and $C_{0'}$, respectively. The cell membrane permeability was calculated according to the following equation:

$$\text{Relative electric conductivity} = \frac{C_1 - C_0}{C_1' - C_0'} \times 100\%$$

The experiment was performed in triplicate.

2.5. Determination of cell swelling rate

S. cerevisiae cells at exponential phase were harvested by centrifugation (4000 × *g*, 5 min). The collected cells were rinsed in water twice and respectively exposed to a challenge with 0%, 5%, 10%, 15% and 20% v/v ethanol for 4 h before counting the cell number with a microscope by being dripped on a hematocytometer. The counted cell number was recorded as $N_{x\%}$. Moreover, the collected cells exposed to 0% v/v ethanol for 0 h were dripped on a hematocytometer for counting the cell number and recorded as N_0. The cell swelling rate was calculated using cell number values according to the following equation:

$$\text{Cell swelling rate} = \frac{N_0 - N_{x\%}}{N_0} \times 100\%$$

The experiment was performed in triplicate. Moreover, Pearson correlation analysis was performed using SPSS13.0 software to determine the connections between membrane permeability