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Modulation of mitochondrial membrane integrity and ROS formation by high temperature in *Saccharomyces cerevisiae*

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ABSTRACT

Background: Yeast strains are exposed to numerous environmental stresses during industrial alcoholic fermentation. High temperature accumulated acetic acid, enhanced the growth inhibition and decreased ethanol production.
Results: In this study the influence of high temperature on the cellular and mitochondrial membrane integrity of *Saccharomyces cerevisiae* as well as reactive oxygen species (ROS) formation was investigated to understand the mechanisms of the high temperature fermentation process. However, increasing the temperature to 42°C resulted in a clear decrease in the cytoplasmic and mitochondrial membrane potential and an increase in intracellular ROS formation. It was also determined that the different thermostability between YZ1 and YF31 strains had a clear correlation with the yeast's intracellular trehalose content of the cell. Finally, random amplified polymorphic DNA (RAPD) was used to explore the genome differences between the YZ1 and YF31 strains.
Conclusions: Thus, the stability of the mitochondrial membrane and subsequently, the clearance ROS ability could be important factors for the viability of *S. cerevisiae* at high temperatures.

1. Introduction

Currently, bioethanol production from micro-organisms has been significantly increasing. Much effort has been devoted to producing more bioethanol at a reduced cost [1]. For example, the simultaneous saccharification and fermentation (SSF) process is often run at 37°C to attain the optimal temperature for the yeast and that for cellulolytic enzymes (between 30–55°C) [1,2]. High-temperature fermentation takes advantage of benefits such as reduction of the risk of contamination and reduction of cooling costs because the chiller unit would not be needed. However, a high temperature fermentation process inhibits cell growth and hampers cell viability. The fermentation efficiency of *Saccharomyces cerevisiae* at high temperatures (>35°C) is low [3]. Hence, although a large amount of research has been conducted on the microorganism fermentation performance at high temperatures, this topic continues to be of interest, particularly the breeding and selection of new yeast strains with enhanced thermostability and increased fermentation performance [4,5].

The thermotolerance phenotype is identified as a quantitative trait which is regulated and controlled by multiple genes [6]. Gaining thermotolerance mainly depends on the regulation of genes related to the synthesis of a specific macromolecule that protects the cell integrity under high-temperature stress. Many genes which have participated in cytoplasmic membrane metabolism have been up-regulated under high temperature stress. In particular, the genes involved in the metabolism of glycerol and ergosterol have been considered to be involved in thermostability. Both glycerol and ergosterol are necessary for the yeast cell to protect and repair cellular structures at high temperatures. Elucidation of the genes and macromolecule role will provide an in-depth mutual understanding of the diverse mechanisms underlying yeast response to high-temperature stress, providing valuable information to improve bioethanol production at higher temperatures.

High-temperature stress causes many changes in the cell that ultimately affect protein structures and function, accumulate denatured and aggregated biomacromolecules, and give rise to growth inhibition or cell death [7]. The high temperature stress disrupts the integrity of cell membranes, increases membrane permeability, and affects the plasma membrane fluidity. It was reported that the specific composition of the cytoplasmic membrane is one of the main reasons for the thermostability of yeast cells. The abnormal proteins are mostly degraded via the proteasome pathway as a defense strategy to guarantee survival [8]. Although many past studies have focused on the function of some candidate genes in some aspects of resistance to

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thermal stress [5,9], the results have not yet been thoroughly understood for any aspect of thermotolerance.

The reactive oxygen species (ROS) is the normal metabolic byproduct of the growth and metabolism of yeast cells. However, excessive ROS accumulation in cells can damage DNA, proteins and other cellular components, leading to cell membrane instability. The excessive ROS accumulates as a consequence of many stresses, including the high temperature. The past studies showed that prevention of ROS formation resulted in an increased thermotolerance. Although much effort has been uncovered regarding the molecular mechanisms, the ROS accumulation in yeast cells still remains unanswered.

In the present study, the effect of high temperature on cellular membrane integrity and ROS accumulation of *S. cerevisiae* YZ1 strain and its thermo-tolerant YF31 strain was investigated. The cytoplasmic membrane permeability and mitochondrial membrane potential were measured. The YF31 cells showed superior ability to maintain their structure and morphology at high temperatures. Moreover, ROS accumulation analysis was also used to examine the impact of high-temperature stress on *S. cerevisiae*.

2. Materials and methods

2.1. Yeast strains and culture media

The *S. cerevisiae* yeast strains used in this study were YZ1 and YF31 strains [10]. In particular, the mutant strain YF31 generated 2.48 times ethanol than the YZ1 strain at 42°C [10]. The YPD media (1% yeast extract, 2% peptone, and 2% glucose on a solid medium containing 2% agar) was used for precultivation. For the main cultivation, a YNB medium (6.7 g L^{-1} yeast nitrogen base without amino acids, 0.77 g L^{-1} complete supplement mixture minus uracil, and 0.02 g L^{-1} uracil containing 20 g L^{-1} glucose) was used. The yeast cells were pre-cultured in a YPD liquid medium at 30°C overnight. Ten OD_{600} overnight cultures were inoculated into the fresh fermentation medium at the indicated temperature (37°C or 42°C) for 72 h. The growth of the cells was determined by monitoring the OD_{600}.

2.2. The viability of cells at a continuous high temperature

The stationary phase cells grown in YPD media at 30°C were washed twice with potassium phosphate-buffered saline (PBS) and resuspended in the fermentation medium with a final OD_{660} of 0.1, and incubated at 42°C for 72 h with shaking. The cells were diluted to obtain a concentration of approximately 2×10^3 cells/mL. Then, approximately 300 cells were spread on YPD plates at 30°C. After 2 d, the colonies were counted. The percentage of cells was identified as a relative value of viable-cell number with temperature shift.

2.3. Detection of yeast cell wall to sodium dodecyl sulfate (SDS) and lysing enzyme sensitivity

The yeast cells were harvested by centrifugation at 5000 rpm for 5 min. After removal of the supernatant, about 10 OD_{600} cells (3×10^8) were resuspended in PBS. This procedure was repeated twice. Then, the cells were mixed with 0.1% SDS or 0.6 mg/mL of lysing enzyme at 120 rpm/min for varying amounts of time. The absorbance of cells at 600 nm was measured.

2.4. Detection of cytoplasmic membrane permeability

The cytoplasmic membrane permeability of *S. cerevisiae* was assessed by carboxyfluorescein diacetate, succinimidyl ester (CFDA) and propidium iodide (PI) fluorescent staining dyes [11]. Cultured yeast cells were harvested by centrifugation at 5000 rpm for 5 min. After the removal of the supernatant, cells were resuspended in PBS twice. A cell suspension of approximately 1 mL was mixed with 1 μL of CFDA (10 μM) and PI (20 μM) solution and incubated at room temperature for 10 min. The samples were analyzed by fluorescence microscopy in triplicate.

2.5. Detection of mitochondrial membrane potential

The mitochondrial membrane potential was measured using 5,5′,6,6′-tetrachloro-1,1′,3,3′-tetraethyl-benzimidazol-ocarbocyanine iodide (JC-1), a cationic dye that reflects membrane potential-dependent formation and emits red fluorescent J-aggregates in mitochondria [12]. The YZ1 and YF31 cells were inoculated at either 37°C or 42°C for 24 and 48 h. The cells were harvested by centrifugation at 5000 rpm for 5 min and washed twice with JC-1 assay buffer. The yeast cells were incubated with 10 μM of JC-1 at 30°C and protected from light for 30 min. Fluorescence images were collected using fluorescence microscopy.

2.6. Determination of ergosterol and trehalose content

The yeast cells were grown in the YPD with shaking at 200 rpm at 30°C for 18 h. Then, the cells were harvested and transferred to the fermentation media at 37°C and 42°C with shaking at 200 rpm for 24 h. The cells were then harvested and total sterol was extracted. Ergosterol concentrations were measured using an HPLC system equipped with a reverse-phase column [13]. Trehalose was extracted from the cells washed with cold 0.5 M trichloroacetic acid and estimated using an established Zheng et al. [13] method.

2.7. The SOD activity assay

The cells were harvested and transferred to the fermentation media at 37°C and 42°C with shaking at 200 rpm for 18 h. The yeast cells were harvested by centrifugation at 5000 rpm for 5 min and washed twice with PBS. Protein levels were determined by a BCA protein assay kit. Total SOD activity (U mg^{-1} protein) was assayed with SOD assay kits (Cayman Chemical Co., Ann Arbor, MI, USA) according to the manufacturer's instructions and determined by the colorimetric method.

2.8. Detection of the intracellular ROS level

The cells were harvested and transferred to the fermentation media at 42°C with shaking at 200 rpm for 24 h. The intracellular ROS levels were measured using dichlorodihydrofluorescein diacetate (DCFH-DA), a fluorescent dye, according to the method used by Zhang et al. [14]. The cells were collected by centrifugation at 10,000 rpm for 10 min, washed three times with PBS (pH 7.4), then stained with 10 μg/mL of DCFH-DA at 30°C for 1.5 h. ROS accumulation was labeled by DCFH-DA, observed and imaged using a CLSM.

2.9. Genomic DNA extraction

The yeast cells were cultured in 100 mL YPD at 200 rpm/min overnight. Then, cells were harvested after centrifugation at 5000 rpm at 4°C for 5 min and washed with 20 mL sterile water three times. Cells were resuspended in 200 μL lysis buffer (100 mM Tris–HCl, pH 8.0, 50 mM EDTA and 0.5% SDS) and transferred to a 1.5 mL microcentrifuge tube. Then, 0.2 g glass beads (0.5 mm) were added to resuspend the cells. The cell suspension was thoroughly mixed at the maximum speed on a high speed vortex mixer. After centrifugation at 5000 × g for 5 min at 4°C, the supernatant was transferred to a new 1.5 mL microcentrifuge tube and 500 μL phenol:chloroform:isoamyl alcohol (25:24:1) was added to the supernatant. This mixture was then briefly mixed on the vortex mixer and was centrifuged again at 12,000 × g at 4°C for 10 min. The upper layer was then carefully withdrawn and transferred to a new 1.5 mL microcentrifuge tube. One