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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.

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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

milliliter ice-cold 95% (v/v) ethanol was added to the supernatant and the solution was briefly mixed by inversion. It was then stored at -20°C for 2 h to precipitate the genomic DNA. After that, the sample was centrifuged at 12,000 × g at 4°C for 10 min and the supernatant was carefully discarded to retain the genomic DNA pellet. Afterwards, 1 mL 75% (v/v) ice-cold ethanol was used to wash the genomic DNA pellet three times, and the DNA pellets were then dried by incubation at 37°C for 1 h. The genomic DNA was resuspended in 200 μL of sterile water and was stored at -20°C until use.

2.10. Random amplified polymorphic DNA (RAPD)

The RAPD reactions were performed using the decamer primers of the OPERON random primer kit (OPA 01, 02, 03, 07, 08, 09, and 10), and the arbitrary primers SOY, RP1-4, RP-2, and RP4-2 listed in Table 1. The amplification was conducted with a predenaturation at 94°C for 10 min followed by 44 cycles of thermal denaturation at 94°C (45 s), primer annealing at 36°C (45 s), and extension at 72°C (2 min). After that, a 10 min final extension at 72°C was conducted to stabilize the amplified DNA products. Such amplified products were separated by electrophoresis in 1.0% agarose gel, 1× Tris-acetate (TAE) buffer (40 mM Trisbase-acetate and 1 mM Na_2EDTA, pH 8.0) and a constant voltage of 120 V. A horizontal electrophoresis (Cleaver, UK) was used, followed by staining with SYBR Safe (ABM) and visualization in a UV transilluminator.

2.11. Statistical analysis

Data presented are the means of the experimental data and are expressed as the mean ± SE. Data were analyzed using by one-way ANOVA with post hoc tests. Means and standard errors were calculated from three replicate tests of every strain.

3. Results

3.1. The fermentation performance of strains and yeast cell viability at high temperatures

Laboratory-scale fermentation using *S. cerevisiae* YZ1 and YF31 yeast cells was performed at 40°C (10). In our previous study, the YZ1 strain was used as the starting strain. The mutants which expressed higher thermotolerance and higher ethanol productivity were selected and designated as YF31. The YF31 strain could grow on plate cultures up to 47°C. In particular, the mutant strain YF31 generated 94.2 ± 4.8 g L^{-1} of ethanol from 200 g of glucose L^{-1} at 40°C, which was 2.48 times the production of the YZ1 strain. The viability of cells was analyzed during continuous exposure to heat. After 24 h of high temperature treatment, both YZ1 and YF31 were still viable at a relatively high level. However, the viability of YZ1 cells had drastically decreased after 72 h of high temperature fermentation, i.e. the survival ratio of YZ1 and YF31 had decreased to 1.3 ± 0.4% and 2.1 ± 0.5%, respectively. Thus, the YF31 strain probably possesses a more prominent ability to resist the adverse environmental stresses in the later fermentation phase.

Table 1
Oligonucleotides used for quantitative RAPD.

	Primer	Sequence (5′ to 3′)
1	OPA01	CAGGCCCTTC
2	OPA02	TGCCGCGCTG
3	OPA03	AGTCAGCCAC
4	OPA07	GAAACGGGTG
5	OPA08	GTGACGTAGG
6	OPA09	GGGTAACGCC
7	OPA10	GTGATCGCAG
8	RP1-4	TAGGATCAGA
9	RP2	AAGGATCAGA
10	RP4-2	CACATGCTTC

3.2. The cell wall fragility to SDS and lysing enzyme

The cell walls are essential components of living yeast cells; many genes involved in cell wall biosynthesis have elevated expression levels during the cell growth phase. Moreover, it is known that cell wall integrity is an important response factor to many stresses, including high temperature.

We measured the sensitivity of the cell walls of YZ1 and YF31 strains to SDS and lysing enzyme sensitivity. The results showed an obvious difference between the mutant YF31 strain and the wild strain YZ1 cell wall to SDS (Fig. 1a) and lysing enzyme sensitivity (Fig. 1b). The wild strain YZ1 was more sensitive to SDS and the lysing enzyme. Thereby, the results also assumed that SDS and the lysing enzyme have a significant impact on the yeast cell wall.

3.3. Effect of high temperature on cytoplasmic membrane integrity

The cytoplasmic membrane integrity of YZ1 and YF31 cells during high-temperature fermentation was analyzed via fluorescence microscopy using CFDA and PI fluorescent dyes (Fig. 2). The dominant portion of the YZ1 and YF31 cells showed low red fluorescence intensity at 42°C for 72 h in Fig. 2c and Fig. 2d, indicating that some of the cells were stained with PI. In contrast, the major portion displayed high green fluorescence intensity via staining with CFDA. As the cells with intact cytoplasmic membranes were not stained with PI, but stained with CFDA, the majority of the YZ1 and YF31 cells appeared to keep their cell membranes intact at 42°C for 24 h. Thereby, it was assumed that fermentation at high temperatures for 24 h has limited impact on cytoplasmic membrane integrity. However, after fermentation at high temperatures for 96 h, the cytoplasmic membrane integrity of YZ1 and YF31 cells (Fig. 2e and Fig. 2f) had drastically decreased, whereas that of the YF31 cells was maintained at a relatively high level.

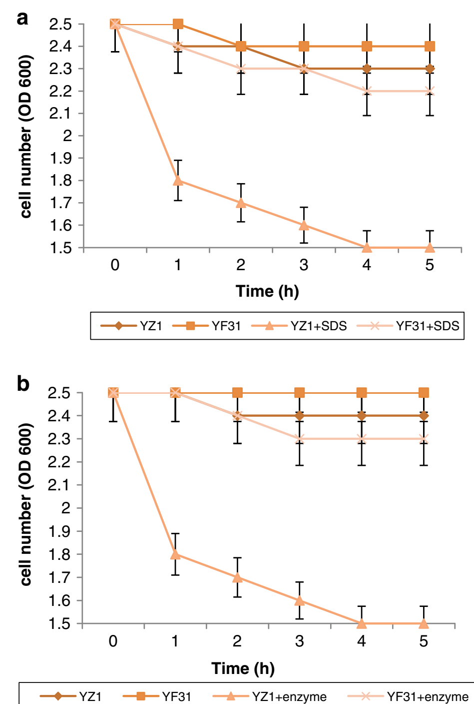

Fig. 1. The cell walls of mutantsYF31 and the wild type YZ1 were sensitive to SDS (a) and lysing enzyme (b).

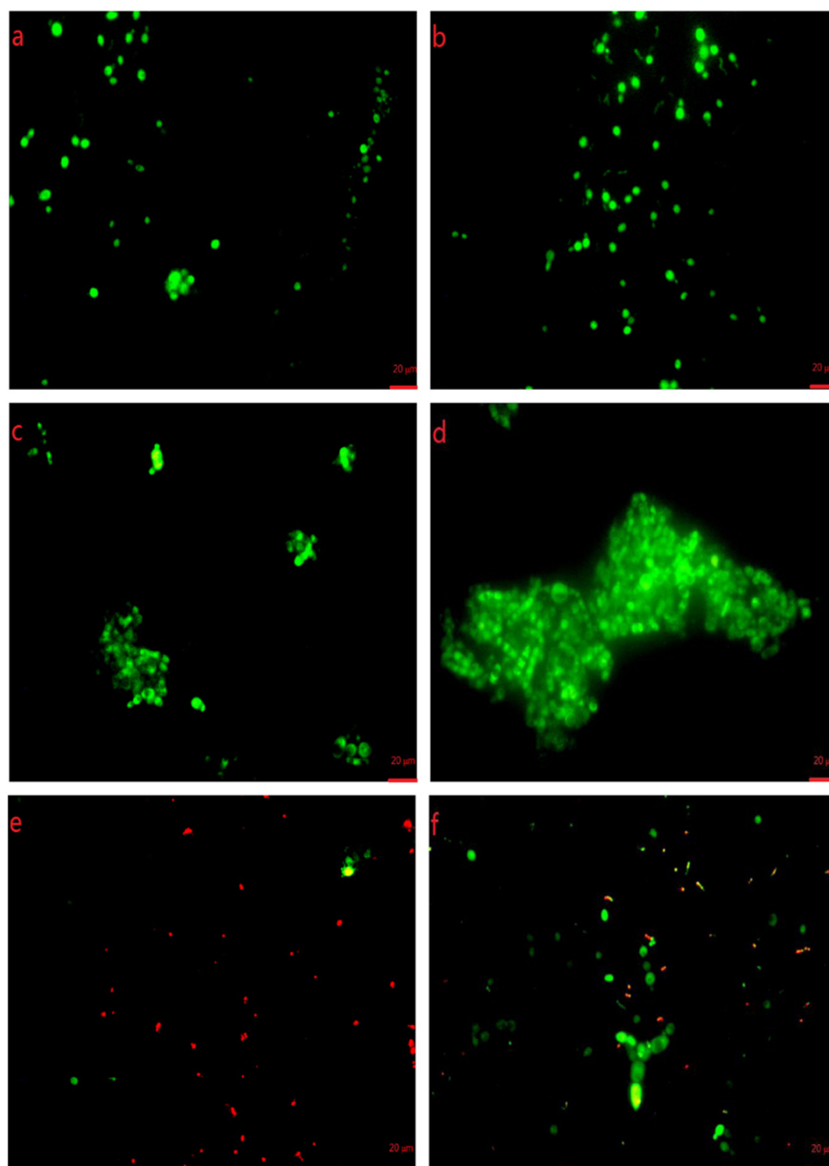

Fig. 2. The YZ1 and the fusant strain YF31 plasma membrane permeability analysis using CFDA /PI. The *S. cerevisiae* strains YZ1 (a, c and e) and YF31 (b, d and f) were sampled at 42°C for 0, 72, and 96 h. Viable cells were stained green with CFDA and the cells with damaged plasma membrane were stained red with PI. Experiments were carried out in triplicate. One representative experiment is shown.

3.4. Effect of high temperature on mitochondrial membrane potential

For a known impact of heat stress on the mitochondrial membrane integrity, the mitochondrial membrane potential of the yeast cells was measured by using a cationic dye JC-1. The JC-1 enters into the mitochondria and forms aggregates emitting red fluorescence in living cells. So, the intensity of red fluorescence of cells reflects the mitochondrial membrane potential. The fluorescence intensities of the YZ1 cells (Fig. 3d) were further reduced than the YF31 cells (Fig. 3c) for 48 h. These results indicated that the mitochondrial membrane potential of the yeast was clearly decreased at high temperatures.

3.5. The relationship between cytoplasmic membrane composition, trehalose, and ergosterol accumulation

Under the fermentation stresses, yeast cells change membrane compositions to maintain the plasma membrane stability. Ergosterol was known as a crucial factor for ethanol production among the various membrane components, because the ergosterol plays a key role in stabilizing the *S. cerevisiae* membrane normal structure and function. The biosynthesis of ergosterol in both YZ1 and YF31 was signally reduced at 42°C (Fig. 4a).

A close link between the amount of trehalose and stress resistance has been confirmed for many types of stresses. Our results showed that the trehalose content of YF31 strain at 42°C was 29% higher than at 37°C. Moreover, the YF31 strain kept more trehalose content compared with YZ1, regardless of temperature which was at 37°C or 42°C (Fig. 4b). These results suggested that yeast cells accumulate more trehalose to resist heat stress.

3.6. The SOD activity

To investigate the effect of high temperature at the level of protein, SOD activity (U mg^{-1} protein) was measured by protein assays in the yeast cells. As shown in Fig. 5, the YF31 cells showed an increased SOD activity of approximately 14.28% compared with the average value of the YZ1 cells at 42°C for 18 h. Heat stress increased SOD activity in all

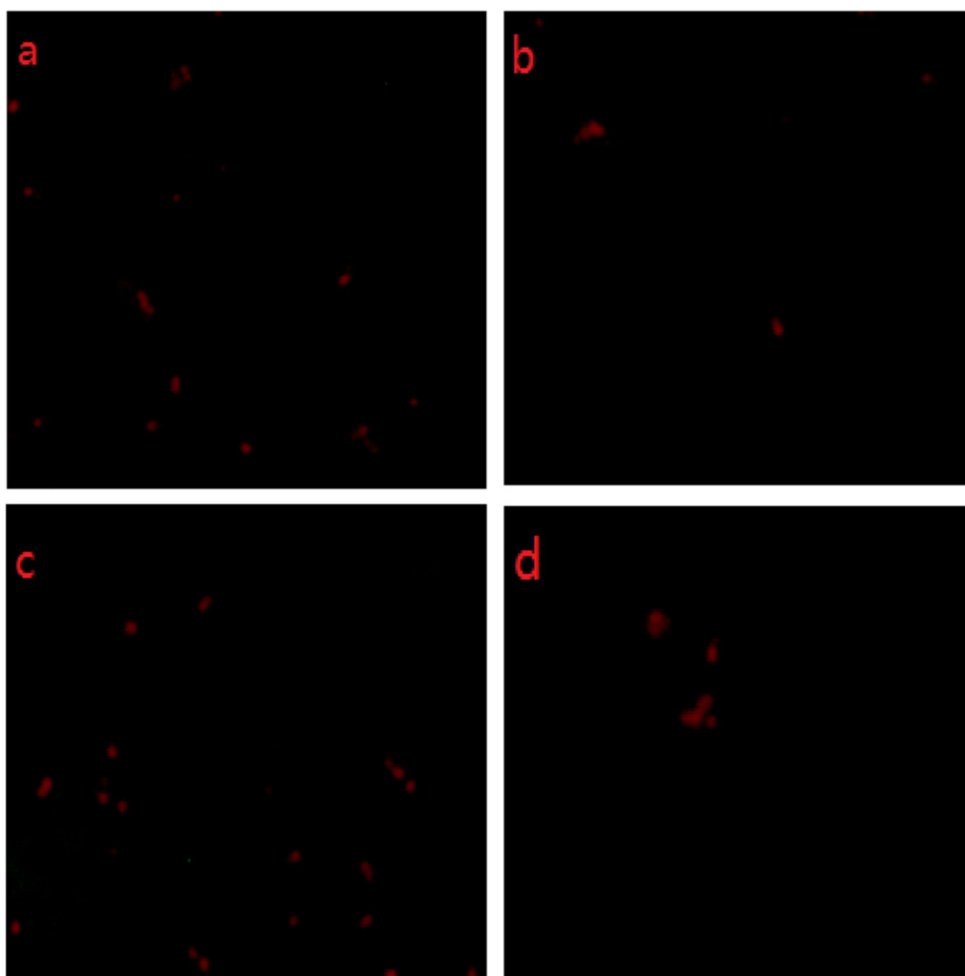

Fig. 3. The mitochondrial membrane potential of the mutant strain YF31 (a, c) and the wild strain YZ1 (b, d) for 24 h and 48 h at 42°C.

of the treated groups. The enhancement of antioxidation capacity may partly account for the resistance to heat stress.

3.7. ROS generation during high temperature stress conditions

It is known that ROS are generated as a result of fermentation stresses. Therefore, we measured the ROS levels in both YZ1 and YF31 strains during high temperature conditions. The fluorescence intensities of both YZ1 and YF31 strains increased during fermentation at 42°C for 24 h (Fig. 6a and Fig. 6b), with YZ1 having the higher fluorescence signal (Fig. 6a). Hence, increased ROS formation in the presence of heat stress was confirmed. Overall, these results suggested that ROS was generated during high temperature conditions, in particular, in the wild strain.

3.8. Random amplified polymorphic DNA (RAPD)

Random amplified polymorphic DNA (RAPD) fingerprints have been used in cultivar identification and genetic relationship analyses of yeast species, and have the advantages of simplicity, efficiency, relative ease of execution, and do not require any previous sequence information. We compared the amplification profiles of parental strain and the potential mutational strains by RAPD. Using 10 selected primers (Table 1), a large number of DNA bands were obtained from the templates of the recombinant yeast strain genomes (Fig. 7). Differences were clearly observed between the RAPD profiles of the YZ1 and YF31 strains (Fig. 7). Consistent RAPD profiles were obtained for YF31 until 50 generations were reached (Fig. 7). Molecular characterization of the mutant YF31 and wild type YZ1 strains confirmed that the mutation is able to bring profound changes in the genetic composition of the yeast.

4. Discussion

Nowadays, bioethanol is a considerably attractive alternative energy source. However, yeast cells currently impose severe environmental stresses, often resulting in stuck fermentation and more formation of metabolic byproducts. A thermo-tolerant yeast strain is necessary for bioethanol fermentation under stress conditions as well in order to save on capital investment and operation costs of the cooling system. Therefore, we have previously obtained a mutant yeast strain, YF31, with multiple stress tolerances. The strain YF31 was mutated by high energy pulse electron beam and protoplast fusion from the parental industrial strain YZ1. The growth and fermentation performance of strains at 40°C have been characterized.

Heat stress adversely affects microorganisms by causing loss of membrane integrity, production of ROS, inactivation and denaturation of proteins, and metabolic and cellular disequilibria, which ultimately lead to cell death. Cells with weakened cytoderms have also been found to display a short life span [15]. Furthermore, the cytoderm checkpoint defected yeast cell was more sensitive to zymolase treatment [16]. We tested the sensitivity of the cytoderms of both YZ1 and YF31 cells to the SDS/lysing enzyme treatment. The YZ1 cell cytoderm was relatively more sensitive to the SDS/lysing enzyme treatment, which is in accordance with the results of the cytoplasmic membrane integrity determination. These results showed that high temperature may not

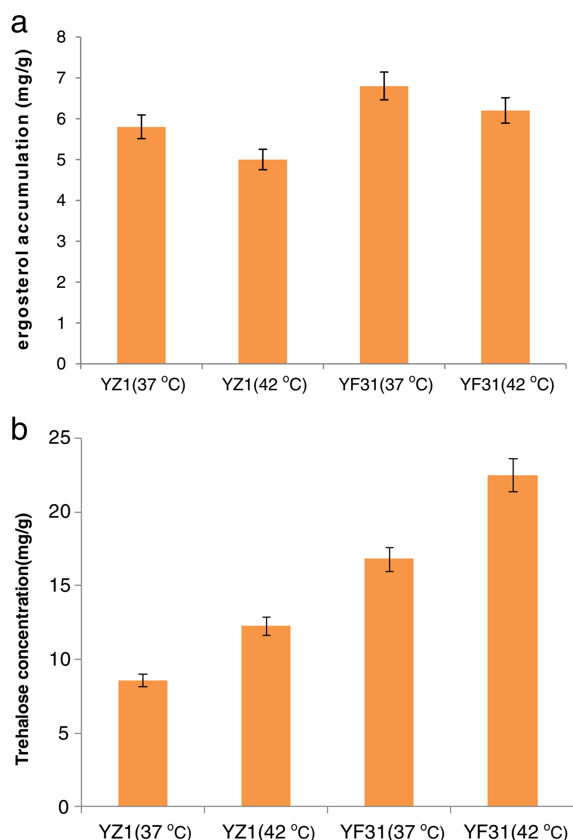

Fig. 4. Ergosterol accumulation and trehalose concentrations of YZ1 and YF31 strains at 37°C and 42°C for 24 h.

only initiate the elevated DNA damage, it may also further trigger other biological abnormalities, including loss of the cell wall integrity. Keeping the cell wall integrity is considered to be important for cell proliferation under many environmental stresses [17].

Past research studies have proven that the thermo-tolerant *S. cerevisiae* strain is considered to be associated with both the DNA repair system [18] and the maintenance of cellular integrity mechanisms [19,20]. We speculated that the thermo-tolerant YF31 strain displayed more resistance to DNA damage caused by heat stress as compared to the wild type strain.

The impact of heat stress on the mitochondrial membrane integrity was evaluated by measuring the JC-1 fluorescence intensities of the yeast. The fluorescence intensities were further reduced with increasing temperature, indicating that both the YZ1and YF31 yeast strains exhibited lower mitochondrial membrane potential when grown in the heat stress environment. These results suggest that the mitochondrial membrane potential of the yeast strain was reduced by the heat stress, mostly via damaging the mitochondrial membrane.

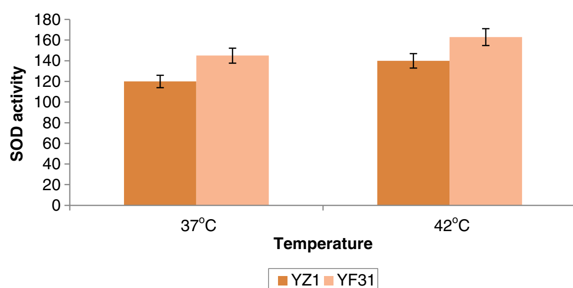

Fig. 5. The SOD enzyme activity of strains YZ1 and YF31 at 37°C and 42°C. The yeast cells were harvested at the stationary phase.

Ergosterol is a lipid component in the yeast membrane which controls the structural membrane features. The past study reported that yeast strains with the highest ergosterol content are the most tolerant of ethanol. However, a clear difference was observed in the ergosterol levels between the YZ1 and YF31 strains in our study.

Trehalose accumulation is identified as a protectant that benefits the yeast cell viability under various stress conditions. We also observed that both YZ1 and YF31 strains contain more trehalose accumulation under heat stress. Moreover, the YF31 strain always had more trehalose content than the wild type at both 37°C and 42°C. The concentration of intracellular trehalose was perhaps a key mechanism for yeast cells to respond to heat stress. In the present study, the YF31 strain showed improved high temperature tolerance via adjusting cell membrane compositions including trehalose content. In brief, a variety of the cell membrane components are identified as the necessary determinants of heat tolerance in the yeast strain.

In this study, there was a significant impact on the permeability of the cytoplasmic membrane of both YZ1 and YF31 at 42°C for 72 h. Moreover, it led to a marked decrease in the mitochondrial membrane potential, and increased the intracellular ROS level. The decrease in the mitochondrial membrane potential and increase in the ROS content could have arisen from the mitochondrial membrane structural instability, which was caused by the high temperature. The high temperature stress-induced mitochondrial membrane instability might lead the membrane-bound electron transport chain to dysfunction, causing a reduction in proton motive force across the inner mitochondrial membrane and finally lead to increased ROS formation. We expected the cell to compensate for the high temperature-induced reduction in proton motive force and to remove the ROS formed. The *S. cerevisiae* cells changed carbon and energy metabolism to meet the demand for energy and redox cofactors.

High temperature tolerance is a complex metabolic control process. It is regulated by multiple genes and is difficult to be controlled by a single gene modification. Enhancing the heat tolerance, as well as relevant physiological and biochemical function, illustrated that large-scale genomic changes happened in the YF31 strain. The RAPD result also confirmed the occurrence of the gene rearrangements between the YZ1 and YF31 strains, with differences in gene lengths. Chromosomal rearrangements were considered to play an important role in yeast evolution and adaptation.

Summarizing all of the results, the high temperature triggered several genes closely related to cell metabolism and thereby increased trehalose, SOD activity, and ROS formation, which subsequently resulted in changed integrity of the cytoplasmic and mitochondrial membrane of *S. cerevisiae* to respond to the heat stress. The RAPD results further prove that the genome rearrangements may have modified the multiple metabolic pathways, and led to the complex phenotypes.

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Conflict of interest

The authors have no conflicts of interest to declare.

Acknowledgments

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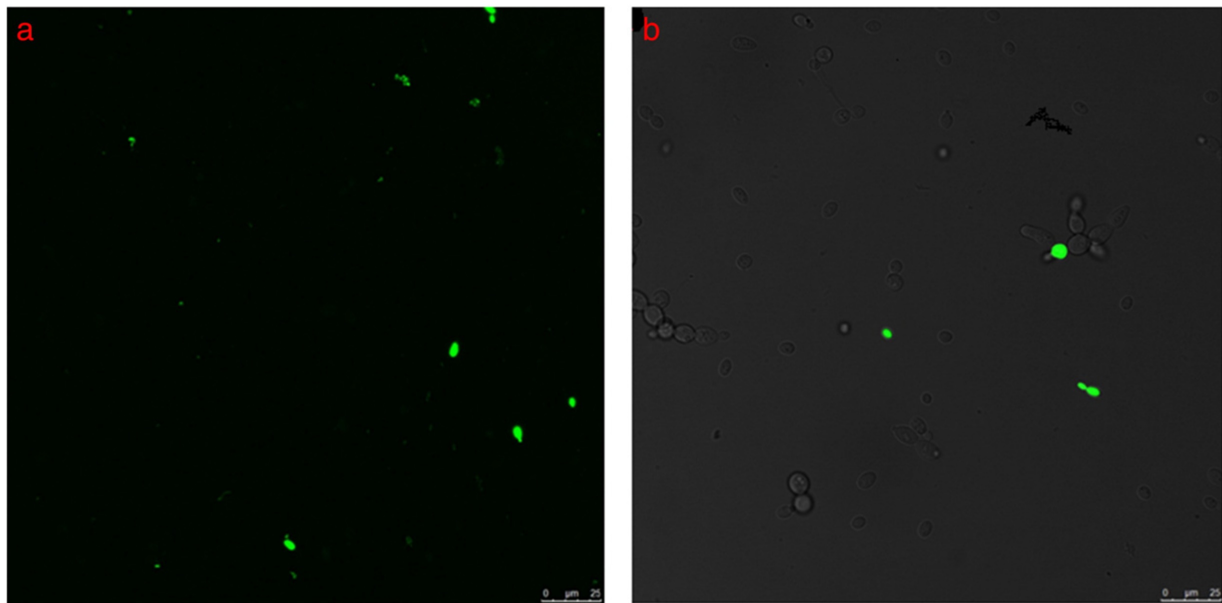

Fig. 6. The ROS accumulation in YZ1 (a) and YF31 (b), which was observed by staining with DCFH-DA at 42°C for 24 h. Three fields of view from each coverslip were randomly chosen, and one representative is shown.

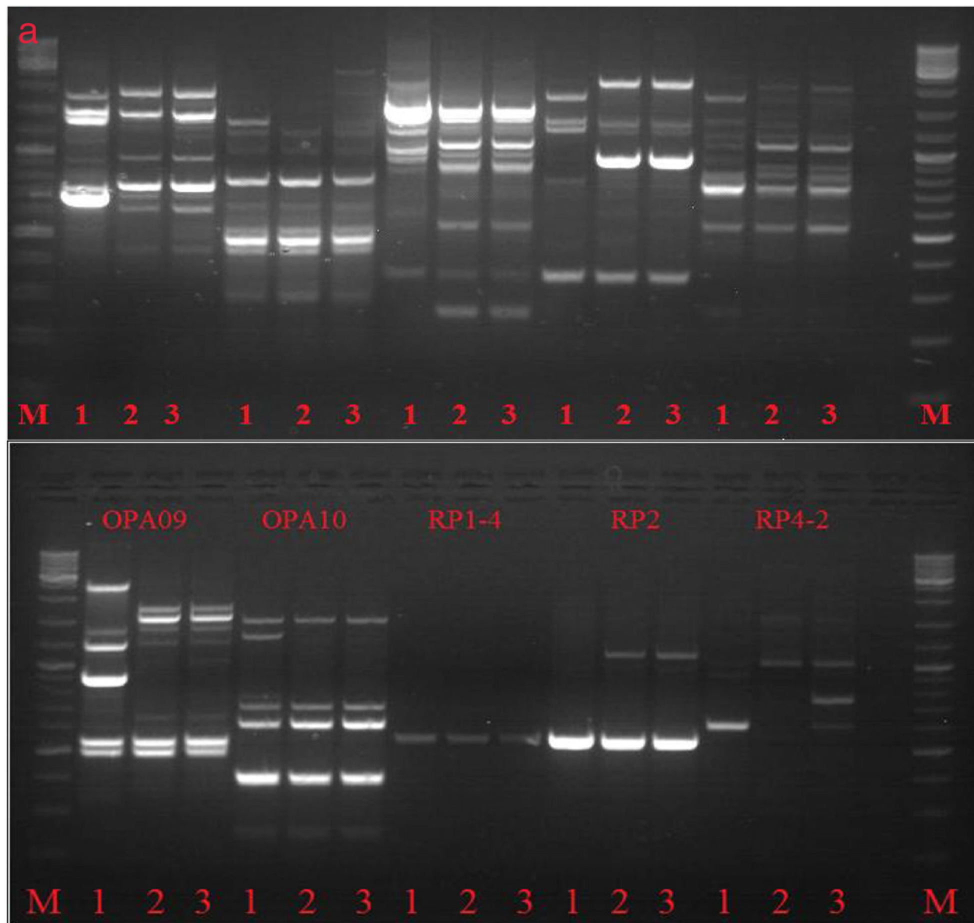

Fig. 7. RAPD-PCR profiles. Lane M, molecular size markers. Lanes 1, 2 and 3, respectively represent the profiles of strains YZ1, YF31 and YF31 until 50 generations.

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