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REVIEW

Advances in mechanisms and modifications for rendering yeast thermotolerance

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Thermotolerant *Saccharomyces cerevisiae* is widely regarded as an attractive strain with which to accomplish the coupling of enzyme saccharification, ethanol fermentation and ethanol distillation in non-grain fuel bioethanol fermentation systems, and it has many advantages for increasing the ethanol yield and reducing production costs. This review provided an overview of the yeast heat-resistant mechanisms from six aspects, including gene expression responses, heat shock proteins, trehalose, ATPase, the ubiquitin-proteasome pathway and heat-induced antioxidant defenses. Innovative methods, such as random and rational strategies for improving yeast thermotolerance, were further discussed, and several special cases were provided. To rationally engineer thermotolerance in yeast, the advances in employing heat-resistant mechanisms of thermophiles were particularly discussed. By designing and constructing heat-resistant devices consists of heat-resistant parts from thermophiles to yeast, a superior thermotolerance of *S. cerevisiae* has been achieved, providing a new system with important applications for research regarding the improvement of the robustness of microbes.

[**Key words:** Non-gain fuel bioethanol; *Saccharomyces cerevisiae*; Thermotolerant mechanism; Thermophiles; Heat-resistant modification; Heat-resistant devices]

Non-grain fuel bioethanol has received considerable attention in recent years as an economic, environmental friendly, practical and renewable alternative to traditional energy sources. Simultaneous saccharification and fermentation (SSF) is an attractive option among all of the bioethanol production processes. Enzyme-induced saccharification of polysaccharides and the subsequent fermentation of sugars to ethanol by yeast take place in the same vessel in SSF, which enhances the enzyme hydrolysis rate of the polysaccharides due to the removal of end product inhibition (1).

Saccharomyces cerevisiae, which possesses high intracellular enzyme activity, has an outstanding capacity to produce ethanol and is fairly resistant to the inhibitors present in biomass hydrolysates. *S. cerevisiae* is currently the most widely used microbial cell factory for producing non-grain fuel bioethanol. In addition, *S. cerevisiae* is relatively tolerant to low pH values and high sugar and high ethanol concentrations (2,3). Despite all of these advantages, there are still several unsolved problems regarding the use of *S. cerevisiae* as an ethanol producer in the SSF process. On the one hand, thermal energy emission during the fermentation process exposes yeast strains to elevated temperatures, which results in significantly reduced cell viability and decreased ethanol production. To address this problem, large quantities of cooling water and cooling power are consumed, leading to high production costs. On the other hand, most of the enzyme saccharifications have optimal temperatures ranging from 45°C to 50°C, which are much higher compared to the optimal temperature for yeast cell growth and fermentation, of approximately 25–30°C, making it difficult to synchronize the SSF process (4). Thus, breeding thermotolerant *S. cerevisiae* has become a necessity in non-grain fuel bioethanol fermentation systems. Performing fermentation at higher temperatures using thermotolerant yeast could not only achieve a higher ethanol production with faster polysaccharide hydrolysis rates and shorter SSF times but could also reduce the cost of cooling and the rate of contamination (5). Moreover, high-temperature fermentation helps with simultaneous ethanol production and distillation (≥45°C) (6). Hence, significant cost reductions and increased production would be achieved if SSF is conducted using the thermotolerant yeast.

An in-depth understanding of the mechanism of yeast thermotolerance provides a theoretical basis for breeding more robust strains. In this review, we highlight and discuss, in detail, the heat-resistant mechanisms of yeast, and review the recent advances in breeding methods aimed at improving yeast thermotolerance, including random and rational strategies. Rational strategies are introduced from two aspects, namely, breeding thermotolerant yeast based on its own heat-resistant mechanisms and based on the heat resistance mechanisms of thermophiles.

YEAST THERMOTOLERANT MECHANISM

The comparison and analysis of the differences between thermophilic and mesophilic yeast in terms of the enzymes, nucleic acids, structure and function of the cell membrane and biosynthetic systems have been the focus of early research on heat-resistant mechanisms. Since the discovery of the heat shock phenomenon in prokaryotes and eukaryotes, heat shock proteins (Hsps) and heat

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shock response (HSR) have become the focal point of research, and a new field has begun to expand in different directions (7). In addition, heat shock can accelerate the production of large amounts of reactive oxygen species (ROS), including superoxide anion (O_2^-), hydrogen peroxide (H_2O_2) and highly reactive hydroxyl radical, resulting in oxidative damage. Subsequently, *S. cerevisiae* produces corresponding antioxidants against heat-induced oxidative stress (8). So far, several molecules have been shown to be involved in heat resistance, including Hsps, trehalose, ATPase, ubiquitin and antioxidant enzymes, all of which play important roles in yeast heat-resistant mechanisms.

Gene expression responses in heat-shocked yeast In *S. cerevisiae*, the expression of Hsps is increased through the mediation of the so-called heat shock factor (HSF) after heat shock, and the phenomenon was described as HSR. Four distinct HSF members have been described in mammals and plants, termed HSF1 to HSF4. However, yeast expresses only a single and indispensable HSF, which is functionally equivalent to HSF1. In most eukaryotes, HSF1 exists as an inactive monomer or dimer with hidden acidic groups in the cytoplasm. Under heat stress, HSF1 forms a homotrimer and binds to the heat shock element (HSE) with repeating units of the sequence nGAAn to upregulate the expression of Hsps. However, unlike in other eukaryotes, Hsf1 in *S. cerevisiae* is constitutively bound to HSE as a trimer under normal temperature. Phosphorylation and other posttranslational modifications stimulate Hsf1 activity and regulate the transcription of Hsps after heat shock (8–10).

All aerobic microorganisms, including yeast, continue to generate ROS during normal aerobic metabolism. ROS are toxic agents that cause membrane lipid peroxidation, nucleic acid damage and protein oxidation toxicity. Heat shock can accelerate the production of ROS, leading to severe oxidative damage to cells, and can induce the oxidative stress response. Thermotolerance in yeast cells is tightly linked to intracellular ROS levels. Yeast cells that are cultured anaerobically display a 10^2- to 10^3-fold increase in thermotolerance than those grown under aerobic conditions (11). The main regulators of the antioxidant response under oxidative stress conditions are the transcription factors Yap1 and Skn7 (12). In the absence of oxidants, Yap1 is primarily found in the cytoplasm. Upon H_2O_2 stress, Yap1 is activated by covalently interacting with a sensor protein, Gpx3, causing it to move to the nucleus and upregulate antioxidant gene expression (13). Contrary to Yap1, Skn7 acts as a transcription regulator that remains in the nucleus. Skn7 has a similar DNA binding domain to Hsf1 and plays a supporting role in combining Hsf1 with HSE (8). In addition to the Hsf1-mediated HSR and Yap1- or Skn7-mediated oxidative stress response, another heat-induced stress response pathway, defined as the environmental stress response (ESR), exists in *S. cerevisiae* and is regulated by the transcription factors Msn2p and Msn4p (14). Both Msn2p and Msn4p have been reported to bind to stress response elements (STREs), which are composed of a conserved pentameric core of CCCCT. ESR responds to a remarkable variety of stressors and induces the expression of ~200 genes with different functions to resist stress (15–17).

Heat shock proteins and yeast thermotolerance Hsps are needed by almost all organisms to resist high temperatures. Hsps protect thermally damaged proteins from aggregation, refold damaged proteins, clear irreversibly aggregated proteins and improve the thermal stability of soluble proteins, SOD and proton pumps in stressed cells. The thermotolerance of most Hsp-deficient strains is recovered by the corresponding gene transfection and expression (18). Moreover, Hsps function in cross protection, that is, Hsps induced by a mild dose of one type of stress can subsequently provide protection against a lethal dose of stress (19). In yeast, the predominant Hsps, as molecular chaperones, mainly include Hsp12, small Hsps (sHsps), Hsp70s, Hsp90s and Hsp100, which are named and classified according to their apparent molecular masses and functions (20,21). The Hsp70 chaperone system is the largest and highly conserved family of Hsps. Most Hsp70s are constitutively expressed, and their expression is further increased in stressed cells. Under non-stress conditions, Hsp70s assist with the *de novo* folding of proteins, whereas under stressed conditions, they participate in the resolubilization of proteins that have aggregated and maintain unfolding proteins in a soluble state (22). Moreover, Hsp70 acts as a sensor for Hsf1-mediated cytoprotection (23). The protection mechanisms of yeast Hsp90s are relatively complex and require a large number of cochaperones to regulate the Hsp90 cycle (24). Hsp90s participate in the maturation and stability of a special class of client proteins, including kinases and transcription factors. In addition, Hsp82 has been reported to be involved in the DNA repair pathway under normal, unstressed conditions (25). The expression of Hsp82 of *S. cerevisiae* increases 20 to 30 times when cells are cultured at 39°C than 25°C; however, at lethal temperatures, the growth of the Hsp82 deficient strain is similar to the growth of the wild strain, illustrating the dispensability of Hsp82 in coping with extreme temperature stress (26). In contrast to Hsp70s and Hsp90s, the yeast protein Hsp104 functions as an oligomeric complex in extreme temperatures. *In S. cerevisiae*, Hsp104 is essential for thermotolerance. *Hsp104*Δ cells grow at the same rate as wild-type cells when cultured at 25°C and 39°C; nevertheless, a 100- to 1000-fold difference was found in the viability of the mutant and wild-type cells after a temperature change to 44°C (27). Hsp104 solubilizes protein aggregates in cooperation with Hsp70 and Hsp40. Recent study has shown that Hsp70 and Hsp40 prevent small aggregates from forming larger aggregates and that the binding of these proteins to Hsp104 stimulates the prolonged association of Hsp104 with aggregates, leading to efficient disaggregation (28,29). Similarly, small heat shock protein Hsp26 interacts with damaged substrates in a mixed oligomeric agglomeration to prevent the irreversible aggregation of its client proteins (30). Hsp12 is a special molecular chaperone that is related with the cell membrane and functions unlike almost all other Hsps, including sHsp. Under physiological conditions, Hsp12 dissolves in the cytoplasm and has an entirely amorphous structure; however, under heat shock conditions, it associates with yeast membranes in a helical structure. The interaction of Hsp12 with the cell membrane stabilizes membrane fluidity and enhances thermotolerance in yeast (21).

Trehalose and yeast thermotolerance When exposed to high temperatures, *S. cerevisiae* can protect itself through the regulation of trehalose biosynthesis. There have been numerous studies on the correlation between thermotolerance and trehalose (31–33). Trehalose is a well-known storage carbohydrate for yeast and contributes to the stabilization of biological membranes, proteins and nucleic acids under stress conditions (34,35). Trehalose biosynthesis involves two steps. First, trehalose-6-phosphate synthase (Tps1) catalyzes the conversion of glucose-6-phosphate and UDP-glucose to trehalose-6-phosphate (T6P). Second, trehalose-6-phosphatase (Tps2) dephosphorylates T6P to trehalose (36). Moreover, the trehalose degradation pathway, catalyzed by neutral trehalases (Nth) and acidic trehalases (Ath), also exists in *S. cerevisiae*, forming the trehalose metabolism cycle. Nth, encoded by the genes *NTH1* and *NTH2*, and Ath, encoded by the gene *ATH1*, can convert trehalose into glucose, which accelerates the return of cells to normal growth after being released from thermal stress (37). The trehalose metabolism in *S. cerevisiae* is an intricate process. The TPS complex in *S. cerevisiae* is composed of two enzyme subunits (Tps1, Tps2)