

Note

Thermotolerant Yeast *Kluyveromyces marxianus* Reveals More Tolerance to Heat Shock than the Brewery Yeast *Saccharomyces cerevisiae*

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The thermotolerant yeast *Kluyveromyces marxianus*, growing at high temperature (45°C), showed stronger survival under heat shock at 50°C than the brewing yeast *Saccharomyces cerevisiae*, which was unable to grow at 45°C. The survival rate of *K. marxianus* decreased to 10% during heat shock at 50°C for 20 min, and to less than 0.01% at 60°C for 20 min. Cells with damaged cellular membranes were infrequently observed at 50°C and had decreased significantly from heat shock at 60°C. The metabolic activity of *K. marxianus* was retained at 50°C, whereas that of *S. cerevisiae* was not. The trehalose content of *K. marxianus* was approximately two times that of *S. cerevisiae*. These results suggest that *K. marxianus* protects itself from heat shock-induced damage through the use of trehalose (a protective molecule in *S. cerevisiae*) as well as other different factors.

Key words : Thermotolerant yeast / Heat shock / Membrane damage / Metabolic activity / *Kluyveromyces marxianus*.

Saccharomyces cerevisiae, widely used yeast in the fermentation and brewing industries, undergoes dynamic changes in its complex biological network of cellular functions during fermentation in response to stressors, such as high temperature, osmosis, and ethanol concentration (Belloch et al., 2008). In particular, high-temperature stress is an important problem in high-temperature cultures producing bioethanol. Fermentations carried out at relatively high temperatures have several advantages, such as protection of the culture from undesirable contamination, faster recovery of ethanol, and considerable savings on capital and running costs required by refrigerated temperature control (Edgardo et al., 2008; Babiker et al., 2010; Benjaphokee et al., 2012). However, stressful culture conditions involving

high temperature usually lead to growth inhibition of *S. cerevisiae*, as well as its loss of cell viability and ability to ferment (Rodrussamee et al., 2011). To address these issues, mutants that can be cultured at high temperature have been used to produce bioethanol (Edgardo et al., 2008; Benjaphokee et al., 2012). In contrast, thermotolerant strains of *Kluyveromyces marxianus* can grow at temperatures higher than the optimal temperature used for culturing *S. cerevisiae* (Fonseca et al., 2008) and are not affected by the high temperatures required for bioethanol production (Babiker et al., 2010; Rodrussamee et al., 2011).

The differences in the cellular responses of the thermotolerant *K. marxianus* and *S. cerevisiae* cultured under high temperature have never been studied. Generally, most cells suffer from biochemical and physical harm after exposure to high temperatures for a short time (called heat shock), resulting in the arrest of certain meta-

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bolic pathways (Mayer et al., 1990; Gasch et al., 2000), damage the membrane structure (Vilaprinyo et al., 2006), inactivation of proteins (Lindquist, 1992; Swan and Watson, 1999), and cleavage of the DNA structure (Grimminger-Marquardt and Lashuel, 2010). Such damage can cause mammalian cells to undergo apoptosis (Paul et al., 2008). However, cells respond to mild heat shock by rapidly altering their global transcription (Trott, and Morano, 2003; Beere, 2004; Guyot et al., 2005) and inducing the *de novo* synthesis of heat-shock proteins (Hsps) become tolerant to the cellular damage (Paul et al., 2008). In *S. cerevisiae*, these responses are executed through activation of a complicated response program to allow the cells to adapt and survive. The transcription of genes involved in the synthesis of trehalose is also activated by heat shock in *S. cerevisiae* (Trott, and Morano, 2003; Hashikawa et al., 2007), and the accumulated trehalose protects the inactivation of proteins and membrane-bound organelles from reactive oxygen species (ROS) generated by the heat shock (Kim et al., 2006; da Costa Morato Nery et al., 2008). In contrast, the biochemical and physiological characteristics of thermotolerant yeasts under heat shock are poorly understood.

Knowledge about the cellular response to the inactivation of proteins, membrane-bounded organelles, etc. in the thermotolerant yeast exposed to heat shock should lead to an understanding of why it is stronger in high-temperature culture and an easily breeding of yeast strain to use in high-temperature fermentation. In this study, we compared the thermotolerant yeast *K. marxianus* with *S. cerevisiae* in terms of their growth at high temperature, cellular inactivation, oxidation, and trehalose accumulation under exposure to heat shock.

The yeast strains used were *S. cerevisiae* BY4741 (*MATa his3Δ1 leu2Δ0 met15Δ0 ura3Δ0*) (Brachmann et al., 1998), one of the parent strains used in the *Saccharomyces* Genome Deletion Project (Open Biosystems, Lafayette, CO, USA), and *K. marxianus* IFO 0482, a thermotolerant yeast strain (Institute of Fermentation, Osaka, Japan). The growth medium, YPD, contained 2% glucose, 1% yeast extract, and 1% peptone. Solid media contained 2% agar. The yeast strains were cultured at the standard temperature of 30°C and high temperature of 45°C. Heat shock was provided at 40°C, 50°C, 60°C, and 70°C for 0, 5, 10, 15, and 20 min, respectively, after culture at the standard temperature for 24 h.

Yeast growth was measured by the absorbance of the culture at the wavelength of 600 nm. The survival rate from heat shock was calculated from the ratio of colony-forming units (CFUs) of heat-shocked cells to non-shocked cells (treated for 0 min), grown on YPD plates for 48 h after heat shock. The number of CFUs of non-

shocked cells was defined as 100% survival. The number of yeast cells with damaged membranes were counted by flow cytometry (Oyane et al., 2009) using a BACTANA flow cytometer (Sysmex Co. Japan, Tokyo, Japan) according to the manufacturer's instructions. In brief, total yeast cells recovered from the various cultures were stained with thiazole orange with the permeation reagent myristyltrimethylammonium bromide (MTAB) for 15 min at room temperature and then counted using the BACTANA flow cytometer. Membrane-damaged yeast cells were stained with thiazole orange for 15 min at room temperature without MTAB and counted using the BACTANA flow cytometer. The fluorescence from thiazole orange was measured by the emission spectrum (650 nm) excited by light of 633 nm wavelength. The percentage of intact cells remaining over time was calculated as follows: $[(\text{total cells} - \text{damaged cells}) / \text{total cells}] \times 100$.

The effect of heat shock on the cellular metabolic activity was determined using the 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) assay (Sigma, St. Louis, MO, USA), which is based on the color produced by the reduction reaction of mitochondrial dehydrogenase in living cells on the MTT reagent (Mosmann, 1983). The assay procedure designed for mammalian cells (Mitrović et al., 2011) was modified as follows: After heat treatment, the yeast cells were seeded in 96-well microtiter plates (10^6 cells per well). Untreated cells served as a control. MTT was added to each well at a final concentration of 5 mg/mL, and the microtiter plate was incubated at 37°C for 4 h. The resulting colored formazan crystals were dissolved in dimethyl sulfoxide, and the absorbance at 450 nm (A_{450}) was measured using an iMark microplate reader (Bio-Rad Laboratories Japan, Tokyo, Japan). The percentage of active cells was calculated by the followed formula: $[A_{450} \text{ of the treated group} / A_{450} \text{ of the control group}] \times 100$.

To assay for intracellular trehalose contents, yeast cells were collected and resuspended in saline (0.85% sodium chloride) to approximately 1.0×10^7 cells/mL. The cell suspensions were boiled for 15 min and then microcentrifuged at $12,000 \times g$ for 10 min at 4°C. The trehalose content of the supernatant was assayed using a trehalose assay kit (Megazyme International Ireland Ltd., Wicklow, Ireland) according to the manufacturer's instructions.

The growth profiles of *S. cerevisiae* and *K. marxianus* at the culture temperatures of 30°C and 45°C were compared (Fig.1). Both yeast strains grew at 30°C and reached the stationary phase at 24 h, where the cell numbers of both strains were approximately 1.0×10^8 cells/mL as measured by the BACTANA apparatus. On the other hand, only *K. marxianus* grew at 45°C, and the

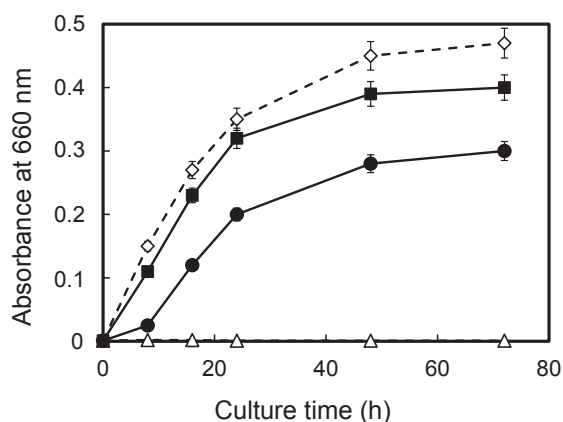


FIG. 1. Growth profiles of *K. marxianus* and *S. cerevisiae* at high temperatures. The *K. marxianus* strain was cultured at 30°C (filled squares) and 45°C (filled circles), the *S. cerevisiae* strain was cultured at 30°C (open diamonds) and 45°C (open triangles). OD values were measured 1/100 volume of every culture. Values are mean \pm SEM of at least three independent assays.

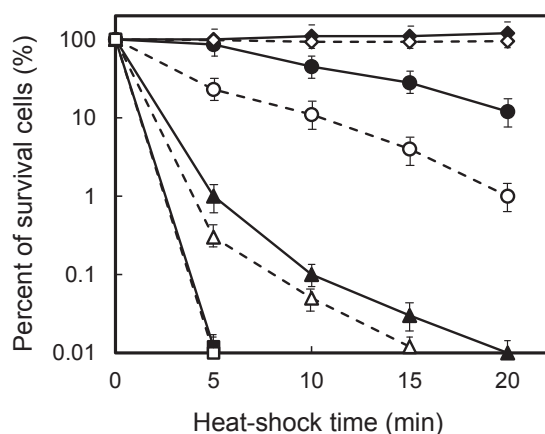


FIG. 2. Effect of heat shock on survival of *K. marxianus* and *S. cerevisiae*. The yeast strains were cultured at 30°C for 24 h and then heated at 40°C to 70°C. *K. marxianus* and *S. cerevisiae* are represented by filled symbols (bold lines) and open symbols (broken lines), respectively. The temperatures used were 40°C (diamonds), 50°C (circles), 60°C (triangles), and 70°C (squares). The percentage of surviving cells was calculated as follows: [CFU of the heat-shocked samples / CFU of the non-shocked sample (0 min)] \times 100. Values are mean \pm SEM of at least three independent assays.

growth level was slightly less than that at 30°C. *S. cerevisiae* BY4741 as well as some other strains of the same species could not grow at 45°C. These results confirm that *K. marxianus* IFO 0482 is a more thermotolerant strain than *S. cerevisiae*, albeit the *K. marxianus* strain could not grow at 50°C (data not shown).

The survival rates of both yeast strains were assayed after heat shock at 40°C, 50°C, 60°C, and 70°C for 5, 10,

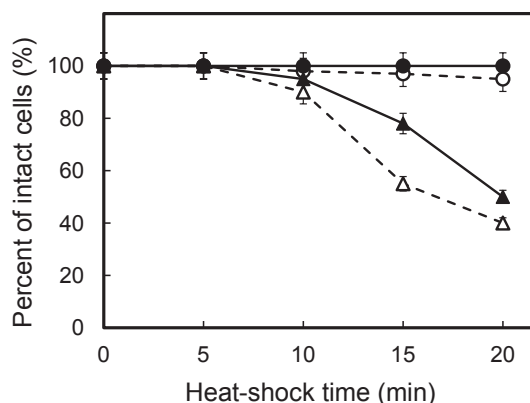


FIG. 3. Effect of heat shock on cellular membrane damage. Both yeast strains were treated at 50°C (circles) and 60°C (triangles) after culture at 30°C for 24 h. *K. marxianus* and *S. cerevisiae* are represented by filled symbols (bold lines) and open symbols (broken lines), respectively. The percentage of intact cells was calculated as follows: [unstained cells of the heat-shocked samples / unstained cells of the non-shocked sample (0 min)] \times 100. Values are mean \pm SEM of at least three independent assays.

15, and 20 min, respectively (Fig.2). Both strains showed stable survival to approximately 100% under 40°C heat shock. During heat shock at 50°C for 20 min, the percentage of surviving *S. cerevisiae* cells decreased gradually to ~1%, at a rate much faster than that of *K. marxianus* under the same conditions (~10%). The survival rate of both yeast strains decreased even further after heat shock at higher temperatures, and was less than 0.01% after 15 min for *S. cerevisiae* and after 20 min for *K. marxianus* at 60°C, and after 5 min for both strains at 70°C. However, the decrease in survival rate of *K. marxianus* was significantly slower than that of *S. cerevisiae* during the heat shock at 60°C. These results suggest that *K. marxianus* IFO 0482 is more tolerant to heat shock than *S. cerevisiae* and is especially remarkable at heat shock of 50°C.

Since the difference in survival rate profiles between the two strains was the most significant at the heat-shock temperature of 50°C, the cellular membrane damage and cellular metabolic (MTT reduction) activity of both strains were analyzed at this same temperature as well as at 60°C (at which both strains were eventually killed). The percentage of intact cells unstained by thiazole orange over heat-shock time is shown in Fig.3. With heat shock at 50°C for 20 min, the percentage of intact *K. marxianus* cells did not decrease, and that of *S. cerevisiae* cells decreased only slightly. However, the percentage of both yeast strains decreased gradually over time at 60°C, although the decrease rate of *K. marxianus* was less than that of *S. cerevisiae* (Fig.3). These results suggest that both strains did not sustain

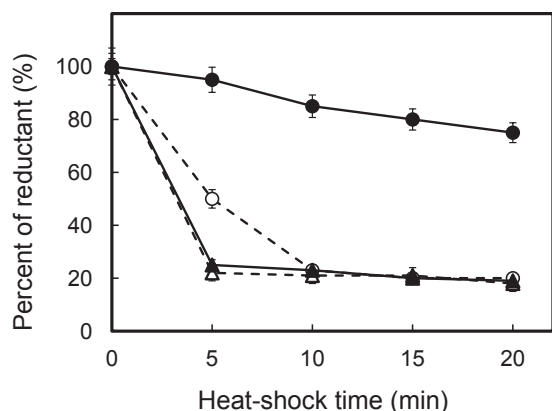


FIG. 4. Effect of heat shock on the cellular metabolic activities of *S. cerevisiae* and *K. marxianus*. Both yeast strains were treated at 50°C (circles) and 60°C (triangles) after culture at 30°C for 24 h. *K. marxianus* and *S. cerevisiae* are represented by filled symbols (bold lines) and open symbols (broken lines), respectively. The reductant generated by the MTT reaction was measured at A_{450} . The percentage of reductant was calculated as follows: [reductant of the heat-shocked samples / reductant of the non-shocked sample (0 min)] \times 100. Values are mean \pm SEM of at least three independent assays.

cellular membrane damage by the heat shock at 50°C, but did so at 60°C, where *K. marxianus* was more tolerant to the damage than the *S. cerevisiae* strain, although both were eventually killed at this higher temperature. These data also imply that the membrane damage is closely related to the decrease in survival rate of both strains at 60°C, as the profiles for intact cell rate and survival rate at this temperature were similar. It is demonstrated that *S. cerevisiae*, cultured at 60°C showed the increase of the permeability of propidium iodide indicating formation of damages within the membrane because it is excluded by the intact membranes of the viable cells (Darvey and Hexlay, 2011). Our results show the possibility that *K. marxianus* forms the similar damage within the membrane at 60°C. In contrast, the more rapid decrease in survival of the *S. cerevisiae* strain at 50°C heat shock does not appear to be correlated with membrane damage, because both strains were barely damaged by the heat shock at 50°C for 20 min. To shed further light on this, the intracellular metabolic activity of both yeast strains were assayed under heat shock at 50°C and at 60°C (the latter as a control of membrane-damaged cells), and the results are shown in Fig. 4.

As revealed in the figure, both strains lost their metabolic activity after 5 min of heat shock at 60°C, suggesting that the loss of metabolic activity is affected by the membrane damage caused by this temperature. With heat shock at 50°C, the metabolic activity of the *S. cere-*

visiae strain was rapidly lost, whereas that of the *K. marxianus* strain was lost slowly. These results suggest that the metabolic activity of *K. marxianus* is more tolerant to heat shock at 50°C than that of *S. cerevisiae*, and may also show that the differences in the tolerance are caused by the different decreasing levels (i.e., greater decrease in *S. cerevisiae* than in *K. marxianus*) of survival rate at 50°C.

Intracellular trehalose, which protects many materials in the *S. cerevisiae* cell from damage by heat shock, was assayed in *K. marxianus* and found to be 45.3 ± 5.8 mg/g wet cells after 24 h culture at 30°C. This value was approximately two times higher than the trehalose content of *S. cerevisiae* (29.5 ± 1.2 mg/g wet cells) under the same culture conditions. This suggests that a higher accumulation of trehalose in *K. marxianus* cells may be the reason for its better tolerance to heat shock relative to *S. cerevisiae*. However, the increase in trehalose accumulation in *K. marxianus* was less than two times, indicating that there are other factors aside from trehalose that may play a role in the yeast's tolerance to heat shock. Now we have been trying to screen the mutants deleted and recombinant highly expressed genes encoding trehalose synthetase and trehalase. The data from those strains are possibly shown some useful information for the tolerance to heatshock in *K. marxianus*.

Our preliminary results for the intracellular oxidant level (ROS contents), showed that it was higher in *K. marxianus* than in *S. cerevisiae* after heat shock at 50°C for 20 min (data not shown), leading to the deduction that *K. marxianus* is more viable than *S. cerevisiae* in this greater oxidative ROS-producing condition generated by the heat shock. These results suggest that *K. marxianus* produces factors other than trehalose to provide tolerance toward the generated oxidants, even though more accumulated trehalose scavenges more ROS.

Most yeast species cannot survive at high temperatures. Our present research shows that whereas *K. marxianus* cells died at temperatures greater than 60°C from physical damage similar to that caused to the cell membranes of *S. cerevisiae*, the *K. marxianus* cells survived at 50°C, unlike *S. cerevisiae* cells. Although both yeast strains did not suffer cell membrane damage at 50°C, only *K. marxianus* was resistant to the heat shock.

Our present results also reveal that the intracellular redox machinery of *K. marxianus* is different from that of *S. cerevisiae*. *S. cerevisiae* eliminates heat-induced ROS (which causes loss of its metabolic activity) by accumulating trehalose at 50°C. In contrast, although the trehalose amount was higher in *K. marxianus* than in *S. cerevisiae*, the sugar was not heavily accumulated and the level of ROS was increased in *K. marxianus*.

Nevertheless, the results of our present study suggest that *K. marxianus* retains metabolic activity at 50°C and eliminates heat-induced ROS by mechanisms not present in *S. cerevisiae*. Recently, some genes encoding heat-shock proteins (Hsps) are reported to express in high temperature condition (Lertwattanasakul et al., 2015). However, it is unknown whether Hsps produced by *K. marxianus* are involved in the redox reaction of the heat-induced ROS. These factors are important to the mechanisms of thermotolerance in yeasts and will be taken into account in our future studies aimed at developing recombinant yeast strains for producing ethanol.

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