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Research Note

Heat resistance acquirement of the spoilage yeast *Saccharomyces diastaticus* during heat exposure

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ABSTRACT

The main fungal cause of spoilage of carbonated fermented beverages in the brewing industry is the amylolytic budding yeast *Saccharomyces cerevisiae* subsp. *diastaticus* (*Saccharomyces diastaticus*). Heat treatment is used to avoid microbial spoilage of the fermented beverages. Therefore, the spoilage capacity of *S. diastaticus* may be linked to its relative high heat resistance. Here, we assessed whether *S. diastaticus* can acquire heat resistance when exposed to heat stress. To this end, ascospores of *S. diastaticus* strain MB523 were treated at 60°C for 10 min followed by growing the surviving spores on a glucose-containing medium. The resulting vegetative cells were then allowed to sporulate again in sporulation medium. This cycle of heat treatment, vegetative growth, and sporulation was performed eight times in three independent lineages. After these eight cycles, the sporulation rate was similar to the start (~75%) but the resulting ascospores were more heat resistant. The time needed to kill 90% of the population at 60°C (i.e. the D_{60}-value) increased from 6.5 to 9.0 min ($p = 0.005$). The vegetative cells also showed a trend to increased heat resistance with an increase in the D_{52}-value from 9.2 to 16.2 min ($p = 0.1$). In contrast, heat resistance of the vegetative cells that had not been exposed to heat during the eight cycles had been reduced with a D_{52}-value of 4.2 min ($p = 0.003$). Together, these data show that *S. diastaticus* MB523 can easily acquire heat resistance by inbreeding while subjected to heat stress. Conversely, heat resistance can be easily lost in the absence of this stress condition, indicative of a trade-off for heat resistance.

Microbial spoilage of beer and beer-related products such as Radler is hampered by the presence of hop, iso-α-acids, ethanol, and CO_2, and by the low nutrient content, the absence of oxygen and low pH. Despite these hurdles, there are bacteria (such as *Lactobacillus*, *Pectinatus,* and *Megasphaera*) and yeasts (such as *Saccharomyces*, *Brettenanomyces*, *Rhodotorula*, *Torulaspora,* and *Zygosaccharomyces*) that can spoil these beverages (Bokulich & Bamforth, 2013; Suiker & Wösten, 2022). The most dangerous spoilage yeast in breweries is *S. cerevisiae* subspecies *diastaticus* (*Saccharomyces diastaticus*) (Hutzler et al., 2012). *S. diastaticus* was considered a subspecies of *S. cerevisiae* distinguished by the presence of the *STA1* gene (Andrews & Gilliland, 1952; Pretorius et al., 1991; Tamaki, 1978; Yamashita & Fukui, 1984; Yamashita et al., 1985). However, *S. cerevisiae* strains that are positive for *STA1* do not form a monophyletic group, but instead are found in several clades (Krogerus et al., 2019; Suiker, 2021). Although *S. diastaticus* is actually not a subspecies, for historical reasons, we will continue to label it as such.

S. diastaticus is not highly prevalent in nature, but it accumulates in breweries in mixed biofilms (Suiker et al., 2021). It spoils beer by producing phenolic off-flavor (POF), haze formation, and superattenuation, leading to increased alcohol percentage, overcarbonation, and weakened body (Meier-Dörnberg et al., 2017). Both vegetative cells and spores can spoil beer products, the latter cell type irrespective of its environmental history (Suiker et al., 2021). However, ascospores are expected to be the main cause of beer spoilage because these cells are more heat resistant than vegetative cells (Milani et al., 2015; Put & de Jong, 1982) and can therefore more easily survive suboptimal thermal pasteurization of beer products.

Breweries have heat sources such as pasteurizers and plate heat exchangers. This raises the question of whether *S. diastaticus* can acquire heat resistance by being exposed to this stress in a brewery environment, thereby increasing its chance to spoil beer and beer products. We indeed here show that ascospores of *S. diastaticus* strain MB523 can easily acquire heat resistance by inbreeding upon repetitive exposure to heat. Results thus imply that the incidence of beer

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spoilage by *S. diastaticus* may increase in breweries due to the presence of heat sources combined with sublethal cleaning and disinfection agents.

Materials and methods

Growth conditions

Wild-type *S. diastaticus* strain MB523 was stored at −80°C in 50 g L^{-1} YPD (yeast extract, peptone, glucose; Sigma, https://www.sigmaaldrich.com) supplemented with 20 g L^{-1} glycerol. Slopes (50 g L^{-1} YPD and 20 g L^{-1} agar) were inoculated with 30 µL of the frozen stock culture, grown for 72 h at 30°C, and stored at 4°C for a maximum of 2 months. To determine survival rates, cultures were inoculated with these cells and grown for 40 h at 20°C and 200 rpm in 5 mL YPD broth. Cultures were grown for 48 h at 25°C and 200 rpm for sporulation. To this end, cells were pelleted at 2000 g for 10 min, washed twice with Milli-Q water, and resuspended in 0.9% NaCl. A total of 1–5 10^7 cells mL^{-1} were transferred to sporulation medium (10 g L^{-1} potassium acetate, 1 g L^{-1} yeast extract, 0.5 g L^{-1} glucose) and incubated for 120 h at 25°C and 200 rpm.

Staining of vegetative cells and ascospores

Cells were transferred to microscope slides, heat fixed, and incubated with malachite green and safranin O to discriminate between vegetative cells and ascospores (Langmead & Salzberg, 2012). To this end, cells were stained with 5% malachite green for 3 min, rinsed with demi water, destained in 96% ethanol for 20 s, rinsed with demi water, stained with 5% safranin O for 30 s, rinsed with water, and air-dried. Metabolically active cells were red (malachite green is removed from cells by destaining, safranin O stains the cell red), immature spores were red, dark blue, or purple since both safranin and, or malachite green stain the spore, while mature spores were bright blue because malachite green but not safranin O diffuses through the mature spore wall. Sporulation rates were determined by counting 400 objects using bright field microscopy at a 1000-fold magnification.

Heat treatment and spot dilution assay

Sporulating cultures with 75% ± 10% ascospores (10^7 cells ml^{-1} 0.9% NaCl) were treated for 10 min at 60°C in a 50 µL volume in a preheated PCR thermocycler (Biometra T3000; http://www.biometra.de). Viable cell concentrations before and after heat treatment were determined by a spot dilution assay. To this end, cells were serially diluted up to 10^7-fold and 5 µL was plated on a YPD medium supplemented with 30 g L^{-1} agar. Cultures were incubated at 25°C for 5 days.

Survival rates and D-value determination

A volume of 200 µL (containing 10^8–10^9 cells) was added to 19.8 mL preheated 0.9% NaCl in 100 mL Erlenmeyers in a shaking water bath. Samples (200 µL) were taken at various timepoints and immediately placed on ice. Vegetative cells and ascospores were treated at 52°C up to 30 min and 60°C up to 60 min, respectively. Viable cell concentrations before and after treatment were determined by a spot dilution assay. The cells were serially diluted up to 10^5-fold with 5 µL plated on YPD medium supplemented with 30 g L^{-1} agar. Cultures were incubated at 25°C for 5 days. Colony forming units (CFUs) before and after treatment were compared. For the eight cycles, survival rates were determined in percentages. Data were analyzed with a two-tailed t test on the percentages of survival using $p \leq 0.05$. At cycle 1 and cycle 8, the D_{52}-values and D_{60}-values were calculated for vegetative cells and spores, respectively. The *D*-value (time needed to kill 90% of a population at a certain temperature) was obtained by taking the reciprocal of the slope of the inactivation curve and multiplying that value by −1. Differences in *D*-values were analyzed with a two-tailed t test using $p \leq 0.05$.

Results and discussion

Understanding the mechanisms underlying heat resistance in *S. diastaticus* will help to design novel preservation methods for beer products. Here, a directed evolution experiment was performed to assess how fast heat resistance can be obtained by inbreeding. To this end, ascospores of *S. diastaticus* strain MB523 were treated at 60°C for 10 min followed by growing the surviving spores on a glucose-containing medium. The resulting vegetative cells were allowed to sporulate again in a sporulation medium. This cycle of heat treatment, vegetative growth, and sporulation was performed with three independent lineages. Similar cycles without heat treatment were performed as a control. The experiment was stopped after eight cycles because the survival rate of the control cultures after heat treatment had become too low to perform another cycle (see below). In addition, there was no significant difference in heat resistance between cycles 6, 7, and 8 of the evolved populations thereby taking away the need for another cycle.

Heat resistance and sporulation rates of the treated and control populations of MB523 were determined after each cycle (Fig. 1). A total of 80% ± 2% of the parental MB523 cells had formed ascospores. Of these spores, 8.7% ± 1.4% survived heat treatment at 60°C for 10 min. Heat resistance gradually increased after cycle 3 of the heat-treated samples, while the sporulation rates remained similar. Increased survival of spores was statistically significant from cycle 6 onwards ($p \leq 0.05$). The percentage of survival of the evolved population was 79.8% ± 15.5% after eight cycles. Thus, directed evolution increased survival almost 10 times. The control cultures showed decreased sporulation rates from cycle 4 onwards as well as decreased heat resistance from cycle 6 onwards ($p \leq 0.05$). The average sporulation rate was 17 ± 16% after eight cycles, while the average survival rate dropped to 0.2% ± 0.2%.

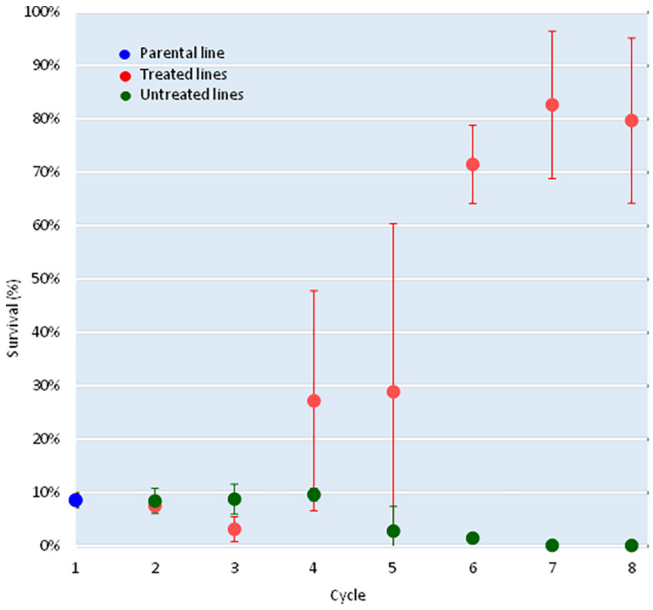

Figure 1. Survival percentages after 1–8 cycles of a 10-min heat treatment at 60°C. Error bars represent the standard deviation of biological triplicates. Heat resistance gradually increased and decreased in the heat and non–heat-treated samples, respectively.

Vegetative cells are less heat resistant than ascospores (Milani et al., 2015; Put & de Jong, 1982). Therefore; D_{52}- and D_{60}-values were determined for the vegetative cells (Fig. 2AC) and the ascospores (Fig. 2BD), respectively. The D_{60}-values of the ascospores increased from 6.5 ± 0.8 min for the parental MB523 strain to 9.0 (±1.1) min after eight cycles of heat treatment (Fig. 2B). The D_{60}-values were not determined for the control cultures since sporulation was impaired when cultures had not been treated with heat. A trend toward increased heat resistance was also observed for the vegetative MB523 cells when spores had been exposed to the heat stress (p = 0.1). The parental strain showed a D_{52}-value of 9.2 ± 2.9 min, while it had increased to 16.2 ± 7.5 min after eight heat treatments (Fig. 2A). In contrast, the D_{52}-value of the three control cultures had decreased to 4.2 ± 2.1 after eight cycles. These results suggest that the heat resistance of ascospores and vegetative cells are linked. We conclude that the increase in D_{60}-value of ascospores is the result of directed evolution and is not caused by adaptation. This is based on the finding that the nontreated cells lost their heat resistance when they were not exposed to the stress. Similarly, cells that had been adapted to spoil lemon lime Radler were still able to spoil this beer product after four doublings in yeast potato dextrose but had lost this ability after 13 doublings (Suiker et al., 2021). In our case, growth in the glucose-containing medium (in between the heat treatments) involved more than 13 doublings. In addition, preliminary experiments have shown a shift in allele distribution from heat treatment cycle 4 onwards (Suiker, 2021), which coincides with increased heat resistance, and thereby is also indicative of a genetic basis of acquired heat resistance.

The fact that non–heat-treated lines show less sporulation and less survival to heat treatment indicates a negative trade-off of this property. Conversely, *S. diastaticus* MB523 strain can rapidly acquire heat resistance of ascospores and vegetative cells by inbreeding. This may take place in breweries by exposure to sublethal cleaning and disinfection agents and by exposure to heat. Notably, *S. cerevisiae* strains have been isolated with D_{60}-values of ascospores of 11.2 and 22.5 min (Milani et al., 2015; Put & de Jong, 1982). Our results indicate that these strains, which had been isolated from beer and a soft drink, respectively, may further increase their heat resistance by inbreeding upon exposure to sublethal cleaning and disinfection agents and heat exposure.

Data availability statement

The data that supports the findings of this study are available in the supplementary material of this article.

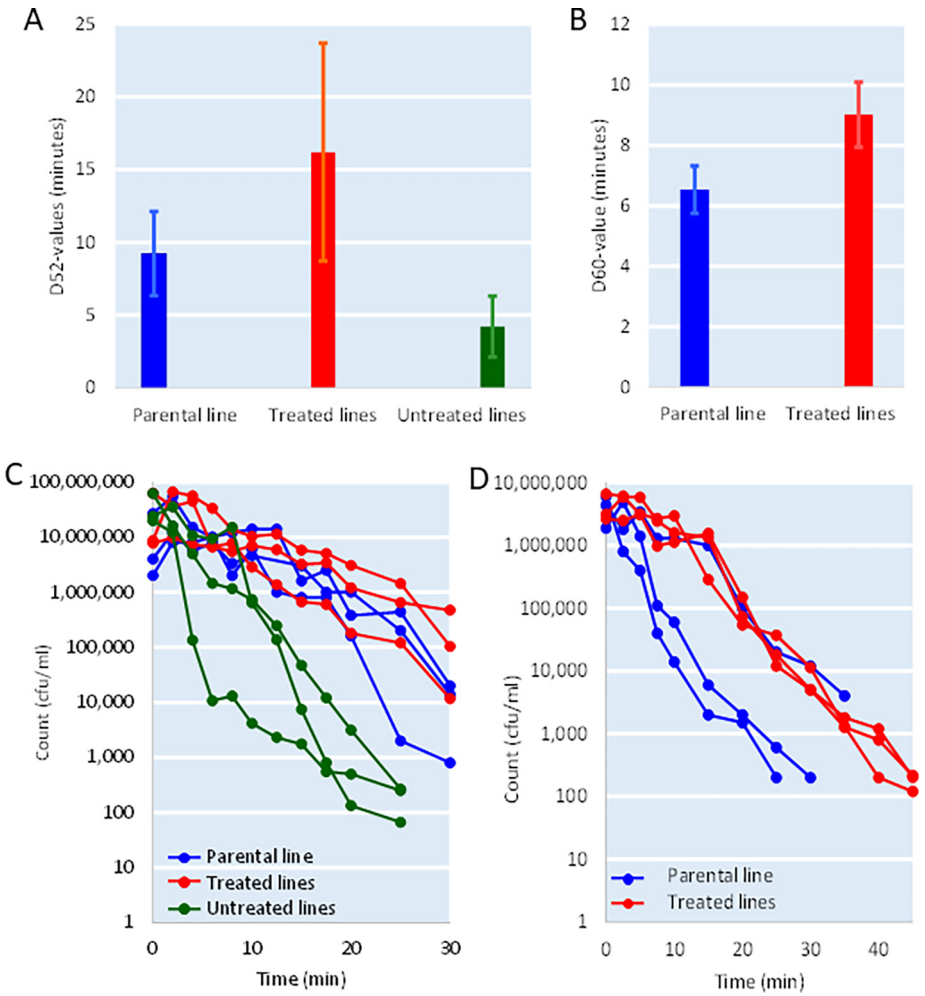

Figure 2. **A.** D_{52}-values for vegetative cells after 40 h growth in YPD for the parental MB 523 line, and the eight times treated and untreated cultures. The three treated lineages had higher D_{52}-values than the parental MB523, whereas the untreated lineages had lower D_{52}-values. Error bars represent the standard deviation of biological replicates. **B.** D_{60}-values for sporulated cultures of MB523 and the average of the three lineages that were heat treated eight times. The D_{60}-value increased from 6.5 to an average of 9.0 min (p value = 0.005 by two-tailed Student's t test). D_{60}-values were not determined for the nontreated cultures, since sporulation in these cultures was impaired. **C** and **D** show the inactivation curves that were used to calculate the D-values of panels **A** and **B**, respectively.

Declaration of Competing Interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

Acknowledgments

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Appendix A. Supplementary material

Supplementary data to this article can be found online at https://doi.org/10.1016/j.jfp.2022.100020.

References

Andrews, B. J., & Gilliland, R. B. (1952). Super-attenuation of beer: A study of three organisms capable of causing abnormal attenuations. *Journal of the Institute of Brewing, 58*, 189–196.

Bokulich, N. A., & Bamforth, C. W. (2013). The microbiology of malting and brewing. *Microbiology and Molecular Biology Reviews: MMBR, 77*, 157–172.

Hutzler, M., Riedl, R., Koob, J., & Jacob, F. (2012). Fermentation and spoilage yeasts and their relevance for the beverage industry – A review. *BrewingScience, 65*, 33–52.

Krogerus, K., Magalhães, F., Kuivanen, J., & Gibson, B. (2019). A deletion in the STA1 promoter determines maltotriose and starch utilization in STA1 + *Saccharomyces cerevisiae* strains. *Applied Microbiology and Biotechnology, 103*, 7597–7615.

Langmead, B., & Salzberg, S. L. (2012). Fast gapped-read alignment with Bowtie 2. *Nature Methods, 9*, 357–359.

Meier-Dörnberg, T., Jacob, F., Michel, M., & Hutzler, M. (2017). Incidence of *Saccharomyces cerevisiae* var. *diastaticus* in the beverage industry: Cases of contamination, 2008-2017. *MBAA TQ, 54*.

Milani, E. A., Gardner, R. C., & Silva, F. V. M. (2015). Thermal resistance of *Saccharomyces* yeast ascospores in beers. *International Journal of Food Microbiology, 206*, 75–80.

Pretorius, I. S., Lambrechts, M. G., Marmur, J., & Mattoon, J. R. (1991). The glucoamylase multigene family in *Saccharomyces cerevisiae* var. *diastaticus* : An overview. *Critical Reviews in Biochemistry and Molecular Biology, 26*, 53–76.

Put, H. M. C., & de Jong, J. (1982). Heat resistance studies of yeasts; vegetative cells versus ascospores: Erythromycin inhibition of sporulation in *Kluyveromyces* and *Saccharomyces* species. *The Journal of Applied Bacteriology, 53*, 73–79.

Suiker, I. M., & Wösten, H. A. B. (2022). Spoilage yeasts in beer and beer products. *Current Opinion in Food Science, 44* 100815.

Suiker, I. M. (2021). *Heterogeneity between and within strains of the beer spoilage yeast Saccharomyces cerevisiae subspecies diastaticus*. Utrecht University. PHD Thesis.

Suiker, I. M., Arkesteijn, G. J. A., Zeegers, P. J., & Wösten, H. A. B. (2021). Presence of Saccharomyces cerevisiae subsp. *diastaticus* in industry and nature and spoilage capacity of its vegetative cells and ascospores. *International Journal of Food Microbiology, 347*. 109173.

Tamaki, H. (1978). Genetic studies of ability to ferment starch in *Saccharomyces*: Gene polymorphism. *Molecular & General Genetics: MGG, 164*, 205–209.

Yamashita, I., & Fukui, S. (1984). Isolation of glucoamylase-non-producing mutants in the yeast *Saccharomyces diastaticus*. *Agricultural and Biological Chemistry, 48*, 131–135.

Yamashita, I., Suzuki, K., & Fukui, S. (1985). Nucleotide sequence of the extracellular glucoamylase gene *STA1* in the yeast *Saccharomyces diastaticus*. *Journal of Bacteriology, 161*, 567–573.