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Trehalose Biosynthesis Enhancement for Six Yeast Strains Under Pressurized Culture

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Abstract Six yeast strains of the commercial brewing yeasts CICC1391 and CICC1471, the commercial baker yeasts CICC1339 and CICC1447, and the commercial alcohol yeasts CICC1286 and CICC1291 have been cultured under 1.0 MPa of pressure with N_2 and CO_2 as pressure media. The concentration of intracellular trehalose and the activity of trehalose synthases complex have been measured. Also, the morphology changes of yeast cells have been observed by scanning electronic microscope. There was a positive correlation between the activity of trehalose synthase complex and the concentration of intracellular trehalose; and there was a negative correlation between the activity of trehalose synthase complex and the viability of yeast strains. Having been cultured for 3 h at high pressure of 1.0 MPa, the concentration of intracellular trehalose and the activity of trehalose synthases complex were improved by 50.1% to 116.4% and 45.2% to 219.1%, respectively, compared to those of atmospheric pressure culture. Under high pressure, many wrinkles appeared on the membrane surface of yeast cells. It has been found that yeasts are more sensitive to high pressure for having more and sharper wrinkles on their cell membranes.

Keywords Pressurized culture · Yeasts · Trehalose · Trehalose synthase complex

Introduction

Pressurized culture is a method in which microorganisms are cultured and propagated (proliferated) under pressurized condition in order to improve the fermentation efficiency. Hiroshi Kuriyama et al. [1] studied the effects of pCO_2 on yeast growth and metabolism under continuous fermentation. They found, under glucose supply limitation, an increase in pCO_2 from 44 to 195 kPa resulting in 25% decrease in cell concentration, 8% increase in ethanol concentration, and 50% decrease in glycerol concentration. Under

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oxygen supply limitation, similar dependency of ethanol and glycerol on pCO_2 was observed; however, no influence of pCO_2 on the cell yield was observed. The change in ethanol yield by pCO_2 appeared to be caused by the equilibrium shift of pyruvate dehydrogenase system.

In addition, the pressure, as a stress factor, which is the same as high temperature, refrigeration, dehydration, and high or low pH, can improve the content of intracellular stress metabolites such as trehalose, glutathione, and ergosterol in yeast cells during cultivation [2–9].

Trehalose is a stable disaccharide, which has some important protective effect on microorganisms or biomacromolecules and can protect organism's activity under some unfavorable conditions such as high temperature, dehydration, or refrigeration [5]. To keep the yeast's viability, relative high content of intracellular trehalose is required for the dry yeast products.

Some previous studies reported that improving the culture pressure can raise the intracellular content of trehalose, ergosterol, and glutathione of yeast cells [2, 6–8]. It is still unknown whether this phenomenon is universal for various yeast strains by different pressure media. Therefore, six kinds of yeast strains were selected to be cultured under 1.0-MPa pressure with N_2 or CO_2 as pressure media. The concentration of intracellular trehalose and the activity of trehalose synthase complex have been examined and the morphology change of six kinds of yeast cells has been observed by scanning electron microscopy (SEM).

Materials and Methods

High-Pressure Apparatus Configuration

A pressurizable reactor system has been constructed [10] in which the pressure can be measured and controlled. The volume of autoclave (made of stainless steel) is 300 ml; broth samples can be injected into it by syringe.

Strains and Culture Conditions

Six yeast strains, commercial brewing yeasts (*Saccharomyces cerevisiae*) CICC1391 and CICC1471, commercial baker yeasts (*S. cerevisiae*) CICC1339 and CICC1447, and commercial alcohol yeasts (*S. cerevisiae*) CICC1286 and CICC1291, were used as trial organisms.

Each strain was incubated in a 500-ml shake flask containing 100-ml YEPD medium under 30 °C and 120 rpm of rotating speed until steady state was reached (in general, about 20 h). Then, the fermentation broth was loaded into the pressured reactor (the initial cell density was $2\sim3\times10^8$ CFU/ml depending on different yeast strains); then, it was CO_2-cultured or N_2-cultured under 30 °C and 120-rpm stirring, while the control atmospheric fermentation was conducted under the same conditions.

A 10-ml sample was taken from the reactor with sampler every hour. The sampler was made of stainless steel with capacity of 10 ml. It was linked with reactor's outlet through a valve only when sampling took place. When the valve of both outlet and sampler were open, the 10-ml sample entered the sampler without decrease of reactor's pressure. Before the sampler was separated from the reactor, the valve of outlet and of sampler had been closed. Samples were released from sampler by opening the valve.

The viability was determined by counting the numbers of colony-forming units obtained by plating suitably diluted samples on YEPD media. The biomass concentration was examined by measuring the dry weight of the yeasts cell pellets produced in a certain volume of broth.

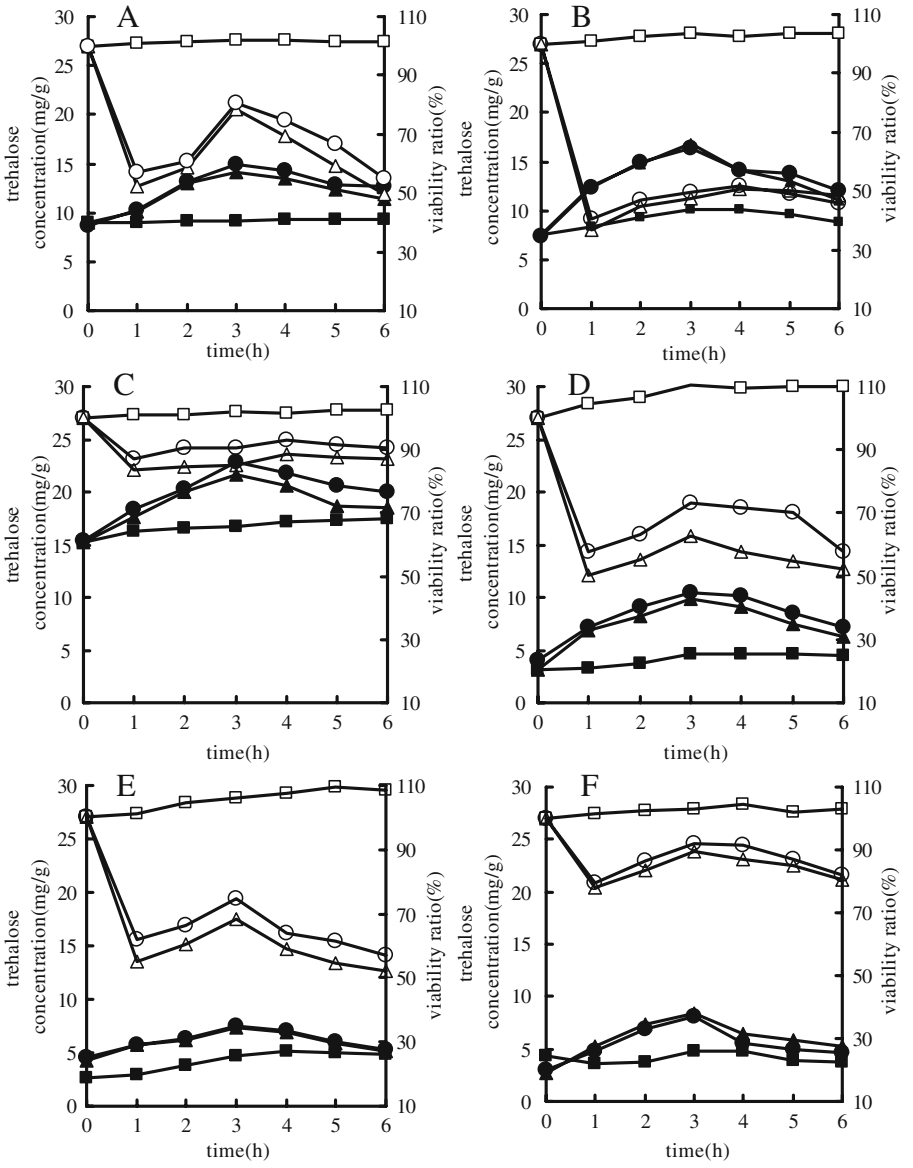

Fig. 1 Effect of time on trehalose concentration and viability ratio of six yeast strains under 1.0 and 0.1 MPa (30 °C, stirring rate 120 rpm). **a** CICC1391; **b** CICC1471; **c** CICC1339; **d** CICC1447; **e** CICC1286; **f** CICC1291; *empty squares*, *empty triangles*, and *empty circles* are viability ratios under 0.1 and 1.0 MPa CO_2 and 1.0 MPa N_2, respectively; *filled squares*, *filled triangles*, and *filled circles* are intracellular trehalose concentration under 0.1 and 1.0 MPa CO_2 and 1.0 MPa N_2, respectively; All measurements were done in triplicate

Trehalose Assay

Trehalose was extracted from cells (dry weight) with 0.5 mol/L trichloroacetic acid at 0 °C and determined by anthrone color method. All tests were done in triplicate.

Determination of the Activity of Trehalose Synthase

The cells were harvested by centrifugation at 4,000 rpm for 10 min and washed with distilled water two times, and then 0.5-g cells (wet weight) were mixed with 10-ml infiltrator which consists of 2.0% (*v*/*v*) toluene and 0.2% (*v*/*v*) EDTA solutions to obtain intracellular trehalose synthase complex without the cell's lysis. The ratio of cells to infiltrator was 0.5 g:10 ml and the mixture was treated for 0.5 h under 35 °C in water bath and then centrifuged at 4,000 rpm for 10 min. To assay the enzyme activity, 1.0% (*w*/*v*) glucose was added into the supernatant as enzyme substrate and reacted at 40 °C for 20 h.

One unit of enzyme activity was defined as the amount of enzyme which synthesizes 1 μg of trehalose in 1 h. All tests were done in triplicate.

Results and Discussion

Effect of Pressure on The Concentration of Intracellular Trehalose

The concentration changes of intracellular trehalose under 1.0 MPa of CO_2 and N_2 for all the trial strains are shown in Fig. 1. For the atmospheric cultivation, the concentrations of intracellular trehalose increased only a little with fermentation duration. However, the concentration of intracellular trehalose increased obviously under pressurized condition. It reached to the maximum at approximately 3 h and then decreased slightly; however, they were still higher than that of control samples. This indicated that yeast cells were induced to synthesize trehalose as a response to the pressure stress. But the viability of the yeast strain and the trehalose concentration showed negative correlation (Fig. 1). For the alcohol yeasts CICC1286 and CICC1447, the concentrations of trehalose were 7.3 and 9.9 mg/g, respectively after 3 h of pressurized treatment; however, the cell viabilities were 86.6% and 60.3% correspondingly. It indicated that the more sensitive the yeast cells were, the more amount of trehalose was synthesized to counteract the influence of pressure. The comparison of trehalose concentration and viability of every yeast strain when it was CO_2-pressurized cultivated for 3 h is shown in Table 1. The same rule was also seen for

Table 1 Trehalose concentrations and viabilities of six yeast strains after 3 h of CO_2 pressurized culture.

	Strains	Trehalose concentrations (mg/g)	Increase ratios of trehalose concentration[a] (%)	Viabilities (%)
Baker yeasts	1339	21.7	29.7	87.2
	1447	9.9	116.4	56.4
Brewing yeasts	1391	14.2	54.2	76.9
	1471	16.9	66.5	46.0
Alcohol yeasts	1286	8.4	80.0	64.2
	1291	7.3	50.1	86.6

[a] Increase ratio of trehalose=[(trehalose concentration under high pressure−trehalose concentration under atmosphere pressure)/trehalose concentration under atmosphere pressure]×100%

baker yeasts and brewing yeasts. This result proved that pressure, as one kind of stress factor, just like starvation, heat-shock, and high osmotic pressure, etc., can also improve the concentration of intracellular trehalose. But further investigation is required to understand the effect of pressure on the metabolic flux of yeast cells.

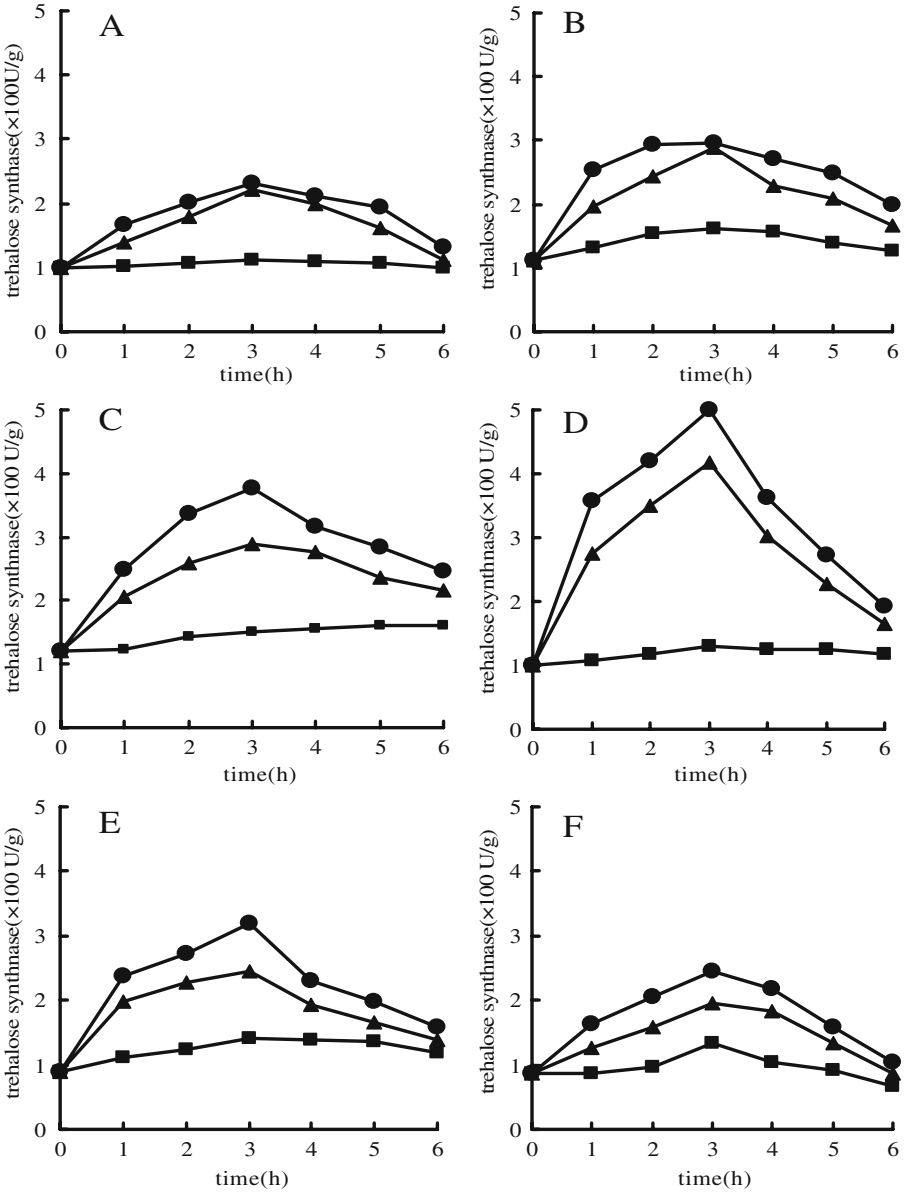

Fig. 2 Effect of time on trehalose synthase activity of six yeast strains under 1.0 and 0.1 MPa (30 °C, stirring rate 120 rpm.). **a**, **b**, **c**, **d**, **e**, and **f** are same as Fig. 1; *filled squares*, *filled triangles*, and *filled* are trehalose synthase activity under 0.1 and 1.0 MPa CO_2 and 1.0 MPa N_2, respectively; All measurements were done in triplicate

Effect of Pressure on the Activity of Trehalose Synthase

It has been found that the activity of trehalose synthase has the similar tendency to the change of trehalose concentration under pressure (Fig. 2). Activities of trehalose synthase of all the six yeast strains increased with pressurized time and came to the maximum and then decreased slightly. However, they were still higher than those of control samples under atmospheric pressure, though there were some differences among the six strains. This result demonstrated that the increase of intracellular trehalose concentrations was attributed to the increased activities of intracellular trehalose synthase. There existed a positive correlation between the trehalose concentration and the activity of trehalose synthase for each kind of yeast.

On the other hand, the pressure medium also influenced the activities of intracellular trehalose synthase. The activities of trehalose synthase complex of yeast cells by N_2 as pressure medium were higher than that by CO_2 as pressure medium. The reason responsible for this could be that the dissolved CO_2 in CO_2-pressurized culture deviated the pH of the ferment broth from the optimal pH 5.5.

In 1993, Welch et al. [11] studied the response of *Escherichia coli* to high-pressure stress and found that at 53-MPa pressure, *E. coli* was induced to produce 55 kinds of pressure-induced proteins, including 11 kinds of heat-shock proteins, four kinds of cold-shock proteins, and other alkali proteins of low molecular weight, while in 2007 Yongsheng Dong

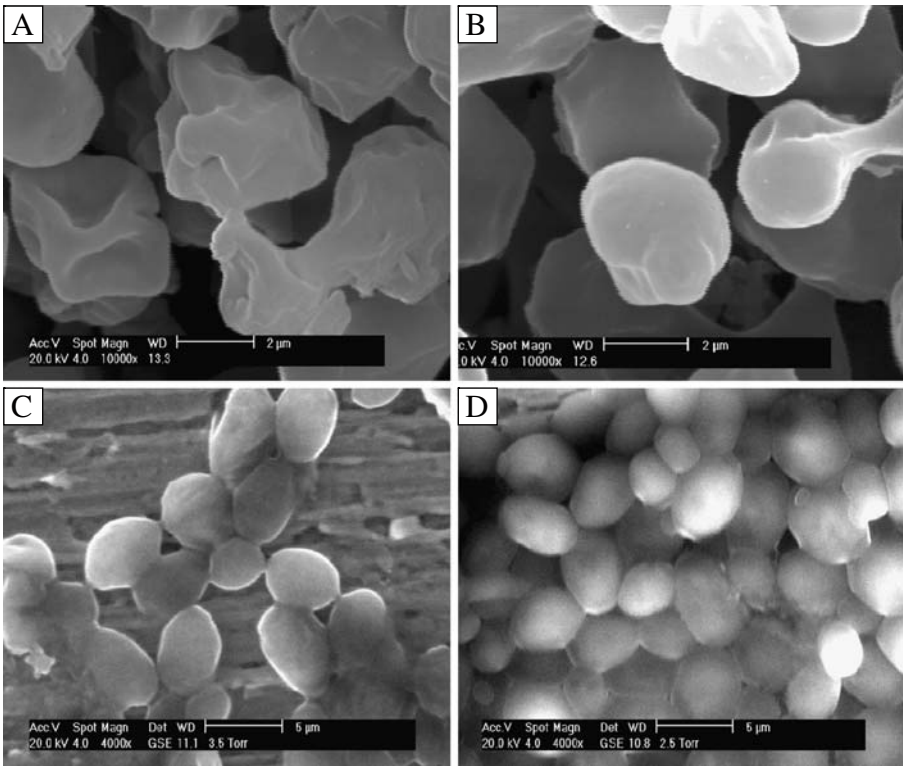

Fig. 3 SEM images of cell morphology of CICC1471 and CICC1339. **a** CICC1471 under 1.0 MPa N_2; **b** CICC1471 under 0.1 MPa N_2; **c** CICC1339 under 1.0 MPa N_2; **d** CICC1339 under 0.1 MPa N_2

et al. [12] studied the effect of 1.0-MPa high-purity air on the accumulation of trehalose and glutathione in yeast cells. Combined with the changes of trehalose concentration and the activity of intracellular trehalose synthase in our experiments, it can be deduced that pressure can regulate or alter the yeast's metabolism.

Effect of Pressure on the Morphology of Yeast Cells

The effects of pressure on the morphology of yeast cells were observed by SEM (Fig. 3). Wrinkles were observed under pressure condition. The shape change of microorganisms, which may be useful for altering the metabolism of yeast cells, is a form of adaptation to environment changes. The yeast strains which had sharper wrinkles were more sensitive to pressure.

The result was similar to the research of Isenschmid et al., Perrier Cornet et al. and Coelho M.A.Z. et al. [13–15] Generally, high pressure affecting yeast cell's metabolism mainly depends on four parameters: (1) pressure magnitude; (2) property of pressure medium; (3) anaerobic environment; and (4) properties of microbial strains. For example, CO_2 can produce the acidic and anaerobic environment. In our experiments, the improvement of the concentrations of intracellular trehalose and the changes of the morphology of the yeast cells may be due to the combined effect from all the aspects mentioned above.

Conclusions

In conclusion, the pressure, as a potential stress factor, can stimulate yeast cells to improve the activity of the intracellular trehalose synthase complex and further more to improve the concentration of intracellular trehalose to protect the yeast cells. Also, the morphology of cells has been altered. This technique deserves further study on the effects of pressure on the structure of cell membrane and cell wall, on the metabolic path, and on the metabolic flux, etc.

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References

1. Kuriyama, H., Mahakarnchanakul, W., Matsui, S., & Kobayashi, H. (1993). The effects of pCO2 on yeast growth and metabolism under continuous fermentation. *Biotechnology Letters*, *15*(2), 189–194. doi:10.1007/BF00133022.
2. Fujii, S., Iwahashi, H., Obuchi, K., Fujii, T., & Komatsu, Y. (1996). Characterization of a barotolerant mutant of the yeast *Saccharomyces cerevisiae*: importance of trehalose content and membrane fluidity. *FEMS Microbiology Letters*, *141*, 97–101. doi:10.1111/j.1574-6968.1996.tb08369.x.
3. Grob, M., & Kosmowsky, I. (1994). Response of bacteria and fungi to high pressure stress. *Electrophoresis*, *15*(8), 1559–1565.
4. Iwahashi, H., Obuchi, K., Fujii, S., & Komatsu, Y. (1997). Effect of temperature on the role of Hsp104 and trehalose in barotolerance of *Saccharomyces cerevisiae*. *FEBS Letters*, *416*, 1–5. doi:10.1016/S0014-5793(97)01141-1.
5. Lillie, S. M., & Pringle, J. R. (1980). Reserve carbohydrate metabolism in: Response to nutrient limitation. *Journal of Bacteriology*, *143*, 1384–1394.
6. Bartlett, D. H. (2002). Pressure effects of in vivo microbial processes. *Biochimica et Biophysica Acta*, *1595*, 367–381.

7. Bartlett, D. H., Kato, C., & Horikoshi, K. (1995). High pressure influences on gene and protein expression. *Research in Microbiology*, *146*, 697–706. doi:10.1016/0923-2508(96)81066-7.
8. Abe, F., Kato, C., & Horikoshi, K. (1999). Pressure-regulated metabolism in microorganisms. *Trends in Microbiology*, *7*(11), 447–453. doi:10.1016/S0966-842X(99)01608-X.
9. Çalik, G., Ünlütabak, F., & Özdamar, T. H. (2001). Product and by-product distributions in glutamic acid fermentation by *Brevibacterium flavum*: effects of the oxygen transfer. *Biochemical Engineering Journal*, *9*, 91–101. doi:10.1016/S1369-703X(01)00132-2.
10. Shiru, J., Aiyou, S., Baoquan, Z., & Hongjun, L. (2003). The effect of supercritical CO_2 on the growth of yeast. *China Biotechnology J*, *23*(7), 94–96.
11. Welch, T. J., Farewell, A., Neidhardt, F. C., & Bartlett, D. H. (1993). Stress response of *Escherichia coli* to elevated hydrostatic pressure. *Journal of Bacteriology*, *175*(22), 7170–7177.
12. Dong, Y., Yang, Q., Jia, S., & Qiao, C. (2007). Effect of high pressure on the accumulation of trehalose and glutathione in the *Saccharomyces cerevisiae* cells. *Biochemical Engineering Journal*, *37*, 226–230. doi:10.1016/j.bej.2007.04.004.
13. Coelho, M. A. Z., Belo, I., Pinheiro, R., Mota, M., Coutinho, J. A. P., & Ferreira, E. C. (2004). Effect of hyperbaric stress on yeast morphology: Study by automated image analysis. *Applied Microbiology and Biotechnology*, *66*, 318–324. doi:10.1007/s00253-004-1648-9.
14. Isenschmid, A., Marison, I. W., & von Stockar, U. (1995). The influence of pressure and temperature of compressed CO_2 on the survival of yeast cells. *Journal of Biotechnology*, *39*, 229–237. doi:10.1016/0168-1656(95)00018-L.
15. Perrier Cornet, J. M., Hayert, M., & Gervais, P. (1999). Yeast cell mortality related to a high-pressure shift: occurrence of cell membrane permeabilization. *Journal of Applied Microbiology*, *87*, 1–7. doi:10.1046/j.1365-2672.1999.00779.x.