

Chapter 6

Heat-resistant ascospores

Jan Dijksterhuis

Applied and Industrial Mycology, CBS Fungal Biodiversity Centre, Uppsalalaan 8, 3584 CT, Utrecht, The Netherlands

INTRODUCTION

Fungal spoilage is a cause of immense food losses during storage of crops and food products. In order to overcome food spoilage by fungi, mankind has developed an array of methods to make food, which can be regarded as a rich and complex medium, difficult to colonize. This is done, among others, by means of lowering of the water activity by addition of sugars, salt or after drying. In other cases, food products are kept under lowered temperatures. A third possibility is the killing of fungi present in the food matrix by a transient heat treatment (pasteurisation). Currently, novel techniques are introduced as high-pressure treatment or storage of a food product under a modified atmosphere. Although heat treatment is a reliable and historical method, a class of fungal organisms is able to survive prolonged periods of heat and these organisms cause a restricted, but consistent loss of food products. This is especially interesting with modern trends to use minimally processed food where the heat treatment is minimised in favour of the organoleptic properties of the product and vitamin content.

HEAT RESISTANCE IN FUNGI

Vegetative (yeast) cells usually have little heat resistance. When heated in beer (pH 4.0, 5% ethanol) *Saccharomyces carlsbergensis*, *Hansenula anomala* and *Pichia membranaefaciens* had D_{49} -values of less than 2 min. *S. willianus*, was

more heat-resistant with a D_{49} around 15 min (Tsang and Ingledew, 1982). For *Zygosaccharomyces bailii*, *Saccharomyces bisporus*, *H. anomala* and *Z. rouxii* decimal reduction was observed after heating for 1 min in a buffer (pH 5.5) supplied with 400 g/l sucrose, at 56, 55, 54 and 50°C, respectively (Baggerman and Samson, 1988). Yeast species that were heated in grapefruit serum showed similar characteristics (Parish, 1991). The presence of sucrose, sodium chloride or glycerol in the heating menstruum was somewhat protective during heating, while sorbate and benzoate usually reduced the heat resistance of the yeast cells (Beuchat, 1981; Agab and Collins, 1992). Living (and growing) fungal cells exhibit after a sudden increase in temperature of approximately 10 °C above the optimal growth temperature, a phenomenon called heat shock. Increases in temperature lead to a number of changes including protein denaturation, cell cycle arrest and changes in the fluidity of the membrane. One of the most deleterious factors in heat shocked cells is the disturbance (unfolding) of the protein structure that leads to the formation of aggregates of proteins that affect the functioning of the cell lethally (Riezman, 2004). Accumulation of compatible solutes (glycerol, trehalose, mannitol) that protect the cellular membrane and the proteins (Crowe *et al.*, 1984; Prestrelski *et al.*, 1993) is one of the answers of the cell to heat shock. In addition, heat shock proteins play an important role in cooperation with compatible solutes in yeasts (Elliott *et al.*, 1996), but these are scarcely studied in fungal survival structures. Recently, the genome of the

filamentous fungus *Aspergillus fumigatus* was sequenced (Nierman *et al.*, 2005) and 323 genes showed higher expression at 48°C compared to 37°C. The most strongly upregulated genes included three proteins related to compatible solute synthesis and degradation and nine heat shock proteins. This also illustrates the role of different factors during the heat-shock response.

Fungal survival structures as conidia, sclerotia, chlamydospores and ascospores can be regarded as more or less heat resistant when they are compared to actively growing fungal cells. They do not exhibit the metabolism of the vegetative cells, but prevail in a dormant state and often are encompassed by a thicker cell wall.

Conidia are non-motile asexual spores of many fungal species that are often dispersed by air and water and which exhibit some dormancy, namely that they germinate only when proper nutrients are present in the medium. Conidia of the fungal species *Aspergillus niger*, *Penicillium chrysogenum*, *Wallemia sebi*, *Eurotium rubrum* and *P. glabrum*, exhibit a decimal reduction in buffer in the range of 56 and 62°C (Baggerman and Samson, 1988) and *P. roqueforti*, *P. expansum*, *P. citrinum* and *A. flavus* have D-values between 3.5 and 230 min at 54-56°C (Bröker *et al.*, 1987a; Spicher, 1991). The age of the spores at the time of harvest as well as the composition of the growing and heating media influence the heat resistance. Furthermore, conidia could restore from a heat treatment while *P. expansum* showed recovery during a 3-day storage in aerated water at 23 °C after a heat treatment at 54 °C compared to conidia that were plated out directly (Baldy *et al.*, 1970). Numbers of colonies were 20-fold higher after this treatment, indicating that the situation after heating has an effect on the number of survivors.

Ascospores are widely formed sexual spores within the Ascomycetes with often a high survival capability. In an investigation of 20 yeast strains from soft drinks and fruit products, mainly *Saccharomyces cerevisiae*, *S. bailii* (now *Z. bailii*) and *S. chevalieri* strains, the D₆₀-values of ascospores were 25-350 times

higher than those of the corresponding vegetative cells (Put and de Jong, 1980). In a pH 4.5 buffer *S. cerevisiae*, *S. chevalieri* and *S. bailii* ascospores exhibited D₆₀-values of 22.5, 13 and 10 min (Put and de Jong, 1982).

In general, ascospores of filamentous fungi are more heat resistant than mycelia and conidia and more resistant than yeast ascospores. A number of observations suggest that thick-walled hyphal fragments and chlamydospores (*Paecilomyces variotii*, *Fusarium spp* and sclerotia (e.g. in *Eupenicillium*) can perform high thermo-resistance (Splittstoesser and King 1984). But by far the most heat-resistant fungal structures known to date are ascospores produced by some members of the genera *Byssochlamys*, *Neosartorya* and *Talaromyces*. Ascospores of these fungi are extra-ordinary heat resistant and show D₉₀ values of several minutes (Beuchat 1986). In fact, these spores belong to the most resilient eukaryotic structures observed hitherto. A decimal reduction time of 1.5-11 min is observed at 90° C among different species (Scholte *et al.*, 1991, Dijksterhuis and Samson, 2006). Ascospores of the fungus *Talaromyces macrosporus* are able to survive at 85 °C for 100 min, which makes these fungal spores as resilient as some bacterial spores (e.g. *Bacillus subtilis*).

One has to realise that heating of cells in a low moisture environment is much less effective. Even yeast cell show very high inactivation times at low humidities. When heated on aluminium foil at a relative humidity of 33-38%, *P. membranaefaciens* and *Rhodotorula rubra* cells showed little survival after 5 min at 110°C (Scott and Bernard, 1985). With D₁₁₀-values of 1.3, 1.8, 2.9 and 3.6 min, respectively, *Debaryomyces hansenii*, *Kloeckera apiculata*, *Lodderomyces elongisporus* and *H. anomala* cells were more resistant.

HEAT-RESISTANT FUNGI

Heat resistant fungi can survive pasteurising heat-treatments of especially high-acid food products (e.g. fruits, see Silva and Gibbs, 2004). Subsequent germination causes spoilage of

canned and pasteurised fruit products. Fungi that are associated with product recalls that cause a damage of millions of dollars in the fruit-juice branch are *Byssoschlamys nivea* (fulva), *Talaromyces flavus* (macrosporus), *Neosartorya fischeri*, *Eupenicillium brefeldianum* (as reviewed by Tournas 1994). Despite of several decades of research these fungi still represent problems in the food branche. Heat-resistant fungi are basically soil-borne fungi and fruits that develop in contact with soil (like strawberries and pineapple) are more prone to contamination. The fungus *Talaromyces flavus* is found to have a worldwide distribution and was isolated from soils samples from 16 different countries including Bermuda, Tasmania, Pakistan and Finland (Fravel and Adams, 1986).

The first description of the heat-resistant nature of these fungi in literature was the isolation of *Byssoschlamys nivea* from processed fruit by Olliver and Rendle in the 1930s (Olliver and Rendle 1934). In 1963, a heat-resistant *Aspergillus* (teleomorph, *Neosartorya fischeri*) was isolated from canned strawberries (Kavanagh *et al.* 1963) and later two heat-resistant *Penicillium*-like species were isolated from flash-pasteurised apple juice (van der Spuy *et al.* 1975) which became later known as *Talaromyces* (*flavus* and *macrosporus*). In addition, other fungal species are now identified as heat-resistant (e.g. *Talaromyces trachyspermus*, Enigl *et al.* 1993; *Talaromyces helicus* and *stipitatus*, Dijksterhuis and Samson, 2006; *Byssoschlamys spectabilis*, J. Houbraeken, unpublished results).

INACTIVATION OF HEAT-RESISTANT FUNGI

Methods

Heat resistance of cells is expressed by means of the D- and Z-values, as is well documented, but the exact measurement and calculation of these values is not so self-evident. Throughout different studies various attempts are made to heat ascospore solutions instantaneously, which is important for an accurate D-value acquisition. These attempts include small

sealed vessels that are plunged into water or oil baths (King and Whitehand, 1990), or a two phase slug flow heat exchanger (King, 1997) or spiral steel capillary tubes (Engel and Teuber, 1991). A simpler method is the addition of a small aliquots of ascospore suspension to a larger preheated volume of fluid. Dijksterhuis *et al.* (2002) used small diameter Teflon tubing for sampling of the heated solution without opening the water bath. The *D-value*, the decimal reduction time, is the time (usually in min) that is needed to inactivate 90% of the microorganisms at a given temperature. Ideally, a linear curve will be obtained when the number of surviving microorganisms, on a logarithmic scale, is plotted against time. However, many cases of non-linear death-rate kinetics are observed (Put and de Jong, 1982; Splittstoesser and King, 1984, King and Halbrook, 1987). King *et al.* (1979) have provided a mathematical method for these situations. They use the formula: $(\log N_0 - \log N_t)^\alpha = kt + C$, where α is a term that addresses the non-linearity of the death kinetics, and k equals a rate constant and $1/k$ is a measure for the D-value at the used temperature. Apart from non-linear survivor plots, tailing is a phenomenon where a small subpopulation of spores seems to be extra resistant to heat (Bayne and Michener, 1979, Casella *et al.* 1990). One cause of tailing can be that a small subpopulation escapes proper heating. Fujikawa and Itoh (1996) modelled the non-linear thermal inactivation of *A. niger* conidia at 60 °C that included a shoulder, an accelerated decline and a tail. A later report of Fujikawa *et al.* (2000) indicated that the conidia that adhered to the inner wall of test tubes were the cause of tailing in a test tube system. To compare heat-inactivation rates at different temperatures, a second parameter, the z-value, is used. It denotes the increase or decrease in temperature needed, respectively, to decrease or to increase the *D-value* by a factor of 10. For *A. niger* conidia for example, a D_{59} of 3.3 min with a z-value of 4.9°C implies a D_{54} of approximately 33 min (Baggerman and Samson, 1988).

Homogeneity of the ascospore suspension is important for heat-resistance measurements;

Tabel 1. Heat-resistance of ascospores at different temperatures and medium composition.

Fungal species	T	D-value	Medium	Reference
<i>Byssoschlamys fulva</i>	86°	13-14	Grape Juice	1
	90°	4-36*	Buffer pH 3.6 and 5.0, 16°Brix	2
		8	Tomato juice	3
<i>B. nivea</i>	85°	1,3-4,5	Buffer pH 3.5	4
		34,6	15° Brix Strawberry pulp	5
	88°	8-9 sec	Ringer solution	6
	90°	1,5	Tomato juice	3
<i>B. spectabilis</i>	85°	ca. 70	Buffer, pH 6,8	7
<i>Eurotium herbariorum</i>	70°	1,1 – 4,6	Grape Juice, 65°Brix	8
<i>Eupenicillium javanicum</i>	85°	3,7	15° Brix Strawberry pulp	5
<i>Monascus ruber</i>	80°	1,7 – 2,0 0,9 – 1,0	Buffers (pH 3,0 ; pH 7,0) In brine	9
<i>Neosartorya fischeri</i>	85°	13,2	Apple Juice	10
		10,1	Grape Juice	10
		10-60	In ACES-buffer, 10 mM, pH 6.8	11
		10,4	Buffer pH 7.0	10
		14,5	15° Brix Strawberry pulp	5
		15,1	15° Brix Apple Juice	12
		19,6 – 29,5	Dionized water, pineapple juice and concentrate	13
		35,3	Buffer pH 7.0	14
	88°	1,4	Apple Juice	15
		4,2-16,2	Heated fruit fillings	16
		12,4 – 17,0	Dionized water, pineapple juice and concentrate	13
	90°	4,4-6,6	Tomato Juice	3
	91°	<2	Heated fruit fillings	16
<i>N. pseudofischeri</i>	95°	20 sec		7
<i>Talaromyces flavus</i> (<i>macrosporus</i>)	85°	3,3	15° Brix Strawberry pulp	5
	85°	39	Buffer pH 5.0, glucose, 16°	17
		20-26	Buffer pH 5.0, glucose	18
	88°	7,8	Apple Juice	15
		7,1 – 22,3	Heated fruit fillings	16
	90°	2-8	Buffer pH 5.0, glucose	18
		6,2	Buffer pH 5.0, glucose	10
		6,0	Buffer pH 5.0, glucose. Slug flow heat exchanger	10
		2,7 – 4,1	Organic acids	19
		2,5 – 11,1	Sugar content (0-60° Brix)	19
		5,2 – 7,1	PH 3.6-6.6	19
<i>T. helicus</i>	70°	ca. 20	Heated fruit fillings	16
<i>T. macrosporus</i>	85°	30-100		20
<i>T. stipitatus</i>	72°	ca. 85	In ACES-buffer, 10 mM, pH 6.8	21
<i>T. trachyspermus</i>	85°	45 sec		20
<i>Xeromyces bisporus</i>	82.2°	2,3		17
				22

References: 1.Michener and King (1974) 2.Bayne and Michener (1979), * the time needed for a log3-reduction is given here. 3.Kotzekidou (1997) 4.Aragão (1989) 5.Casella *et al.* (1990). 6. Engel and Tueber (1991) 7. J. Houbaken, unpublished data. 8. Splittstoesser *et al.* (1989) 9. Panagou *et al.* (2002) 10. Conner and Beuchat (1987b) 11. J. Dijksterhuis, unpublished data, strain CBS 133.64 12.Gumerato (1995). 13. Tournas and Traxler (1994) 14. Rajashekhara *et al.* (1996) 15. Scott and Bernard (1987) 16. Beuchat (1986) 17. King (1997) 18. King and Halbrook (1987) 19. King and Whitehand (1990) 20. J. Eleveld, unpublished data. 21. Dijksterhuis and Teunissen (2004) 22. Pitt and Hocking (1982).

no other cell types may mask or deform the results obtained.

In some studies pre-treatments are done to kill other cell types in case of very heat-resistant cells (Reyns *et al.*, 2003). For instance *Byssoschlamys* (anamorph, *Peecilomyces*) forms ascospores, conidia, chlamydospores and hyphal fragments in one culture. Further, viable counts of suspensions will show deviations of thermal death kinetics when the cells of the population stick together. Asci (ascus; the structures that contain 8 ascospores and give The Ascomycetes their name) must be broken effectively to measure proper thermal death kinetics of single ascospores. Michener & King (1974) use a French press treatment for the particularly resistant asci of *Byssoschlamys*.

To enumerate the surviving colonies, in many studies, the stain Bengal Rose (typically 10 mg/L) is added to the agar medium. The stain causes some more distinct boundaries of the fungal colony (King and Halbrook, 1987) and in time a nice purple dot is formed beneath the centre of the colonies; these factors facilitate the counting of the colonies.

Conditions that affect heat-resistance

The majority of the studies on heat-resistant spores in the past is done with respect to factors that correlate with the extent of heat-resistance. A large part of these studies is summarised in Table 1 (Modified after Dijksterhuis and Samson, 2006). Heat-resistance varies with different external and endogenous factors.

External factors include the presence of sugar (Beuchat 1988a; King and Whitehand 1990; Splittstoesser and Splittstoesser 1977) in the heating medium, the influence of pH and the presence of organic acids. The presence of sugars invariably has a protective action on the survival of spores within experiments, while, between studies sometimes confusing values are seen. Several organic acids counteract heat resistance of ascospores, but only at low pH's (lower than 4). This is most prominent for fumaric acid (Beuchat 1988b; Conner and Beuchat 1987a; Splittstoesser and Splittstoesser 1977). Conner and Beuchat

(1987a) state that pH, type, molarity of the undissociated form of the organic acid act synergistically with heat to inactivate ascospores. Benzoic and sorbic acid had also clear effects on *T. flavus* and *N. fischeri* (Beuchat 1988b; Rajashekara *et al.* 1998). Tartaric and malic acid however correlated with a higher heat resistance of spores of *B. fulva*. The combination of different factors may lead to some puzzling variations in heat resistance observed in literature. For instance, *N. fischeri* is more heat resistant in apple juice compared to grape juice (Conner and Beuchat, 1987a) and both *B. fulva* and *N. fischeri* exhibit a markedly lower heat resistance in cranberry juice compared to grape, apple or tomato juice (Splittstoesser and Splittstoesser, 1977). *N. fischeri* in 0.1 M phosphate buffer (pH 7.0) exhibited a far higher heat resistance than in grape jellies with large amounts of cane sugar at pH 3.1-3.3 (Beuchat and Kuhn 1997). *B. nivea* and *fulva* and *N. fischeri* were approx. twice as heat resistant in tomato juice (pH of 4.2) as in phosphate buffer (pH 7.0, 0.1 M, Kotzekidou 1997).

Endogenous factors that influence heat resistance are the age of the culture for *N. fischeri*, *T. flavus* and *B. nivea* (Beuchat 1988a; Casella *et al.* 1990; Conner and Beuchat 1987b; Dijksterhuis and Teunissen, 2004) that positively correlates with the resilience of the spores, the composition of the medium and the growth temperature of the fungus (Conner and Beuchat 1987b; King and Whitehand 1990). The latter reported higher heat resistance of *T. macrosporus* when the fungus was grown on solid medium. *T. flavus* showed higher heat resistance when grown on oatmeal agar compared to malt extract agar. Finally, the individual isolates used showed variation in *B. nivea*, *T. macrosporus* and *flavus* and *N. fischeri* (Bayne & Michener 1979; Beuchat 1986; King and Whitehand, 1990). To our knowledge, the only study on the nature of heat resistance was done by Conner *et al.* (1987). They studied younger (11 days) and older (25 days) ascospores of *N. fischeri* with different heat resistance (D_{82} of approx. 23 and >60 min respectively). Ascospores showed changes in the inner cell wall region at the lateral ridge

during aging. They observed qualitative differences in extractable proteins, but did not see changes in fatty acid or lipid content. Older spores contained 2.8% (dry weight) of mannitol and 0.6% of trehalose which could not be measured inside 11 days old spores. Polyols and disaccharides may play an important role in heat protection. Heat resistance of ascospores not only increased with the age of the fungal culture, but also harvested and washed ascospores showed maturation in case of *T. macrosporus* (i.e. increase of heat resistance in time, Dijksterhuis and Teunissen, 2004) when stored at 30° C. This phenomenon did not occur at lower temperatures (10° C) suggesting a temperature dependent acquisition of resistance.

More recent reports on heat-resistant ascospores include the fungus *Monascus ruber* from brine containing thermally processed canned green olives (Panagou *et al.* 2002). The spores are moderately heat-resistant in a phosphate buffer (pH 7,0; $D_{75} = 7,1$ min) and not much higher in citrate buffer (pH 3,0, $D_{75} = 7,8$ min). In brine (pH 3,8, 5,6% NaCl) the D_{70} was somewhat increased, but the D_{75} markedly lowered (4,9 min). In brine with higher salt content (10,5% potassium chloride) the D_{70} is markedly higher and the D_{75} similar as in the buffer situations. The D_{80} is lower (0,9-1,0 min) in the salty environments than in buffer (1,7-2,0 min). Taken together these results show a complex behaviour of the spores with respect to the presence of salt in the bathing medium. At lower temperatures salt is correlated with an increase of heat resistance, but this effect inverses at higher temperatures. The authors suggest that potassium chloride might have a detrimental effect if damage occurs to the spores. While other heat-resistant fungi show initial activation of germination by heat, this behaviour is not reported in this study.

GERMINATION OF ASCOSPORES AFTER HEAT ACTIVATION

The phenomenon of dormancy

Dormancy can be regarded as a form of hypometabolism, which includes any rest

period or reversible interruption of the phenotypic development (Sussman and Halvorson, 1966). Exogenous dormancy (quiescence) includes delayed development due to physical or chemical conditions of the environment. Constitutive dormancy is a condition in which development is delayed as an innate property such as a barrier to the penetration of nutrients or water, a metabolic block, or as a result of the action of a self-inhibitory compound. There are clear indications that ascospores of the heat-resistant fungi described here exhibit constitutive dormancy and need a robust physical signal (as heat, but also high-pressure) for breaking of dormancy. In most of the studies done on heat-resistant fungi, activation of spores to germinate is observed where the number of viable counts after a short heat treatment is increased several log cycles (e.g. *Byssoschlamys nivea* (5 min, 75°C), Yates *et al.*, 1968; *Eurotium herbariorum* (15 min, 60°C), Splitstoesser, 1989; *Neurospora tetrasperma*, (5 min, 65°C), Lingappa & Sussman, 1959; *Talaromyces flavus*, Katan, 1975; *Neosartorya fisheri*, Beuchat, 1986, Gomez *et al.*, 1989; *T. macrosporus*, (7-10 min, 85° C, Dijksterhuis and Teunissen, 2004). The fungus *T. flavus* shows heat activation at 80 and 85°C and activation is followed by killing during prolonged treatment at the higher temperature (Beuchat, 1986). At lower temperatures activation fails and only low numbers of germinated spores are observed. Recent observations at our laboratory indicate that with *T. macrosporus* no activation was observed on Malt Extract Broth below 60 °C during a one-hour activation period (J. Dijksterhuis, unpublished results). When suspensions of *N. fisheri* were heated at different temperatures for 10 min a very steep activation (nearly 1000 times) occurred between 60 and 70° C (Gomez *et al.*, 1989). When the cells were dry-heated at relatively low humidities (50%) at 95 °C for 60 min this trajectory was starting at lower temperatures (50° C) and completed at 65° C. Both *T. flavus* and *N. fisheri* isolates show marked variations in the temperature and the extent of heat activation. (Katan, 1985; Splitstoesser *et al.*, 1993).

The speed of activation

Remarkably, the speed of activation increases with higher temperatures in the case of *T. flavus*, although *N. fisheri* exhibits constants rates of heat activation between 70-85°C (Beuchat, 1986; King and Halbrook, 1987). In our laboratory we observed that ascospores of *T. macrosporus* added to preheated buffer of pH 6,8 were fully activated within 2 min. Kikoku (2003) activated ascospores of *T. macrosporus* in a citrate-phosphate buffer (pH 6.5) within 100 s at 81° and 82.5°C. Shorter activation times were observed at higher temperatures, namely 60 s at 86.5 and 87°C, and 35 s at 91°C. From these data the author calculated rate constants of heat activation (expressed as k), which range from 1.2 to 4.1/min between 81° and 91°C. At 84 °C no difference in k was observed between pH 3.5 or 6.5 (2.9 and 2.8/ min respectively) and also in phosphate buffer (pH 6.6) the k value was 2.8/min. However in grape juice (5 °Brix) a very high k value was observed (7.7/min). Thus, the presence of the sugars or organic acids or some other compound in the fruit juice resulted in a very rapid activation at this temperature (100% in 20 s). The activation energy (E_a) of a certain reaction was calculated using the Arrhenius plot where $\ln(0.23303.k)$ is plotted against $1/T$. If activation is confined to a conformational or chemical change of one compound in the ascospore, for instance a receptor protein or structures inside the (plasma) membrane, the E_a reflects the energy needed to convert 1 mole of such a compound. Changes in proteinaceous compounds do need a different energy absorption than lipid compounds and then the E_a could give clues about the nature of activation. In case of systemic changes inside the ascospore, many different alterations absorb the energy delivered by the heat and the E_a calculated does not give much information. Recent findings suggest that heat activation includes the release of a protein, alterations of different fractions inside the cell wall and an increase in the permeability of the ascospore cell wall (J. Dijksterhuis, unpublished results). All these different processes will make a precise indication of the activation factor with the Arrhenius plot more difficult.

Dry heat treatments of ascospores

Apart from heat, also a drying treatment can result in activation, but differences seem to occur between species. For *N. fisheri* the dormant state can be broken by a drying treatment of 18h at 40 °C (Beuchat, 1992), but *T. flavus* ascospores did not show a release of dormancy. After a subsequent storage period under dry conditions ($a_w = 0.23$) up to 30 months or more, the *T. macrosporus* ascospores did not need heat activation anymore and germinated after wetting. Remarkably, in case of storage in blueberry, grape or strawberry fruit powders, full activation was obtained within 8 months of storage, after 20 months in pineapple powder, but only partial activation after 30 months in apple powders. As stated earlier, heating at 50% r.h. (dry heat treatment) at 95° C (for 30 or 60 min) activated *N. fisheri* ascospores, but the temperature of the wetting or recovery buffer was crucial for the viable count obtained. (Gomez *et al.*, 1989, 1993). The latter could hint into a de-activation phenomenon where ascospores are reversibly brought back into the dormant state. It could also hint into the direction of imbibitional damage to the cells where rewetting in a cold fluid may cause the occurrence of different phases in the membrane at the same time resulting in leakage and cell damage (van Bilsen *et al.*, 1994). Glycerol had a protective action on the ascospores after drying during recovery in ice water, which indeed suggests that these resilient ascospores undergo imbibitional damage. Surprisingly, water vapour alleviated the detrimental effect of temperature on recovery.

Later research of the same group (Gomez *et al.*, 1994) indicated that the ascospores were more heat resistant as the relative humidity had decreased up to four orders in magnitude compared to the resistance during wet heat.

Recovery in hot buffer (80 ° C) was higher than in cold buffer (0° C) only in ascospores that were heated at 30 and 40% RH and subsequently vapour treated, but this "recovery" was interpreted as a result of heat activation

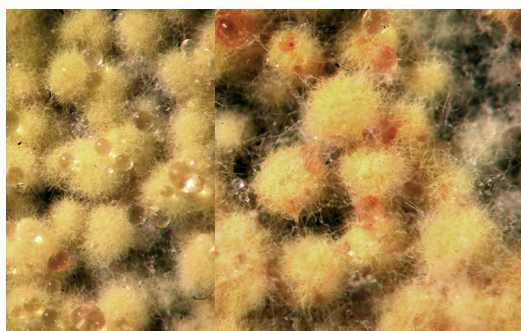


Figure 1. Formation of fruit bodies of the fungus *Talaromyces macrosporus*; numerous yellow coloured ascomata (fruiting bodies) can be observed after 7 (left) and 14 days (right) after inoculation of the fungus.

Can spores be de-activated?

T. macrosporus is a suitable model system for the study of heat-resistant ascospore biology. The fungus has a worldwide distribution, is a homothallic fungus (forms sexual fruit bodies without the need of different mating types) on oatmeal agar at 30 °C (Figure 1). Ascomata and ascospores can be harvested by a simple procedure to a dense homogenous suspension. The fungus also produces Penicillia with elongated phialides and conidia (Figure 2). Ascospores do not germinate when left in malt extract broth for prolonged times. As stated above, a 7-10 min treatment at 85 °C, however, results in activation and synchronisation of germination of the majority of the cells. HPLC studies showed that these cells contain very high concentrations of trehalose, up to 15-20% of the wet cell weight (that is 24-32% of the dry weight, Dijksterhuis *et al.*, 2002). The low water content of the spores (38%) introduces a very high viscosity inside the spores which is recently measured by means of EPR (Electron Paramagnetic Resonance) studies and cryo-electron microscopy (J. Dijksterhuis *et al.*, in press). Furthermore these cells are encompassed by a very thick ornamented cell wall (Figure 3) that is covered with numerous spikes (Figure 4).

A sudden lowering of the temperature or a reduction of the water content might introduce a glass transition situation inside the cell. The glassy state is an amorphous phase characterized by very low movement speeds of

the molecules inside the cytoplasm. This state inside the cell can be considered as a "biological glass", a term introduced by Buitink (2000). Thoroughly dried glasses, especially those containing trehalose are characterised by a high melting temperature (Wolkers *et al.*, 1996).

Could activated ascospores resume dormancy again when the cytoplasm is brought into a glassy state, for instance, by plunging of the cells in liquid nitrogen (a sudden lowering of the temperature) or controlled drying below 3% water? We observed that heat activated spores when plunged in nitrogen or kept at -20 °C directly germinated upon introduction into conducive conditions (Dijksterhuis and Samson, 2006). Further, ascospores were dried according to the procedures used at the CBS, and ascospores remained dormant after drying and could be effectively activated by a heat treatment after resuspension in buffer.

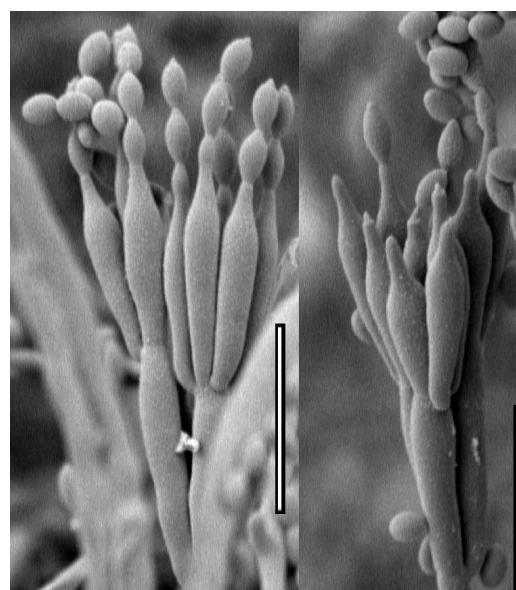


Figure 2. Two examples of conidiophores formed by *T. macrosporus* as viewed by means of cryo-SEM. Not the elongated phialides (arrow) and conidia (arrowhead).

Activated spores however, germinated directly upon rewetting, but these cells showed similar tolerance to the drying treatment as dormant cells. These cells were also very good able to

survive another heat treatment and no additional increase or decrease of cell numbers occurred compared with undried activated cells.

This indicated that these cells had retained their activated state, but still showed heat

resistance. Both drying tolerance and heat resistance had decreased markedly after keeping the cells at 2h at 30°C. These combined observations suggest that important phase transitions of the inner cell may not change the status of activation.

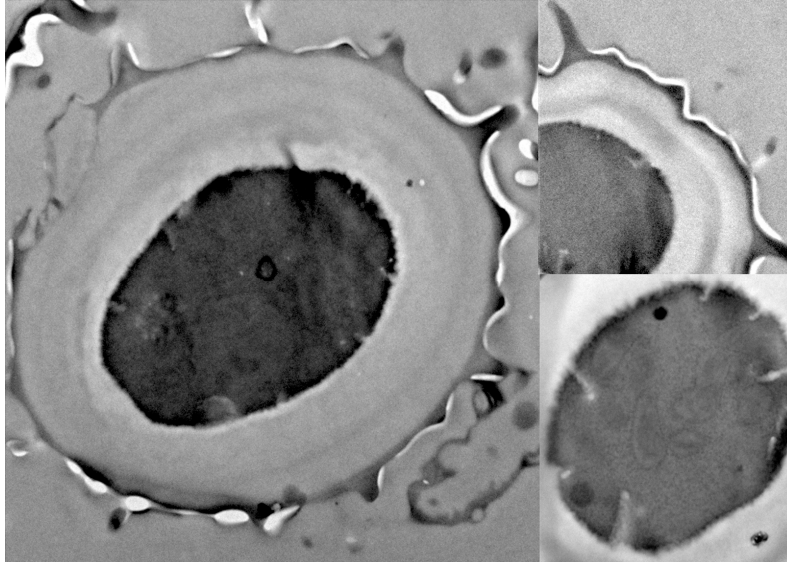


Figure 3. The ascospores of *T. macrosporus* as observed by means of transmission electron microscopy. It clearly illustrates the very thick outer cell wall of the spore which contains different layers and ornamentations (top right) and deep indentations of the cell wall into the cell (lower right). Photograph made by Kenneth van Driel, CBS, Utrecht.

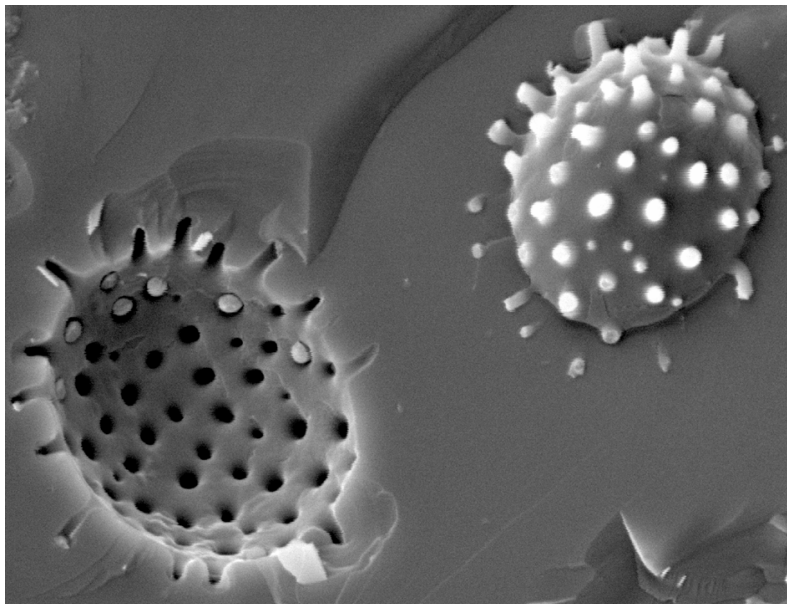


Figure 4. The ornamentations are clearly visible on this freeze break cryo- scanning electron micrograph. Markedly, much ornamentation did not break during the quick removal of the cell during breaking (see arrow). Photograph made in collaboration of Jaap Nijse, Plant Physiology, Wageningen.

An ecological function for heat resistance ?

The ecological function of heat activation is unknown, but could be related to survival of fungal cells after fires followed by a rapid colonisation of soils. *Talaromyces* is a fungal genus worldwide isolated from soil (Fravel and Adams, 1986) and postfire ascomycetes are described before as *Daldinia loculata*, a fungus that produces fruit bodies on trees killed by fire (Johannesson *et al.*, 2000). Remarkably, this fungus produces ascospores that germinate through a stage including a sudden opening of the thick outer cell wall, which enables the germ tube to grow out. (Beckett, 1976a,b), which is similar as the phenomenon of prosilition in case of *T. macrosporus* (see below).

Indeed, activated germinating spores could quickly fill empty ecological niches that are formed as a result of the fire. Fire related germination is well known with plant seeds (e.g. *Eucalyptus*), but attempts at our laboratory to activate *T. macrosporus* with smoke extracts, as is observed in case of plant seeds (Flematti *et al.*, 2004) failed. It is also possible that heat activates spores because it result in changes in the cell that are also addressed by the still hidden activation signal in nature.

A last possibility might be that the dormancy of these ascospores works as a clock to enable the spores to survive long periods and henceforth these spores can be regarded as "dispersed" in time.

BREAKING THE DORMANCY BY ULTRA-HIGH PRESSURE

Ascospores can be activated by a novel non-thermal activation method

Heat treatment of food products is a classical and very important method for preservation, but it also influences firmness, taste and vitamin content. Modern consumers tend to prefer "fresh" food and therefore shorter treatments at lower temperatures gain popularity. In addition, a new generation of non-thermal preservation techniques is under development (Barbosa-Cànovas *et al.*, 1998; Smelt 1998). One of these alternative methods is the application of high-pressure that results

in death of micro-organisms, but structure and vitamin content of the food stay relatively unchanged.

Vegetative (growing) microbial cells are sensitive to high-pressure treatments at 200-400 MPa (0,1 MPa = 1 bar, Barbosa-Cànovas *et al.*, 1998). Fungal and bacterial spores are inactivated at much higher pressures (Maggi *et al.*, 1994; Butz *et al.*, 1996; Wuytack *et al.*, 1998) and ascospores of *B. nivea* are inactivated above 600 Mpa combined with high temperatures (60 °C, Butz *et al.*, 1998), but these treatments were more effective when the treatments were repeated shortly after each other (designated as oscillatory treatments, Palou *et al.*, 1998). Since pressurization is already commercially used to pasteurize fruit juices and other products, pressure-resistance of these fungi is of relevance for the food industry.

Two independent recent studies showed that ascospores of *T. macrosporus* were activated by high-pressure treatments (Reyns *et al.*, 2003; Dijksterhuis and Teunissen, 2004). Both studies showed that even a very short treatment at high pressure caused maximal activation. Dijksterhuis and Teunissen (2004) observed full dormancy after 200 MPa treatments and activation of part of the spores (up to 7% of the cells) between 400 and 800 MPa, while Reyns *et al.* (2003) observed partial activation at 200 MPa and full activation at 600 MPa following 15 seconds of treatment. The following aspects seem important for this difference. Firstly, the cultivation of the fungus was different including temperature, medium and harvesting time of the ascospores. Dijksterhuis and Teunissen (2004) report that the age of the fungal culture during harvesting of the ascospores correlated with the heat resistance and that considerable increase of heat resistance occurred between 20 and 40 days of culturing.

So, the combined results of the papers hint in the direction that the extent of maturity of the ascospores may also influence the ability of the cells to remain dormant (see Dijksterhuis and Samson, 2006). Further, Reyns *et al.* (2003) had a pretreatment of the ascospore suspension for 20 min at 65 C to kill the vegetative cells. In

a buffer at a pH 6,8, this treatment will not activate ascospores, but 70° C gives a strong increase of germination (J. Dijksterhuis, unpublished results). At lower pH (3.0), Reyns *et al.* (2003) observed that near complete activation occurs after the heat treatment at 65° C. At our laboratory we observed activation at even lower temperatures in low pH solutions (J. Dijksterhuis unpublished results). Thirdly, the actual high-pressure treatments are done in a buffer (Dijksterhuis and Teunissen, 2004, 10 mM ACES, pH 6,8) or in distilled water (Reyns *et al.*, 2003). It is known that these treatments lower the acidity of the medium temporarily, which will be less extensive in a buffer and also this could have an effect on activation. Dijksterhuis and Teunissen (2004) performed cryo-electron microscopy on the spores and observed changes of the cell wall after short treatments at 600 MPa, where Reyns *et al.* (2003) illustrated that treated spores collapsed after air drying, while untreated spore maintained their shape. These observations indicate that structural changes occur in the cell wall and that these have a direct influence of the process of activation.

All these factors learn that activation of the ascospores of *T. macrosporus* is influenced by many different cues and that profound knowledge will be needed of activation and germination in order to tackle the growing problem of heat-resistant fungi in food products.

The relation between different treatments

Ascospores of *Eurotium herbariorum* (also sometimes designated as *E. repens*) are mildly heat resistant (Splittstoesser *et al.*, 1989). Eicher and Ludwig (2002) showed that 8% of these spores were activated from dormancy after 60 min at 200 MPa (and not above or below these pressure values, H. Ludwig, personal communication) and that 50% germinated after 8 min at 60° C. After 18 hours approx. 15% of the cells showed signs of germination at room temperature. Ascospores that were heat activated (15 min, 60°C) were more sensitive to a subsequent high pressure treatment at 500 MPa and the effect was much stronger when the heat treatment was applied immediately

after pressurization. The spores recovered 10-fold during storage at 20°C in an isotonic salt solution; a phenomenon designated by the authors as “re-stabilisation”. When pressurisation was first (500 MPa, 30 min), heat activation did not enhance germination, but slightly reduced viability. A pause between these treatments reduced the counts further.

These relations between treatments indicate that germination and inactivation are complex phenomena ruled by many different parameters. In case of *T. macrosporus*, full heat activation occurred after short treatments at 800 MPa (Dijksterhuis and Teunissen, 2004). However, the experiments of Reyns *et al.* (2003) show clear heat sensitivity of ascospores for the activation heat treatment (30 min, 80°C) after all pressure treatments at 600 MPa and 700 MPa.

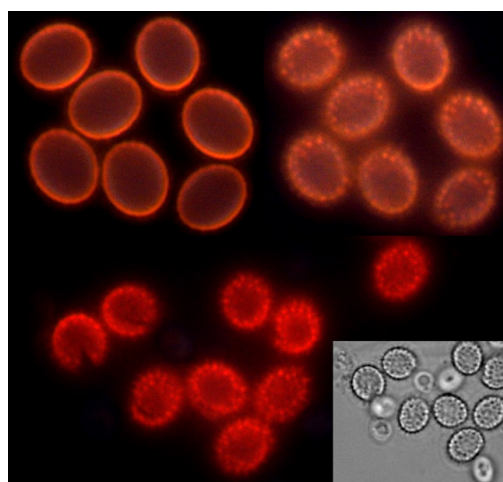


Figure 5. Ascospores of *T. macrosporus* exhibit a strong autofluorescence in a broad range of excitation wave-lengths. Top panel show two focal planes through the spores, illustrating the spikes on the surface of the cell wall. The lower panel show prosilited ascospores with ruptured cell walls where ejection has taken place (for an image with transmitted light, see the inset).

HEAT-RESISTANT ASCOSPORES OF
TALAROMYCES MACROSPORUS

Heat activation is as earlier stated a process that includes different mechanisms including a change in permeability in the ascospore cell wall (J. Dijksterhuis *et al.*, in preparation) and a possible influx of water into the inner cell. Upon heat treatment, the disaccharide trehalose is degraded by a high trehalase activity and the product of hydrolysis, glucose, is accumulated inside the spore, but resides inside the cell for only a very short time (Dijksterhuis *et al.*, 2002). Then it is released into the bathing medium till no glucose could be detected inside the spore after breaking of the cells. These observations, indicate a massive release of glucose from the germinating cell. It is not clear if glucose transporters are involved in this process, the spores show only very low respiration during these stages of germination (J. Dijksterhuis and D. Molenaar, unpublished results).

After 150 min or more the inner cell jumps through the outer cell wall, which becomes ruptured. The emptied outer cell wall remains attached to the protoplast which is encompassed by the inner layer of the ascospore cell wall by means of a third, fibrillar layer (Dijksterhuis *et al.*, in press). This process is very sudden, it only takes a second or less, and is termed prosilition (Lat: *prosilire*, to jump out). After this remarkable phenomenon, the respiration of the cells increases strongly and isotrophic growth (swelling) is followed by polarised growth (germ tube formation). The time needed from heat activation to germ tube formation is approx. 7 hours following the heat treatment. Figure 5 shows prosilited and unprosilited cells by means of the fluorescence microscope.

Recently, prosilition was also confirmed in other species of *Talaromyces* namely *T. stipitatus*, *T. helicus* and *T. bacillosporus*. However, ascospores of *Neosartorya* species seem to germinate by a slow separation of the two shell-like ornamented halves and subsequent formation of a germ tube. Apparently, the two genera show different modes of ascospore germination.

HEAT-RESISTANT ASCOSPORES OF
*NEUROSPORA***Earlier studies on *Neurospora tetrasperma***

One of the most important model systems in fungal biology is *Neurospora crassa* and the very related *N. tetrasperma* forms ascospores that survive temperatures at and above 60°C for long times (> 1 h) and these ascospores do need a "heat flash" (65 °C, 5 min) for activation of germination (Lingappa and Sussman, 1959). These ascospores are not relevant in the food context, but research at these spores is done in the past and summarised here. After heat activation the spores can be best grown at 27 °C. During this incubation the heat resistance of the spores decreases. The latter is also the case when the exospore (the thin outer layer of the spore cell wall, which is actively shed during germination) is removed by using Clorox, but it was not clear if the compound itself did have side-effects (Lingappa and Sussman, 1959). Remarkably, chemical compounds including furfural and phenetyl-alcohol were able to break the dormancy of these spores (Sussman *et al.*, 1959; Eilers and Sussman, 1970). It was observed that furfural was taken up by the ascospores and that the compound bound to the cell wall of the spores. In 1976 Sussman hypothesized that these compounds may act by causing an alteration in lipid moieties of the spore and thereby break dormancy.

Upon activation trehalose inside the ascospores is rapidly degraded, which was at that time interpreted as carbon storage compounds usage for energy metabolism and biosynthesis. Hecker and Sussman (1973) suggested that the trehalase needed for this degradation was associated with the inner cell wall of the ascospore which was based on immunofluorescence studies (were the cytoplasm had a strong autofluorescence) and the observation that trehalase was stabilised, that means protected from drying followed by a heat treatment, by cell wall preparations of the spores (and also by a fraction of it). Belmans *et al.* (1983) observed that the activation temperature of *N. tetrasperma* ascospores shifted upwards with 4K/100 MPa

(1000 bar) during high-pressure treatment. They argued that this is typical for proteins and not for lipids, which have melting characteristics from 20 K/100 MPa and concluded that the activation trigger in these spores is transduced by a protein conformational change.

Later studies

During sexual development in *N. crassa* (perithecium development and also ascospore germination) the amount and also composition of the fatty acid population in cultures changes (Goodrich-Tanrikulu *et al.*, 1998). In developing asci oleate (18:1) becomes the predominant fatty acid, while vegetative tissue contains mostly linoleate (18:2). The amount of polar lipids inside ascospores before heat activation was 8 pg/spore on a total fatty acid content of 44 pg/spore. In the first 6 hours after activation, the amount of linoleate increases markedly in the polar lipids. The content of triacylglycerol inside spores increases from 60% of the total to a maximum of 80-90% between 3 and 12 h after activation (after 4-6 the first hyphal tips appear from the spores in *N. tetrasperma*). Upon activation, ascospores show a biphasic increase in respiration. The first phase lasts for 90 min and is independent of (mitochondrial and cytoplasmic) protein-synthesis. Radioactive methionine is incorporated into the spores only after 90 min when the first signs of germ tube formation occur (Hill *et al.*, 1992) and the second stage of increase in respiration starts. However, it is possible that the spores do not take up the labelled compound while the cell wall is not permeable. Isolated mRNA was hybridised with cDNA for mitochondrial ATPase. In dormant cells some transcripts were present, but had disappeared at 30-90 min after activation. Then transcription obviously started as judged by reappearance of mRNA. In another study, germinating ascospores were exposed to a heat shock. Only cells reacted with accumulation of hsp30 after 480 min, cells at 90-300 min did not react (Plesofsky-Vig *et al.*, 1992).

All these data suggest that ascospores germinate through an initial stage characterised by lower respiration and

biosynthesis of proteins and nucleic acids. Recent studies on *T. macrosporus* ascospores show that also these spores exhibit such a stage, which is also characterised by high internal viscosity of the cytoplasm, which drops suddenly after prosilition (Dijksterhuis *et al.*, in press). Obviously, the very stress resistant ascospore needs time to renormalize to a vegetative cell as the cell constituents become "less protected".

FUTURE DEVELOPMENTS

Heat-resistant ascospores remain a concern in food industry due to the tendency for minimal processing of food products. More recently, these fungi are also reported from raw pectin and dairy products, which may mean that their occurrence is increasing. More research has to be done on the precise mechanism of germination to find alternative methods to prevent spoilage with these fungi. Apart from this, these unique resistant eukaryotic cells may give new insights in unique ways of protection of biological compounds.

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