ACTIVATION OF ASCOSPORES BY NOVEL FOOD PRESERVATION TECHNIQUES

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1. INTRODUCTION

Most fungal survival structures can be regarded as heat resistant to some extent: sclerotia, conidia and ascospores can survive temperatures between 55 and 95°C. *Byssochlamys*, *Neosartorya* and *Talaromyces* are the most well known heat-resistant fungal genera. Ascospores of these fungi are the most resilient eukaryotic structures currently known. A decimal reduction time of 1.5-11 min at 90°C has been reported for some species (Scholte et al., 2004). Recently, Panagou et al. (2002) reported moderate heat resistance (D_{75} 4.9-7.8 min) in ascospores of *Monascus ruber* isolated from brine of a commercial thermally processed can of green olives. There appeared to be a complex interaction between pH and salt content of the heating menstruum and decimal reduction time for this fungus.

During recent work undertaken in our laboratory, moderate heat resistance has been observed for *Talaromyces stipitatus* and *T. helicus* (J. Dijksterhuis, unpublished results), and studies involving ascospores of *Byssochlamys spectabilis* have resulted in a calculated decimal reduction time at 85°C of 47-75 min (J. Houbraken, unpublished results). Table 1 shows a compilation of heat resistance data for many known heat resistant species and their D values in various heating menstrua (modified from Scholte et al., 2004).

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

Fungal species	D-value (min)	Medium	Reference
Byssochlamys fulva	86°C, 13-14	Grape Juice	Michener and King (1974)
	90°C, 4-36 (3 $\log_{10}$ reduction)	Buffer pH 3.6 and 5.0, 16 °Brix	Bayne and Michener (1979)
	90°C, 8.1	Tomato juice	Kotzekidou (1997)
Byssochlamys nivea	85°C, 1.3-4.5	Buffer pH 3.5	Casella et al. (1990)
	88°C, 8-9 sec	Ringer solution	Engel and Teuber (1991)
	90°C, 1.5	Tomato juice	Kotzekidou (1997)
Byssochlamys spectabilis	85°C, 47-75	Buffer, pH 6.8	Authors' unpublished data
Eurotium herbariorum	70°C, 1.1-4.6	Grape Juice, 65 °Brix	Splittstoesser et al. (1989)
Eurotium chevalieri	70°C, 17.2	Plum extract (pH 3.8,	Pitt and Christian (1970)
	80°C, 3.3	20 °Brix, a_w 0.98)	
Monascus ruber	80°C, 1.7-2.0	Buffers (pH 3,0 ; pH 7,0)	Panagou et al. (2002)
	80°C, 0.9-1.0	Brine	
Neosartorya fischeri	85°C, 13.2	Apple Juice	Conner and Beuchat (1987b)
	85°C, 10.1	Grape Juice	Conner and Beuchat (1987b)
	85°C, 10-60	In ACES-buffer, 10 mM, pH 6.8	Authors' unpublished data CBS 133.64
	85°C, 10.4	Buffer pH 7.0	Conner and Beuchat (1987b)
	85°C, 35.3	Buffer pH 7.0	Rajashekhara et el. (1996)
	88°C, 1.4	Apple Juice	Scott and Bernard (1987)
	88°C, 4.2-16.2	Heated fruit fillings	Beuchat (1986)
	90°C, 4.4-6.6	Tomato Juice	Kotzekidou (1997)
	91°C, < 2	Heated fruit fillings	Beuchat (1986)
Neosartorya pseudofischeri	95°C, 20 sec		Authors' unpublished data
Talaromyces flavus (macrosporus)	85°C, 39	Buffer pH 5.0, glucose, 16 °Brix	King (1997)
	85°C, 20-26	Buffer pH 5.0, glucose	King and Halbrook (1987)

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

Fungal species	D-value (min)	Medium	Reference
	85°C, 30-100	ACES-buffer, 10 mM, pH 6.8	Dijksterhuis and Teunissen (2004)
	88°C, 7.8	Apple Juice	Scott and Bernard (1987)
	88°C, 7.1-22.3	Heated fruit fillings	Beuchat (1986)
	90°C, 2-8	Buffer pH 5.0, glucose	King and Halbrook (1987)
	90°C, 6.2	Buffer pH 5.0, glucose	King (1997)
	90°C, 6.0	Buffer pH 5.0, glucose Slug flow heat exchanger	King (1997)
	90°C, 2.7-4.1	Organic acids	King and Whitehand (1990)
Talaromyces flavus	90°C, 2.5-11.1	Sugar 0-60 °Brix)	King and Whitehand (1990)
	90°C, 5.2-7.1	pH 3.6-6.6	King and Whitehand (1990)
	91°C, 2.1-11.7	Heated fruit fillings	Beuchat (1986)
T. trachyspermus	85°C, 45 sec		Authors' unpublished data
T. helicus	70°C, approx. 20		"
T. stipitatus	72°C, approx. 85		
Xeromyces bisporus	82°C, 2.3		Pitt and Hocking (1982)

2. HEAT-RESISTANT ASCOSPORES OF *TALAROMYCES MACROSPORUS*

Germination of heat-resistant ascospores of different species is activated and also synchronised by a heat treatment (see for a survey, Dijksterhuis and Samson, 2002). Germination of these spores has been studied in detail by Dijksterhuis et al. (2002) in *Talaromyces macrosporus*. This fungus is a candidate to become a model system for research on heat-resistant ascospores. On oatmeal agar at 30°C *T. macrosporus* forms numerous yellow ascoma (Figure 1) within a few weeks and a dense homogenous suspension of ascospores can be harvested by a simple procedure. These cells do not germinate when left in malt extract broth for prolonged times. A 7-10 min treatment at

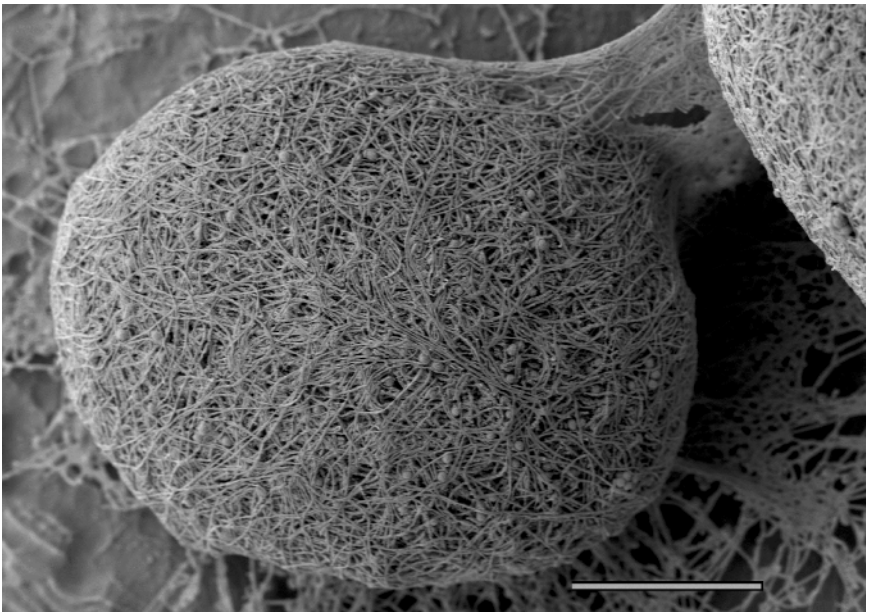

Figure 1. Ascoma of *Talaromyces macrosporus*. Note the intricate network on the outside of the structure. The ascospores visible on the outside of the fruit body have been released from other broken ascomata. Bar = 100 µm.

85°C, however, results in germination of the majority of the cells. The ascospores contain high levels of trehalose, low amounts of water and are bound by a very thick multi-layered cell wall. Upon heat treatment the trehalose is broken down by a very active trehalase and the product of hydrolysis, glucose, is accumulated inside the spore. Figure 2 shows

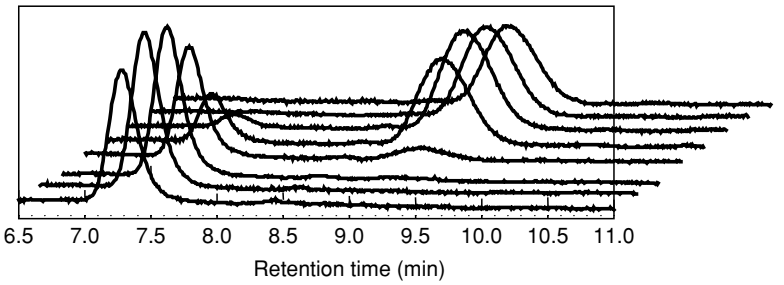

Figure 2. HPLC profiles of cell free extracts of broken spores during early germination of ascospores. The left peak shows trehalose, the right peak co-elutes with a glucose standard. The different profiles are at 0, 24, 39, 56, 73, 90, 106 and124 min after the start of the heat treatment (from front to rear).

the degradation of trehalose and the simultaneous formation of glucose early in the germination process. The glucose is present in the cells for only a short time. After that it occurs in measurable quantities in the substrate, indicating a massive release of glucose from the germinating cell.

After 150 min or more the inner cell emerges rapidly from within the outer cell wall, which is ruptured. The emptied outer cell wall remains attached to the protoplast, which is encompassed by the inner layer of the ascospore cell wall. 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 quickly and the cells swell and form a germ tube 6 hours after the heat treatment. Figure 3 shows snap frozen cells that are in various stages of the process, as observed by Cryo-Scanning Electron Microscopy using a JEOL JSM 840 scanning electron microscope (Dijksterhuis et al., 2002).

Recently, prosilition was confirmed in other species of *Talaromyces* namely *T. stipitatus, T. helicus* and *T. bacillosporus* (our unpublished observations). 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, these two genera show different modes of ascospore germination.

3. FUNGI IN FOOD AND HIGH PRESSURE TREATMENT

Ultra high pressure is a suitable candidate for non-thermal treatment of food products. These treatments have the benefit that the organoleptic properties of the food are less affected compared to pasteurisation or more severe heat treatment. In addition, vitamins are better preserved after the application of this alternative preservation technique. While vegetative microbial cells are inactivated at relatively low pressures (200-300 MPa), spores are more resistant to these treatments. Bacterial spores even are activated to germinate at a treatment of 200 MPa, but bacterial spores germinate so quickly that they are killed during prolonged treatment. When higher pressures are applied the germination sequence of the bacterial spores is blocked rendering the spores less vulnerable to ultra high pressures

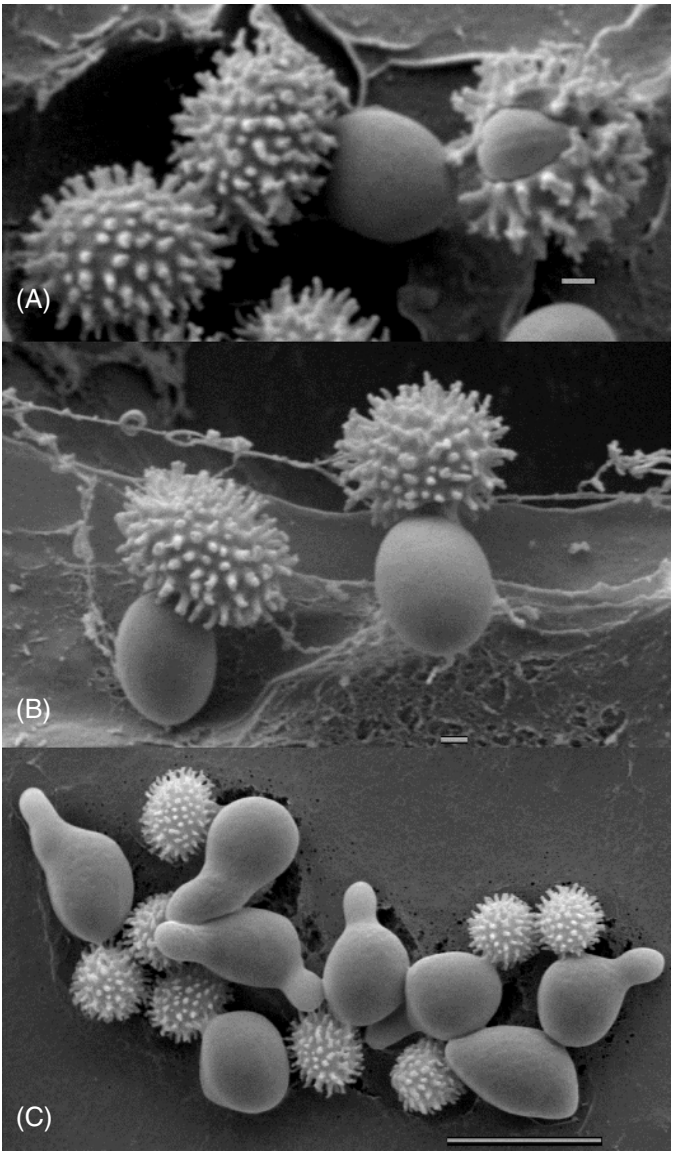

Figure 3. Germinating ascospores of *T. macrosporus*. In the top panel (A) an unprosilited cell (left) and a fully prosilited spore (middle) are shown. At the right a spore in the process of prosilition is captured. The outer cell wall has opened and the smooth inner cell wall is visible. In the middle panel (B) two fully prosilited spores are shown. Note the connection between the released cell and the empty outer cell wall. In the bottom panel (C), spores 6 h after heat treatment are shown, most of the swollen cells exhibit a germ tube, in one case two germ tubes are formed. Bars are 1 μm (A and B) and 10 μm (C).

(above 600 MPa), but also with a higher sensitivity for heat treatments (Wuytack et al., 1998).

Do resistant fungal spores such as those produced by heat-resistant fungi show extended endurance against this novel food treatment? Ascospores of *Byssochlames nivea* survive pressures at or above 600 MPa for many minutes (Butz et al., 1996), but are killed after repetitive treatments at these pressures, which are designated as "oscillative treatments" (Palou et al., 1998). These authors also describe that the application of elevated temperatures in the presence of high pressures effectively kills the ascospores. However, care is necessary with such applications to ensure that the organoleptic properties of the food are changed as minimally as possible. Probably a "happy medium" approach is important with such treatments.

4. BREAKING ASCOSPORE DORMANCY BY ULTRA-HIGH PRESSURE

Is heat the only factor that can break the dormancy of these ascospores? Recently activation of ascospores of *T. macrosporus* by high-pressure treatments was reported by Reyns et al. (2003) and Dijksterhuis and Teunissen (2004). Both studies used the same strain of fungus and a similar pressurisation equipment. The most important shared observation was that activation of ascospores occurred after a pressure treatment and that even a very short treatment at high pressure caused maximal activation. This is relevant for the food industry, because short treatments are important for economic reasons.

Dijksterhuis and Teunissen (2004) observed no activation at 200 MPa and activation of only part of the spores (up to 7% of cells) between 400 and 800 MPa. This could indicate that treatments at approx. 300 MPa would be of interest for the food industry to prevent contamination with microorganisms without activating these fungi. Reyns et al. (2003), however, observed partial activation of *T. macrosporus* at 200 MPa and activation of all spores at 600 MPa after 15 sec treatment.

A number of factors could have caused of these differences between the studies. Firstly, the growth conditions of the fungal cultures were different. As well as age of the culture, growth temperature (Conner and Beuchat, 1987a) and the growth medium (Beuchat, 1988a) all influence the heat resistance of spores. Beuchat (1988a) reported that ascospores harvested from malt extract agar (which was used by

Reyns et al., 2003) exhibited a somewhat lower heat resistance than spores formed on oatmeal agar (used by Dijksterhuis and Teunissen, 2004). These factors also may have a bearing on the extent of activation of the spores. The spores used by Reyns et al. (2003) were younger and grown at a lower temperature. Dijksterhuis and Teunissen (2004) report that the age of the fungal culture from which ascospores are harvested correlates with the acquisition of heat resistance, and that the major increase in heat resistance occurs between 20 and 40 days incubation. The combined results of the two papers point to the importance of ascospore maturity in influencing the ability of the cells to remain dormant.

The second parameter that may affect activation is the treatment of the spores before and during pressurisation. Reyns et al. (2003) pretreated their ascospore suspension for 20 min at 65°C to kill vegetative cells. In a buffer at a pH 6.8, this treatment should not activate ascospores, although at 70°C a significant increase in activation is observed in our laboratory (J. Dijksterhuis, unpublished results). At lower pH (3.0) Reyns et al. (2003) observed that nearly complete activation occurred after heat treatment at 65°C. This indicates a clear lowering of the heat activation temperature at low pH. Recently, we observed activation in a low pH medium at room temperature or only slightly elevated temperatures (J. Dijksterhuis, unpublished results). Heat resistant ascospores cause problems in fruit juices, and this lowering in heat activation could also occur in these food products. The lower pH of the environment in fruit juices may reduce the heat resistance of the ascospores somewhat, but the protective effect of the increasing the sugar content is much greater (King and Whitehand, 1990). Organic acids, particularly fumaric acid and to a lesser extent sorbic, benzoic and acetic acids have clear effect on heat resistance below pH 4.0 (Beuchat, 1988b). We have observed that the spores also become more resistant to high pressure under these conditions (J. Dijksterhuis, unpublished results).

It is also possible that menstruum in which the spores are suspended during the high pressure treatments has an effect. Dijksterhuis and Teunissen (2004) used buffer (10 mM ACES, pH 6.8) whereas Reyns et al. (2003) suspended ascospores in distilled water. During high pressure treatment the acidity of the medium decreases, but this drop would be less extensive in a buffered system. Spores suspended in distilled water may be confronted with a temporary drop in pH, resulting in more extensive activation.

The authors of both papers conclude that activation by high pressure might be related to the barrier function of the ascospore cell wall.

Dijksterhuis and Teunissen (2004) performed cryo-electron microscopy on the spores and showed alterations of the cell after very short treatments at high temperature. Reyns et al. (2003) illustrated that the spores collapsed when air dried after a high pressure treatments, whereas untreated spores 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.

5. THE CONNECTION BETWEEN THE DIFFERENT TREATMENTS

Ascospores of *Eurotium herbariorum* are recognised as heat resistant, albeit less so than *Byssochlamys, Neosartorya* and *Talaromyces* species (Splittstoesser et al., 1989). Eicher and Ludwig (2002) showed that a proportion of the spores of *Eurotium repens* (8%) is activated from dormancy by a treatment of 200 MPa for 60 min. The ascospores were also activated by a heat treatment: after 8 min at 60°C approximately 50% of the spores germinated (on Sabouraud agar) after 5 days. At 50°C, 60 min were needed to activate this population of cells. At room temperature in an isotonic salt solution ascospores did germinate, though after a delay: after 18 hours approximately 15% of the cells showed signs of germination. Ascospores that were heat activated (15 min, 60°C) were more sensitive to a subsequent high pressure treatment at 500 MPa. A 30 min treatment at 500 MPa reduced the number of colony forming units even compared with the unactivated spore suspensions. When the pressure treatment was applied immediately after a heat treatment at 60°C for 15 min the number of colony forming cells reduced by a factor of 40. When a pause was introduced between the treatments during which the spores were stored at 20°C in an isotonic salt solution, the number of colony forming units restored 10-fold. This phenomenon was designated "re-stabilisation" (Eicher and Ludwig, 2002). After treatments with high pressure (500 MPA, 30 min), heat activation did not result in any enhancement of germinating cells of *E. repens*. In fact, heat treatment resulted in some further reduction of colony forming units, viability counts only reduced further when a pause was present between the treatments (Eicher and Ludwig, 2002).

In the case of *T. macrosporus,* Dijksterhuis and Teunissen (2004) described near total activation of germination by heat (7 min, 85°C) after 5 min pressure treatments up to 800 MPa. Short pressure

treatments at least did not lead to heat sensitisation in their experiments. However, Reyns et al. (2003) show clear sensitisation of ascospores for the activation heat treatment (30 min, 80°C) after all pressure treatments at 600 MPa and 700 MPa.

6. DORMANCY REVISITED

The observed re-stabilisation phenomenon of *E. repens* ascospores poses the question of whether ascospores can return to their dormant state once activated. We addressed this question in a number of experiments where heat activated ascospores of *T. macrosporus* were confronted with a sudden lowering of the temperature or with drying conditions. The high amount of trehalose (10-20% wet weight) and the low water content of the spores (38%) may introduce a very high viscosity inside the spores. This has recently been confirmed by electron-(para)magnetic-resonance studies (J. Dijksterhuis, unpublished results). A sudden lowering of the temperature or a reduction of the water content will most likely introduce a glass transition situation inside the cell. The glassy state is an amorphous phase characterized by very low movement speeds of the cell components. Reduction of the water content or lowering of the temperature are two factors that favour the glass transition of the ascospores. Plunging of the cells into liquid nitrogen or controlled drying below 3% water-content especially will introduce a glassy state in these cells. This transition also may re-establish the dormancy of these cells. "Biological glasses", a term introduced by Buitink (2000) are characterised by a melting temperature that can be high (Wolkers et al., 1996) and cells may need to be exposed to high temperatures again in order to re-activate them.

A number of experiments were done by storing heat-activated ascospores (7 min, 85°C) on ice for 15 min or plunging the cells into liquid nitrogen and keeping them there for 15 min. The samples were allowed to warm to room temperature and subsequently plated out. In addition, activated ascospores were incubated at 30°C for 1 h before the cold treatment. Table 2 summarises these experiments and shows that dormant ascospores showed only low levels of germination whether they are cooled or not, while heat activated cells under all cases showed very high percentages of germinating cells.

Ice-treatment directly after or 1 h following heat activation did not show any effect. These experiments indicate that a sudden lowering of

Table 2. Influence of a sudden cooling treatment on extent of germination of activated ascospores of *T. macrosporus*. Numbers represent the ratio between the treated samples and the untreated controls.

	Cooled on ice		Cooled in liquid nitrogen	
	Experiment 1	*Experiment 2*	*Experiment 1*	*Experiment 2*
Controls	1	1	1	1
Controls, cooled	1.2	0.3	0.5	0.2
Activated	150	9.7	11	13
Activated, cooled	248	11.2	7.4	14.1
Activated, cooled after 1 h	198	31	12.9	10.2

Heat activation of ascospores was in 3-5 ml suspensions at 85°C for 7 min and shaken at 140 rpm in a waterbath. When spores were incubated for 1 h this was at 30° C (140 rpm). Spores were placed in Eppendorf tubes and put on ice or into liquid nitrogen. Samples were plated out in duplicate and colonies counted. The ratio between treated samples and untreated controls is given in this table.

the temperature, irrespective of how fast and large it might be, does not "reset" the ascospores to the dormant mode.

In a further experiment, ascospores were dried according to the procedures used at the CBS. The latter includes controlled freezing to –40°C (1°C/min), storage at –80°C and drying under vacuum. Dried dormant ascospores remained dormant after drying and could be effectively activated by a heat treatment after resuspending them in buffer. Dried freshly activated ascospores produced the similar numbers of activated spores and the cells showed similar tolerance to the drying treatment as dormant cells. When these cells were heat treated (in case dormancy had re-established) no additional increase or decrease of cell numbers occurred. 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 incubating the activated cells for 2 h at 30°C. These combined observations show that important phase transitions of the inner cell do not change the status of activation or dormancy in *T. macrosporus*.

7. THE SPEED OF HEAT ACTIVATION

According to Sussman (1966) dormancy is defined as a hypometabolic state; i.e. a rest period or reversible interruption of (phenotypic) development. He discerns exogenous dormany (quiescence) which include delayed development due to physical or chemical cues.

Constitutive dormancy is a condition in which development is delayed as an innate property such as a barrier to the penetration of nutrients, a metabolic block, or a self-inhibitory compound. In case of *T. macrosporus,* ascospores can be activated and also synchronised to germinate by a robust physical signal such as heat and/or high pressure. Careful examination of the data provided by Beuchat (1986) suggests that the speed of activation is increased at higher temperatures. Ascospores of *Neosartorya fischeri* exhibited constant rates of heat activation between 70° and 85°C (King and Halbrook, 1987, Figure 1). In our laboratory we observed that ascospores added to preheated buffer were fully activated within 2 min.

Kikoku (2003) reported full activation of *T. macrosporus* ascospores in a citrate-phosphate buffer (pH 6.5) within 100 s at 81° and 82.5°C. Above this temperature this time became even shorter, namely 60 s at 86.5 and 87°C, and 35 s at 91°C. From these data the author extracted 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).

Activation energy (Ea) can be calculated using an Arrhenius plot where ln (0.23303.k) is plotted against 1/T. When activation is the result of the conformation or chemical change of one defined compound in the ascospore, for instance a compound of the (plasma) membrane or a receptor protein, the Ea reflects the energy needed to convert 1 mole of such compound. Changes in proteinaceous compounds do need a different energy absorption than lipid compounds and the Ea could give clues about the nature of activation. However, when more systemic changes in the ascospore absorb the energy delivered by the heat, the Ea calculated does not give much information. Recent findings at our laboratory show changes in the ascospore during heat activation involving both proteins and cell wall components, and this multitude of changes may make a interpretation of the Ea value difficult.

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