Dormant ascospores of *Talaromyces macrosporus* are activated to germinate after treatment with ultra high pressure

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**ABSTRACT**


**Aims:** Ascospores of *Talaromyces macrosporus* are constitutively dormant and germinate after a strong external shock, classically a heat treatment. This fungus is used as a model system to study heat resistance leading to food spoilage after pasteurization. This study evaluates the effect of high pressure on the germination behaviour of these spores.

**Methods and Results:** Ascospore containing bags were subjected to ultra high pressure and spores were plated out on agar surfaces. Untreated suspensions showed invariably very low germination. Increased germination of ascospores occurred after short treatments at very high pressure (between 400 and 800 MPa). Activation is partial compared with heat activation and did not exceed 6-9% (65 times that of untreated suspensions) of the spore population. Maximum activation was attained shortly (10 s–3 min) after the pressure was applied and accompanied by cell wall deformations as judged by scanning electron microscopy. The spores observed in this study were harvested from cultures that were 39–58 days old. The maturity of spores at similar developmental stages was measured by assessing the heat resistance of ascospores. Between 20 and 40 days heat resistance increased 2–4-fold, but only an additional increase of 1–3-fold was observed at later stages (40–67 days).

**Conclusions:** Our investigations show that high pressure constitutes a second type of shock that can activate heat-resistant ascospores to germinate. Activation is maximal after very short treatments and accompanied with changes in the cell wall structure. High-pressure activation is not the result of immaturity of the ascospores.

**Significance and Impact of the Study:** These observations are relevant for the application of high pressure as a novel pasteurization method.

**Keywords:** ascospore, dormancy, food spoilage, germination, high pressure, *Talaromyces macrosporus*.

**INTRODUCTION**

Heat-resistant ascospores cause spoilage outbreaks in food products after pasteurization treatments (Tournas 1994). As an alternative, high hydrostatic pressure can be used for inactivation of spoilage organisms. High pressure is most effective when applied to vegetative (growing) microbial cells at 200–400 MPa (0.1 MPa = 1 bar, Barbosa-Cánovas et al. 1998). Fungal and bacterial spores are inactivated only at much higher pressures (Butz et al. 1996; Nakayama et al. 1996; Palou et al. 1998; Zook et al. 1999). The heat-resistant fungus *Byssochlamys nivea* forms spores that are only inactivated above 600 MPa in combination with temperatures above 60°C (Butz et al. 1996). Inactivation is more effective when pressure treatments are repeated in close succession (the so-called ‘oscillatory treatments’, Palou et al. 1998). As pressurization is already commercially used to pasteurize fruit juices and other products, the...
pressure-resistance of food-spoiling fungi that are heat resistant is of relevance for the food industry.

Talaromyces macrosporus is such a heat-resistant fungus (Van Der Spuy et al. 1975; Beuchat 1986; Dijksterhuis et al. 2002) and used as a model system to study heat resistance and heat activation of ascospores. The fungus was earlier classified as T. flavus var. macrosporus, but later described as a separate species (Frisvad et al. 1990). The fungus produces numerous ascospores that can be easily obtained in dense homogenous suspensions. These ascospores (and those of other fungi, such as Neurospora, Byssochlamys and Neosartorya) need a heat treatment for germination (Lingappa and Sussman 1959; Katan 1985; Beuchat 1986). Many studies have been performed on the heat resistance of these spores (Van Der Spuy et al. 1975; Splittstoesser and Splittstoesser 1977; King et al. 1979; Beuchat 1986; Conner and Beuchat 1987a,b; Beuchat 1988; King and Whitehand 1990), but activation of germination by heat has rarely been addressed. Recently, the germination process of ascospores of T. macrosporus was studied after heat activation (Dijksterhuis et al. 2002). Trehalose makes up to 20% of the wet weight of the cells and it is rapidly degraded to glucose monomers upon activation. The glucose is released from the cell into the medium. Three hours after heat activation, the interior mass of the spore including its inner cell wall(s) is rapidly ejected through the outer ornamented cell wall in a process termed prosilition.

Here, we report that ascospores of T. macrosporus are not only capable of surviving heat, but that they also survive ultra high-pressure treatments. Moreover, these spores are activated to germinate by high pressure. Activation by high pressure is associated by damage to the (outer) cell wall without cell death.

**MATERIALS AND METHODS**

**Organism, cultivation and isolation of ascospores**

*Talaromyces macrosporus* Stolk and Samson (Frisvad et al. 1990) CBS 130-89 was grown on oatmeal agar (30°C). The oatmeal agar was prepared from 30 g oatmeal flakes, boiled for 2 h in 1 l of water and filtered. Then 1-5% (w/w) agar was added and the mixture was sterilized for 15 min. Cultures were flooded with 5 ml ACES-buffer (10 mM pH 6-8, N-[2-acetamido]-2-aminoethane-sulphonic acid; Sigma, St. Louis, MO, USA) supplemented with 0-05% Tween-80 and ascospores were harvested according to the method described by Dijksterhuis et al. (2002). Ascospores were obtained from cultures that were 39–58 days old. Only fresh preparations were used for experiments. The density of the suspension was counted with a haemocytometer and ranged from 0-3 to 1 x 10^8 spores ml\(^{-1}\).

**Application of high isostatic pressures**

A heat sealer was used to make small bags (0-5-1 x 6-8 cm) out of Sterile Stomacher® Model 400 polyethylene bags (Sewad Ltd., London, UK). Ascospore suspension was injected aseptically into the bags and the presence of air was kept to a minimum during closing with the sealer. Bags were then subjected to high pressure. The pressure treatments were carried out in two different pressure units (Resato, Roden, The Netherlands). The units differed only in that one had a single pressure vessel of 172 ml volume and the other had six 8-ml vessels. All vessels were equipped with a thermocouple to monitor temperature. In addition, the vessels were jacketed to facilitate external heating or cooling. The bags were pressurized in a medium consisting of partially polymerized glycol (Resato). Different high-pressure treatments were performed. First, the effect on the ascospores of the degree of the pressure was evaluated. Pressures of 200, 400, 600, 800 and 1000 MPa were applied to the bags for 5 min. Secondly, we studied the effect on ascospores of the duration of exposure to high pressures. In these experiments pressures of 200 and 600 MPa were used. After the pressure treatment the ascospore suspension was removed from the bag and used for dilution plating.

**Low temperature scanning electron microscopy of ascospores**

For electron microscopy, small volumes of ascospore suspension (5 µl) were placed on excised malt extract agar (5 x 5 mm) cubes. The cubes were glued in a special copper holder and quickly frozen in nitrogen slush as described by Dijksterhuis et al. (1991). The samples were examined under a JEOL JSM 840 scanning electron microscope. Pictures were acquired using Semafore 3.62 software (JEOL, Sollentuna, Sweden).

**Measurement of heat resistance**

As a positive control, ascospores of different age were used for heat activation and heat resistance measurements. Heat resistance of the spores was assessed by means of a measuring flask (10 ml, flat bottomed) in a Julabo water bath at 85°C (140 rev min\(^{-1}\)). The flask was filled with 8 ml ACES-buffer and covered with an aluminium cap. Thin silicon tubing (diameter ca 1 mm) was used for sampling with no need to open the water bath. The buffer was heated to 85°C and the temperature was checked with an identical flask with a thermometer in it. Ascospore suspension (880 µl) was injected via the silicon tubing into the heated buffer. Ascospore samples were taken at different time intervals and used for dilution plating.
Dilution plating of the samples

Ascospore suspensions after high pressure or heat treatment were analysed by dilution plating on plate count agar (Oxoid). The plate count agar was supplemented with rose bengal (10 mg l\(^{-1}\), Sigma; King and Halbrook 1987) to promote formation of distinct, countable colonies. Fungal colonies were counted 48–96 h following incubation of the plates at 25°C. Each sample was plated out in threefold.

For heat resistance experiments, values of the time needed to reduce the viability of the spore suspension to 10%, were calculated according to the method of King et al. (1979). These calculated values can be regarded as analogues of the D-values used for linear logarithmic thermal death rate kinetics.

RESULTS

Pressurization activates germination of ascospores

During the pressurization procedure a temperature increase occurred and we had to be sure this could not activate the ascospores to germinate. The rate of increase of pressure at the start of the procedure was 4–5 MPa s\(^{-1}\) (it took 3 min 40 s to reach 1000 MPa). The pressure vessels were cooled to 15°C to prevent an increase of temperature during the build up of pressure. Maximum temperatures reached during pressurization did not exceed 35°C and were elevated only for short times (maximally 1–2 min). As a control, experiments with ascospores subjected to 40°C did not show any heat activation after 10 min of treatment. During depressurization at the end of the pressure treatment, temperatures shortly dropped (maximally 1–2 min) below 0°C, the extent and time being dependent on the magnitude of the applied pressure. Control experiments showed that these low temperatures did not induce germination in ascospores.

Heat activation of ascospores was performed to compare the amount of heat activation with pressure activation. Germination of spores was assessed after dilution plating of treated suspensions. Untreated spore suspensions invariably showed low germination. Only a small proportion of the number of cells present in the suspension formed colonies on the agar surfaces (0·15 ± 0·04%, \(n = 8\)) upon dilution plating. After heat activation for 7 min at 85°C, this number was dramatically increased to 69 ± 13% \((n = 8)\) of the cells.

Suspensions of ascospores were subjected to high isostatic pressures in the range of 200–1000 MPa. Suspensions treated for 5 min at 200 MPa showed decreased numbers of colonies after dilution plating (0·05 ± 0·03%, \(n = 7\)) compared with untreated spore suspensions. In another series of experiments the effect of prolonged treatment (20 min) at 200 MPa was tested and germination did not significantly decrease between 5 and 20 min of exposure (Table 1). When these samples were subsequently heat activated, very high viable counts were obtained, which shows that ascospores were not killed by treatment at 200 MPa. These results indicate that cells other than ascospores, namely hyphal fragments and conidia, are sensitive to these pressures and are killed.

Treatments at higher pressures (400–800 MPa) enhanced germination of the ascospore suspension, which was six to 65 times that of untreated suspensions during different experiments (47 ± 1±4%, \(n = 7\)). Pressure activation was less effective than heat activation. The amount of germination after heat activation seen in replicate trials was 11 to 23 times the amount obtained for pressure-activated cells. Figure 1 summarizes the data of four experiments where spores were treated with a pressure of 600 MPa, the pressure that resulted in highest germination, for 10 s and compares these treatments with untreated and heat-activated spores. Germination was significantly different in all treatments as judged by ANOVA of Log (viable count) values.

Figure 2 shows the effect of the degree of pressure on spore germination in two typical experiments. Bags containing ascospore suspensions were treated for 5 min at different pressures and showed maximum germination at 600 MPa. A decrease of counted colonies after 200 MPa treatments was notable. A small number of ascospores survived a 5-min treatment at 1000 MPa (Fig. 2a). When pressurized spores were subsequently treated with heat (7 min, 85°C), the extent of germination increased strongly. Figure 2b indicates that even spores treated for 5 min up to 800 MPa remained for the major part alive, though dormant.

Pressure activation and cell damage

When ascospores were pressurized for different time periods at 600 MPa the highest germination is observed, surprisingly, after very short treatments (10 s–3 min, Fig. 3).

| Table 1 Resistance of ascospores of *Talaromyces macrosporus* to a pressure of 200 MPa |
|-----------------------------------|---|---|---|
|                                   | No treatment* | 5 min at 200 MPa | 20 min at 200 MPa |
| Before heat treatment             | 1             | 0·1 ± 0·02       | 0·1 ± 0·02       |
| After heat treatment              | 524 ± 98      | 462 ± 98        | 413 ± 2          |

*The ratio is given between the number of CFU obtained after dilution plating of treated samples and the untreated control sample. The average of two experiments with ascospores is given. The ascospores are isolated from 40-day-old cultures.
Longer treatments showed lower numbers of germinating spores, which suggests that ascospores were inactivated.

The nature of pressure activation and inactivation of ascospores at 600 MPa was studied further by light and electron microscopy. When the number of prosilited (ejected) cells of each sample at 24 h after pressure treatment and the number of colonies formed after dilution plating were counted, both enumerations showed a similar trend towards a decrease in germination after prolonged pressurization at 600 MPa (Fig. 4a). From this it was clear that short treatments at high pressure (up to 8 min) activated spores above the level obtained in untreated cell suspensions (solid line in Fig 4a). Longer pressurization caused lower germination rates.

Ascospores were observed by cryo-scanning electron microscopy after different treatments. Untreated ascospores showed a coherent ornamented cell wall (Fig. 4c). After heat activation the spores germinated normally with ejection of the cell through the outer cell wall (Fig. 4d). After short pressure treatments (10 s) marked alterations of the cell wall structure were observed compared with untreated or heat-activated cell walls (Fig. 4e). These include indentations of the cell wall or a characteristic bending of the ornamentation on the outer cell wall as if the spines were compressed together around a cavity in the (outer) cell wall. When the

Fig. 1 Viable counts of ascospores suspensions of *Talaromyces macrosporus*. Values are given for untreated cells and after heat activation (7 min, 85°C) or high-pressure treatments (600 MPa, 10 s). Averages of four experiments are shown. ANOVA of the Log (viable count)-values showed that all three treatments showed significantly (*P* < 0.05) different germination.

Fig. 2 Pressure induced germination of ascospores of *Talaromyces macrosporus*. (a) A representative experiment in which ascospores were treated for 5 min at different pressures. (b) In another experiment ascospores were subjected to different pressures (closed squares) followed by a heat activation (open squares).

Fig. 3 Pressure induced germination and holding time. Ascospores were subjected to a pressure of 600 MPa for different time intervals. Data from three different experiments are shown. Cultures were 39 (squares, circles) and 49 days (triangles) old.
spores were pressurized for 60 min, the shape of the cell wall showed extensive deformation (Fig. 4f).

Those cells which were damaged during prolonged pressure treatment were indicated by the formation of hyphae from ascospores that had prosilited. Ascospores that had prosilited with higher numbers after short pressure treatments (3 min or lower) seldom formed hyphal outgrowths within 24 h. The ratio of the cells that grew hyphae and branches increased steeply after longer treatments (8 min or more, Fig. 4b). Of course, the absolute number of ejected cells had decreased strongly in these samples. This indicated that cell damage results in the release of cell compounds into the nutrient-poor ACES-buffer causing development of hyphae and branches on the spores that succeeded to germinate.

**Acquisition of stress resistance**

We have now compared the relation between heat activation and pressure activation. Is there any relation between dormancy and pressure activation? In other words, is pressure activation only confined to unripe ascospores that are not fully dormant? To evaluate this, heat resistance was measured to determine the extent to which ascospores matured. To study the acquisition of stress-resistance during maturation of the ascospore we measured the thermal death-rate kinetics of spores originating from cultures of different ages.

Prolonged exposure of ascospores to 85°C resulted in inactivation; the extent of which accelerated with time. In Fig. 5, the calculated time needed for reduction in germination with 90, 99 and 99.9% is depicted for ascospores from fungal cultures of different age. For each time point a Log (survivor) plot was made and the corresponding time needed for inactivation calculated. The time needed to reduce the viability of the spore suspension with 90% is analogous to the $D$-values, which are calculated from the linear parts of survival curves (where log surviving fraction is plotted against time). It is this value that we used to
express heat resistance. A clear correlation was found between heat resistance of ascospores and ascospore age. The calculated values showed that the heat resistance of ascospores increased in time from 33 min for spores from 20-day-old cultures to 100 min for spores from cultures that were 67 days old. The larger part of this increase (70%) was reached within 40 days of cultivation. The maximal time measured for a 99.9% reduction in viability was 160 min! Ascospore germination after dilution plating of spore suspensions that were not treated with heat was within the same range as described above (0.2 ± 0.1%; n = 8), as was increased germination after heat activation (50 ± 19%; n = 8).

In another series of experiments, ascospores were isolated from cultures that were 18–20 days old and stored in buffer at 10 and 30°C, respectively. After 1–2 weeks the heat resistance of spores stored at the higher temperature had increased in all cases, but at low temperatures no increase could be observed (Table 2).

**DISCUSSION**

**Fungal ascospores can be activated to germinate by high-pressure treatments**

Ascospores of *T. macrosporus* were very resistant to high-pressure treatments, some spores survive up to 5 min treatments at 1000 MPa (data not shown). For comparison vegetative, growing microbial cells are killed after treatments at 200–400 MPa (Barbosa-Cánovas *et al.* 1998). The pressure resistance of the ascospores of *T. macrosporus* is comparable with that of another heat-resistant fungus namely, *Byssolchlamys nivea*, which also survives treatments above 600 MPa (Butz *et al.* 1996).

Our investigations show that high-pressure treatments at 400–800 MPa activate ascospores of *T. macrosporus* to germinate. High pressure, besides heat, constitutes a second type of shock that can break the dormancy of these ascospores. High-pressure activation is not as dramatic as the heat effect, but a significant increase in germination was observed. This observation is very relevant for the application of high pressure as a novel pasteurization technique. Heat-resistant fungi that can be activated by a pasteurization treatment have the potential to cause similar problems after high-pressure treatment.

Now we can ask if heat or pressure activation is confined only to immature spores that have not developed full dormancy. The acquisition of heat resistance (and dormancy) can be regarded as a process of maturation. There is evidence that ascospores of these stress-resistant fungi mature (Conner and Beuchat 1987b; Beuchat 1988; Casella *et al.* 1990), but the length of this process is not known. Our observations show that the acquisition of full stress resistance during maturation of ascospores of *T. macrosporus* takes several weeks. Extensive examinations of heat resistance of ascospores harvested from fungal cultures of different ages shows that heat resistance increased dramatically during 4 and 6 weeks of growth. Cultures older than 6 weeks provided ascospores that showed a less marked increased heat resistance. Remarkably, maturation of ascospores also can occur autonomously that is outside the context of the fungal culture. Ascospores from stored suspensions in buffer also show an increase in heat resistance in time. Moreover, maturation of heat resistance is temperature dependent and it does not occur at lower temperatures (10°C). The spores observed in this study were harvested from cultures that were 39–58 days old and were fully able to exhibit dormancy, heat activation and germination. We conclude that pressure activation is not a phenomenon confined to only immature spores.

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**Table 2** Heat resistance of ascospores of *Talaromyces macrosporus* after storage of cells in buffer at different temperatures

<table>
<thead>
<tr>
<th>Time and heat resistance at harvest</th>
<th>After storage at 10°C*</th>
<th>After storage at 30°C</th>
</tr>
</thead>
<tbody>
<tr>
<td>Experiment 1 20 days, 32-8</td>
<td>7 days, 33-7</td>
<td>7 days, 44-5</td>
</tr>
<tr>
<td>Experiment 2 19 days, 31-1</td>
<td>14 days, 25-6</td>
<td>14 days, 35-2</td>
</tr>
<tr>
<td>Experiment 3 18 days, 27-0</td>
<td>14 days, 28-1</td>
<td>15 days, 36-6</td>
</tr>
</tbody>
</table>

*Time of additional storage is given. Heat resistance is calculated according to King *et al.* (1979).
The mechanism of activation of ascospores

Thevelein et al. (1979) studied the relation between moderate high-pressure treatments and heat activation of sporangiospores of the fungus Phycomyces blakesleeanus. The dormant spores of this Zygomycete need activation by short (3 min) heat treatments at 50°C (Van Assche et al. 1972). At pressures between 100 and 250 MPa the temperature of maximal activation decreased by 9°C (to ca 40°C). Above 250 MPa the pressure was lethal to the spores. For the high pressure used with T. macrosporus ascospores, a much larger decrease in the temperature of activation could then be expected. Taking a difference between 200 (no activation) and 600 MPa (maximal activation) we then should expect a decrease in the activation temperature from 24°C. During the high-pressure treatments the temperature did reach temperatures of 30°C and we have estimated that the minimal activation for T. macrosporus as 70°C (J. Dijksterhuis, unpublished data). So, in case of the pressure treatments the activation must have been lowered by 40°C. But does such a relation between high pressure and heat exist in T. macrosporus? With P. blakesleeanus, the authors hypothesize that protein conformation changes may play a role in activation and that these changes are invoked by the high-pressure treatment. This premise might be based on studies on germination of bacterial spores, which are also induced to germinate by high pressure. Bacillus cereus and B. pumulis were activated after 30 min at 100 MPa (Gould and Sale 1970). At moderate pressures (ca 250 MPa) activation was followed by death of the germinating spore (Wuytack et al. 1998; Wuytack et al. 2000). At higher pressures, the germination sequence of the spore was blocked. Interestingly, these spores became heat sensitive, but retained pressure resistance.

However, bacterial spores have different properties than fungal spores. First, the speed of germination is much slower in ascospores of T. macrosporus with germ tubes formed within 6 h. Bacterial spores germinate within short time and killing of these spores occurs when they are germinating during the pressure treatment. So, the fungal spores are not killed as a result of their germination. Secondly, there is no direct evidence for the involvement of receptors in germination of fungal ascospores. In case of bacterial spores receptors for sugars and/or amino acid play an important role during germination. Ascospores of T. macrosporus do not germinate in the presence of complex media, which contain all these compounds. Previous studies on T. macrosporus (Dijksterhuis et al. 2002) report that the ascospores have a low water content, which is maintained in liquid. Obviously, the ascospore cell wall isolates the internal cell from the environment during dormancy. This is also suggested by the observation that ejected cells show a tenfold increase in respiration (Dijksterhuis et al. 2002).

Damage to the (outer) ascospore cell wall at higher pressures may cause activation when the physical barrier of the outer cell wall is damaged and a water influx occurs.

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