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DPA Release and Germination of *Alicyclobacillus acidoterrestris* Spores under High Hydrostatic Pressure

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Abstract

High thermoresistance combined with the ability to grow under acidic conditions, which are unique among spore formers, make *Alicyclobacillus acidoterrestris* one of the most serious problems for the fruit processing industry. Dipicolinic acid (DPA) is an important factor in spore resistance to many environmental stresses, and in spore stability.

The aim of the study was to determine the relationship between DPA release and the germination of *A. acidoterrestris* spores, initiated by high hydrostatic pressure (HHP).

Samples of the spores of two *A. acidoterrestris* strains suspended in apple juice and pH 4.0 and pH 7.0 McIlvain buffers were treated with pressure of 100-500 MPa, at a temperature of 20-75°C for 15 min. The total amount of DPA in *A. acidoterrestris* spores was 50.3 μM for the TO-169/06 and 42.7 μM for the TO-117/02 strain.

The amount of DPA released in apple juice treated with 300 MPa was 29.3 μM at 50°C and 35.8 μM at 75°C for the TO-169/06 strain, and 24.6 μM at 50°C and 27.8 μM at 75°C for the TO-117/02 strain. DPA release in the pH 7 buffers and at 20°C was inhibited. The amount of DPA released correlated to the amount of the germinated *A. acidoterrestris* spores.

Keywords: *Alicyclobacillus acidoterrestris*; Spore germination; High hydrostatic pressure; Dipicolinic acid

Introduction

The extreme resistance of bacterial endospores to chemical and physical treatments makes them a significant problem for the food industry. One of this spore-forming bacterium is *Alicyclobacillus acidoterrestris*. This acidothermophilic, soil-borne microorganism has the ability to survive commercial pasteurization, and may thus cause fruit juice spoilage, producing compounds associated with a disinfectant-like and smoky odour, such as 2-methoxyphenol (guaiacol), 2,6-dibromophenol and 2,6-dichlorophenol [1-6].

The survival and growth of *Alicyclobacillus* in an acidic pH and at high temperatures are attributed to the unique composition of its cellular membrane, which contains cyclohexane fatty acids. These structures are closely packed in the core membrane and stabilize it, influencing spore heat resistance. Therefore it is necessary to look for new alternative methods of food preservation, including the use of high hydrostatic pressure [7-18].

Knowledge about the spore germination process has increased considerably during the past two decades. This has allowed the development of novel strategies to inactivate bacterial spores in a two-step process, i.e., germination followed by inactivation [19]. Data in the literature indicate that during the germination stage of the spore development cycle, which consists of three steps: activation, initiation and outgrowth [20], their sensitivity to inactivation via physical or chemical factors increases significantly [21,22].

The germination signal from the germination receptors is transduced and amplified in some manner by the GerD protein, and this leads to the release of monovalent cations, and then dipicolinic acid (DPA), the main factor responsible for the resistance of the spores to external conditions. During the initiation step, the spores lose their heat resistance due to the release of DPA, absorb water, and the metabolic processes are activated. Within the core of an endospore, DPA forms a 1:1 chelate with calcium ion (Ca-DPA) that excludes water, which contributes to the thermal resistance [23-26].

DPA is released from its core as the endospore germinates. DPA is also released when an endospore's structural integrity is compromised by chemicals, heat, high pressure, so by hydrolysis of the large peptidoglycan cortex of the spore. The release of DPA is connected with the activation of the spore cortex by lytic enzymes (CLE), which are responsible for degradation of the cortex [24,27-29].

DPA is synthesized in the mother cell by SpoVF and SpoVA proteins are also involved is some way in Ca-DPA release during spore germination. The Bacillales release of DPA through a DPA channel, presumably composed at least partly of SpoVA, leads to the activation of CwIJ, whereas changes in the cortex strain might activate SleB.

These two redundant CLEs degrade the peptidoglycan cortex, allowing completion of germination and the initiation of spore

outgrowth [30-32]. The DPA released by *A. acidoterestres* could be a potential indicator of injury to the spores [24]. Ca-DPA is important in spore resistance to many environmental stresses and in spore stability, and its level in spore populations can vary with the spore strains, as well as with the sporulation conditions [33,34].

In general spore resistance could be related to the high levels of DPA, α/β SASP (small acid soluble proteins) and low amount of water. In addition, DPA was also implicated in the spore resistance of Bacillus sp. to UV radiation [35] and high pressure homogenization [36], as well as in maintaining dormancy since spores containing lower amounts of this compound are rather unstable and germinate spontaneously [37].

Changes in spore sensitivity to heat and high pressure were used to differentiate the stages in the germination process using the plate count method [9,38] or by measuring the decrease in optical density [28,39,40]. Various methods such as the visualization of protein mobility [41] and monitoring of dipicolinic acid release [27,33,42,44] can also be used to analyses the germination and sporulation process.

There are only a few articles concerning the germination of *A. acidoterrestris* spores induced by HHP, describing the use of the plate method for studying this process [9,16,40].

The aim of this study was to characterize the process of the germination of spores of two strains of *A. acidoterrestris*, initiated by an innovative food preservation technique - High Hydrostatic Pressure (HHP) and to evaluate the relationship between DPA release and the germination of *A. acidoterrestris* spores after HHP treatment.

This study is an attempt to expand the current state of knowledge concerning the mechanism of the *A. acidoterrestris* spore germination process, the variations in spore population and the factors stimulating this process.

Materials and Methods

Tested organisms

The A. acidoterrestris strains TO-169/06 and TO-117/02 used in this study were isolated from Polish concentrated apple juice, using the International Federation of Fruit Juice Producers' method (2004/2007). These strains were chosen from among eight wild strains tested in our previous study [10]. TO-117/02 was the strain highly resistant to HHP and TO-169/06 was the sensitive one.

Spore production

Spores were produced based on the method described by Sokolowska et al. [11]. Just before the experiments, the spores (>95% phase bright - ungerminated) were suspended in apple juice (11.2°Bx, pH 3.4) or in a McIlvain buffer solution of pH 4.0 and pH 7.0. The number of spores in the suspensions was approximately 6 log cfu/mL for determining spore germination using the plate method, and determining the release of dipicolinic acid.

High pressure treatment

Samples of A. acidoterrestris spores were subjected to high pressure at the Institute of High Pressure Physics, The Polish Academy of Science, using U 4000/65 (Unipress) apparatus. The volume of the treatment chamber was 0.95 L and the maximum pressure 600 MPa. The pressure-transmitting fluid used was distilled water and polypropylene glycol (1:1). A pressure of up to 500 MPa was generated in 70-80 s; the release time was 2-4.

Thirteen millilitre samples in polyethylene tubes (Sarstedt) were exposed to HHP treatment with 100–500 MPa at temperature 20, 50 or 75 °C for 15 min. The pressurization times reported do not include the come-up and come-down time. The assays were performed using two independent samples. Unpressurized samples were used as controls.

Determination of releasing dipicolinic acid

Quantification of the DPA concentration in the samples was performed using the HPLC method with modification [45]. A Waters 2695 Separations Module with Waters 2996 Photodiode Array Detector system and SunFire C8 Column, (5 µm, 4.6 mm x 250 mm) with SunFire C8 Guard Pre-column, (5 µm, 4.6 mm x 20 mm) were used.

To determine the total amount of DPA in the spore suspensions, 3 mL of each individual batch (in 0.05 M PBS buffer pH 7), was sterilized at 121°C for 20 min and then analysed [43].

Determination of the germination of spores by plate method

The spread plate method on BAT-agar (Merck) with incubation for 5 days at 45°C was used. Pressure-induced germination was the difference between the plate count before HHP treatment and after HHP, followed by heat treatment at 80°C for 10 min [9,16,40], expressed as log (cfu/mL).

Phase microscopy observation

The presence of brightness (un-germinated) and darkness (germinated) spores before and after pressure treatment were observed using an MN-800 F (OPTA-TECH) microscope.

Data analysis

An analysis of the variance and Duncan's multiple-range test, using StatSoft® Statistics 7.1 was used to test the significance of the differences ($p<0.05$). The assays were performed using two independent samples from two independent processes. The bars on the figures indicate the mean standard deviation for the data points. Microsoft Office Excel 2007 was used for linear regression and to calculate the coefficient of determination (R_2).

Results and Discussion

The strains used in this study were selected due to their different response to external factors. As was shown in previous studies *A. acidoterrestris* spores of the strain TO-169/06 were sensitive to temperature [46], nisin and lysozyme [11] and to HHP while the spores of the strain TO-117/02 were resistant to these agents.

To study the effect of moderate pressure and temperature on DPA release and germination of the spores of these strains, temperatures of 20, 50 and 75°C and a pressure of 100, 200, 300, 400 and 500 MPa were used.

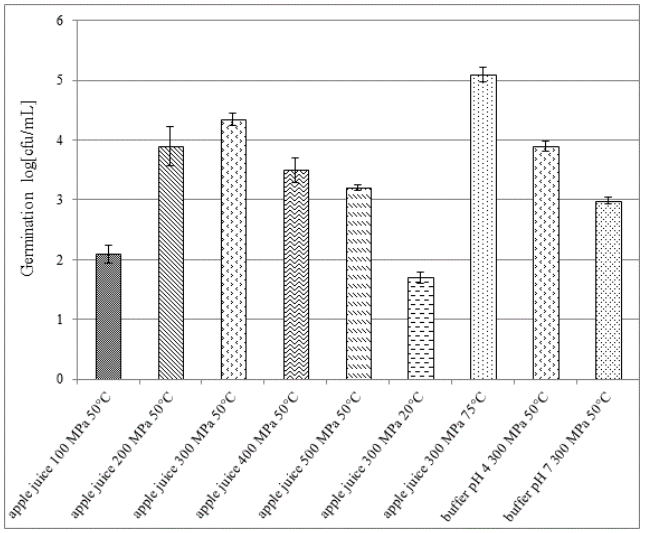

Figure 1: Germination of *A. acidoterrestris* TO-169/06 spores after 15 min HHP treatment.

Also for the TO-117/02 strain spores, the effect of pH on the germination was observed. In buffer pH 4.0, germination achieved 3.1 logs when 300 MPa at 50°C was used. A neutral pH inhibited germination, and only 1.3 logs of spores germinated under the same conditions (Figure 2).

The results in Figure 1 indicate that the germination of *A. acidoterrestris* TO-169/06 spores in apple juice were dependent on pressure and temperature. It transpired that pressure of 100 MPa applied at 50°C was not efficient for spore germination, which was 2.1 log in these conditions. Better results were achieved at 50°C when the apple juice was treated with pressure 200 and 300 MPa, compared to 400 or 500 MPa.

After 15 min at 200 MPa/50°C, germination achieved 3.9 log, and 4.4 log when the pressure was 300 MPa, while at a higher pressure 400 and 500 MPa, germination at a lower level-3.5 log and 3.2 log respectively-was observed ($p<0.05$) (Figure 1). At 20°C germination was significantly smaller than at 50°C and after 15 min at 300 MPa achieved only 1.7 logs in apple juice ($p<0.05$).

Studies by Lee et al. [7], showed that the inactivation of *A. acidoterrestris* spores in apple juice were strongly dependent on the process temperature: at 22°C no reduction was achieved, at 45°C a max. 3.5 log reduction occurred and total reduction (>5.5 log) was observed after 5 min treatment with 207 MPa at 71°C.

Therefore we applied an elevated temperature of up to 75°C to study germination. The highest germination of 5.1 log ($p<0.05$) (Figure 1) was achieved when the process was conducted at 300 MPa and 75°C, which is also consistent with the results obtained by other researchers [9].

The results achieved in this part our study show that the nutrients present in commercial apple juice can promote the germination of *A. acidoterrestris* spores during pressurization using HHP.

This could be associated with the acidophilic nature of these bacteria. The same phenomenon was observed previously in apple juice [16,40] and in tomato juice [9].

The results obtained confirm once again that the resistance of *A. acidoterrestris* to high pressure and elevated temperature is strongly strain-dependent. The spore germination was also assessed using phase contrast microscopy, since the germinated spores become phase dark while the dormant ones remain phase bright.

In the next part of our study, we focused on examining the process of DPA release. The high levels of DPA in bacterial endospores are an important factor in their resistance to chemical and physical stressors, and the pressure-induced release of DPA is considered a trigger for nutrient receptor-independent spore germination.

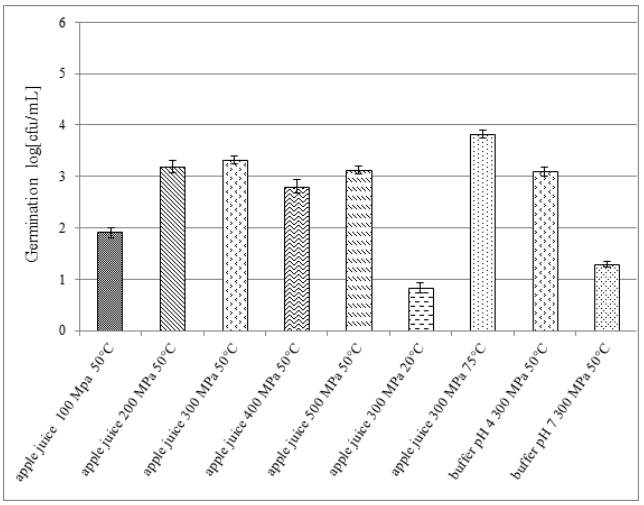

Figure 2: Germination of *A. acidoterrestris* TO-117/02 spores after 15 min HHP treatment.

To study the effect of pH on the germination of *A. acidoterrestris* TO-169/06 spores, a temperature 50°C and pressure of 300 MPa were selected. The low (4.0) and neutral (7.0) pH buffer and real food-apple juice were compared (Figure 1). In the apple juice the germination obtained was 4.4 logs. For comparison, in the buffer pH 4.0 germination achieved 3.9 logs under the same conditions but in the buffer pH 7.0 only 3.0 log of spores germinated ($p<0.05$).

The same experiments were conducted with the second strain of *A. acidoterrestris* TO-117/02, giving similar results with regard to spore germination trends in response to HHP (Figure 2). At 20°C only 0.8 logs germinated after 15 min at 300 MPa in apple juice.

Treatment at 50°C significantly supported germination in apple juice, which resulted in 3.2-3.3 log germinated spores after processing at 200 or 300 MPa ($p<0.05$). The highest germination of 3.8 logs was achieved in apple juice, when 300 MPa were used at 75°C. When the process was conducted at 400 and 500 MPa, the germination was slightly lower and reached 2.8 logs and 3.1 logs respectively ($p<0.05$).

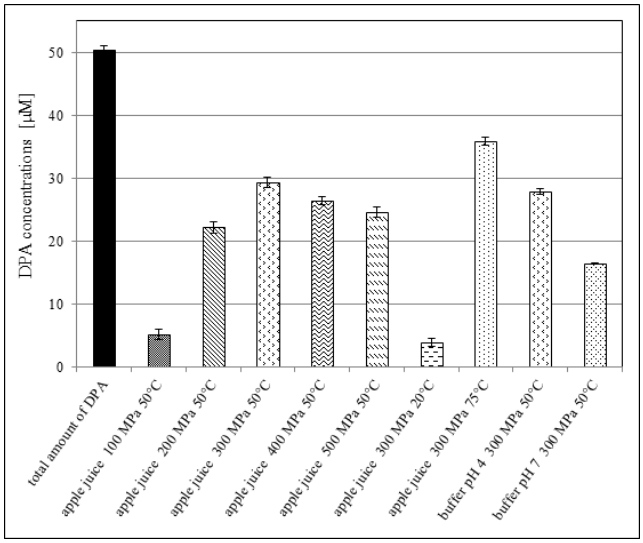

Figure 3: DPA released from the *A. acidoterrestris* TO -169/06 spores after 15 min HHP treatment.

The data presented in Figures 3 and 4 derived from the processes conducted under the same conditions as the processes in which the germination experiments were carried out (Figures 1 and 2).

The total amount of DPA present in TO-169/06 *A. acidoterrestris* spores (released during sterilization) was 50.3 µM (Figure 3), and 42.7 µM for the TO-117/02 strain (Figure 4).

The results obtained indicate that the amount of DPA released from the spores after HHP processing was strongly affected by the pressure and temperature and corresponded with the degree of germination of the spore population (Figures 1 and 2).

When the processes were conducted at 50°C in apple juice, the highest amount of released DPA-29.3 µM (58.3% of total DPA) was observed at 300 MPa (Figure 3). When higher pressures at 400 and 500 MPa were used, the amounts of DPA released were slightly lower and reached 24.6 µM and 23.8 µM respectively. Also lowering the pressure to 100 and 200 MPa resulted in decreasing the amount of DPA released.

Temperature strongly stimulated DPA release, and it achieved 3.84 µM at 20°C and increased up to 35.8 µM at 75°C (71.2% of the total DPA) when the process was conducted at 300 MPa.

The effect of pH on the release of DPA was observed. Acidic environments stimulated DPA release as well as germination. At pH 4.0, the DPA released achieved 27.9 µM, but only 15.2 µM at pH 7.0 (Figure 4).

The same trends were observed for the TO-117/02 strain spores, but the amounts of DPA released were smaller. After 15 min treatment with 300 MPa at 50°C, the amount of DPA released was 24.6 µM (57.6% of the total DPA).

Similarly to the TO-169/06 spores, after treatment with pressures of 400 and 500 MPa at 50°C, the amount of DPA released from the spores decreased up to 21.3 µM and 16.4 µM respectively (Figure 5).

The temperature affected the DPA release process. The amount of DPA released in apple juice at 20°C after 300 MPa treatment was 1.68 µM DPA and increased up to 27.8 µM at 75°C (65.1% of the total DPA).

Acidic environments also stimulated DPA release from the TO-117/02 spores as well as germination. At pH 4.0 the amount of DPA released achieved 17.5 µM and 4.5 µM at pH 7.0 (Figure 5).

The data obtained on DPA release corresponded with the level of *A. acidoterrestris* spore germination, hydrolysis of the spore cortex. Similar results were obtained by Reineke et al. [43], for Bacillus subtilis spores. The relationship between DPA release after HHP treatment and pressure-induced germination of *A. acidoterrestris* spores is presented in Figure 5. A strong positive correlation (R^2=0.8992) was observed.

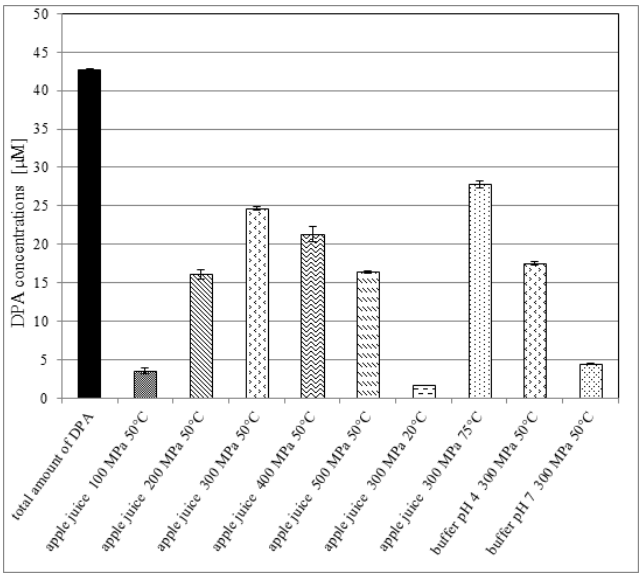

Figure 4: DPA released from the TO-117/02 *A. acidoterrestris* spores after 15 min HHP treatment.

The significant variations of the DPA released from the population observed in the present study for different *A. acidoterrestris* strains are consistent with the results of a study by Huang et al. [33] and Molva et al. [47], who reported a significant variation in the DPA levels between the populations of spores of different Bacillus species and between the spore populations prepared under different sporulation conditions.

One possible explanation is that there may actually be a significant variation in the size of the spores in a population, with larger spores having more total Ca-DPA. Variation in the volume of *B. anthracis* spores was observed by other authors [44].

So far, no studies have been reported on the DPA release of *A. acidoterrestris* spores under HHP. This is the first study which confirms DPA release during pressure-induced germination of *A. acidoterrestris* spores.

Conclusion

Moderate hydrostatic pressure can induce the germination of *A. acidoterrestris* spores. Some process parameters, mainly temperature and low pH, strongly affected the spore germination. The ability of

spores to germinate under HHP depended on the strain. The nutrients present in apple juice probably promoted the germination of *A. acidoterrestris* spores after pressurization using moderate HHP. The process of DPA release from the spores depended on the strain, pressure and temperature. The amount of DPA released correlated to the amount of germinated *A. acidoterrestris* spores.

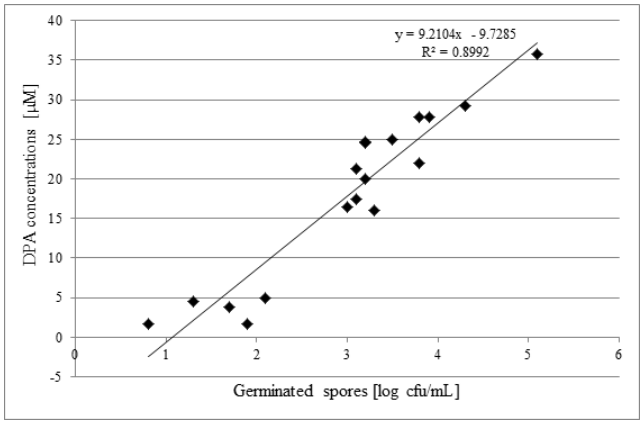

Figure 5: DPA released from of the spore suspensions vs number of germinated spores of *A. acidoterrestris* after HHP treatment.

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