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THE GERMINATION OF *ALICYCLOBACILLUS ACIDOTERRESTRIS* SPORES AND THE RELEASE OF DIPICOLINIC ACID UNDER SUPERCRITICAL CARBON DIOXIDE

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K e y w o r d s: *Alicyclobacillus acidoterrestris*, spore germination, supercritical carbon dioxide, dipicolinic acid.

A b s t r a c t

Alicyclobacillus acidoterrestris (AAT) is an acidothermophilic spore forming bacterium that causes the contamination of pasteurized fruit and vegetable juices. Since it survives typical heat treatment, the use of more effective techniques, such as supercritical carbon dioxide (SCCD), are considered for preserving juices.

Dipicolinic acid (DPA) is a universal component of bacterial spores and its release can serve as an indicator of spore germination.

The aim of this study was to determine the relationship between the release of DPA and the germination of AAT spores, initiated by SCCD. ąSamples of the spores of two AAT strains suspended in apple juice and pH 4.0 and pH 7.0 McIlvain buffers were treated with pressure of 10–60 MPa, at a temperature of 35–75°C for 30 min. The results showed that some of the process parameters, mainly the temperature and pH, strongly affected spore germination. The amount of released DPA correlated strongly (R = 0.928) to the number of germinated AAT spores.

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KIEŁKOWANIE PRZETRWALNIKÓW *ALICYCLOBACILLUS ACIDOTERRESTRIS* I UWALNIANIE KWASU DIPIKOLINOWEGO POD WPŁYWEM NADKRYTYCZNEGO DITLENKLU WĘGLA

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S ł o w a k l u c z o w e: *Alicyclobacillus acidoterrestris,* kiełkowanie przetrwalników, nadkrytyczny ditlenek węgla, kwas dipikolinowy.

A b s t r a k t

Alicyclobacillus acidoterrestris (AAT) należy do bakterii acidotermofilnych, tworzących przetrwalniki, które powodują psucie się pasteryzowanych soków owocowych i warzywnych. Ze względu na zdolność do przetrwania procesów pasteryzacji poszukuje się alternatywnych metod do ich inaktywacji, m.in. ditlenku węgla w stanie nadkrytycznym (SCCD). Kwas dipikolinowy (DPA) jest związkiem swoistym tylko dla przetrwalników, a jego uwalnianie do środowiska jest wskaźnikiem procesu kiełkowania przetrwalników. Celem badań było określenie korelacji między ilością uwalnianego DPA a procesem kiełkowania przetrwalników AAT, zainicjowanego przez SCCD. Przetrwalniki zawieszone w soku jabłkowym oraz w buforach McIlvain o pH 4,0 i pH 7,0 poddawano działaniu SCCD o ciśnieniu 10–60 MPa, w temperaturze 35–75°C, w czasie 30 min. Zaobserwowano, że niektóre parametry procesu, m.in. temperatura i pH, miały znaczący wpływ na proces kiełkowania przetrwalników. Ilość uwolnionego DPA była silnie (R = 0,928) skorelowana z liczbą kiełkujących przetrwalników AAT.

Introduction

The presence of *Alicyclobacillus acidotersestris* (AAT), a thermoacidophilic and spore-forming bacterium, in pasteurized acidic juices poses a serious problem for the processing industry. The typical sign of spoilage in contaminated juices, mostly apple and orange-is a characteristic phenolic off-flavour associated with the production of guaiacol (GOCMEN et al. 2005, JENSEN et al. 2003, SPLITTSTOESSER et al. 1994).

AAT spores demonstrate extremely high thermal resistance. The values of D95 in apple juice, which can be found in literature, vary from 1.85 to 15.1 min. The standard pasteurization process, which uses temperatures of 85–95°C, is therefore not effective against these bacteria (SPLITTSTOESSER et al. 1994, BAUMGART et al. 2000, STEYN et al. 2011 KOMITOPOULOU et al. 1999, SOKOŁOWSKA et al. 2008).

Generally spores are a unique dormant form of many types of bacteria, which develop through a remarkable series of stages to render the vegetative cells into forms that are naturally resistant to environmental conditions. Their resistance is clearly due to the cumulative effect of structural, chemical and biochemical features. The most important part of the spore is its numerous layers, which constitute up to 50% of the dry weight of the whole spore. These layers are composed of proteins containing large amounts of cysteine. The structure which is particularly important to the spore's resistance is the spore cortex. The spores are also highly dehydrated; water constitutes only 15% of the cells. Compared with vegetative cell spores, they contain more protein and 75% less carbohydrates. The structure of the spore contains a lot of dipicolinic acid, associated primarily with calcium ions, as well as other bivalent elements. Complex Ca^{2+} – DPA (calcium dipicolinate) may constitute up to 10% by dry weight of the spore. The resistance of the spores is also connected to the family of proteins known as SASP (small amide-soluble proteins). They alter the structure of the DNA, stiffening and straightening it by saturating the biomolecules on the outer side of the DNA helix (LEGETT et al. 2012, SETLOW et al. 2006).

To effectively kill spores, a temperature of 121°C is commonly used in a steam autoclave, whereas many other vegetative bacteria are killed at temperatures of between 60 and 100°C. To enhance the effectiveness of decontamination processes, it is recommended that spore germination be induced and that the spores be transformed into a vegetative form which greatly increases their susceptibility to inactivation with the use of physical or chemical agents, while their metabolic activity remains unchanged. It is a commonly accepted and well-documented theory that pressure triggers spore germination and during this process, the spores progressively lose their typical resistance and more readily become inactivated (SETLOW 2003, NGUYEN et al. 2010). Therefore, at present, the hope of a final solution to the *Alicylobacillus* problem is seen to be in unconventional preservation methods, based on elevated pressure, such as high hydrostatic pressure (HHP) or SCCD (supercritical carbon dioxide).

SCCD is considered a promising technique for food preservation because it requires much lower pressures than those used in HHP. The mechanism of inactivating vegetative bacteria with the use of SCCD has been widely investigated. The lethal action of highly pressurized CO_2 on bacteria can be explained by cell-membrane modification, reduction in the internal pH of the bacterial cell, the effects of CO_2 and HCO_3^- on metabolism, alteration of the intracellular electrolyte balance, and the extraction of vital constituents from cells and cell membranes. CO_2 is physiologically safe, inexpensive and easily available in high purity and in large quantities (BAE et al. 2009).

The deactivation effect of SCCD has been evaluated on spores of numerous species (SPILIMBERGO et al. 2003, WHITE et al. 2006, ZHANG et al. 2006A, 2007). Only a few researchers have found this technique effective for killing AAT spores in combination with heat. So far there has only been one approach to applying this technique for the deactivation of AAT spores which has been quite successful. A reduction of above 5 log was obtained after 20-min treatment at 70°C and 10 or 12 MPa (BAE et al. 2009).

The mechanism of destroying bacterial spores using supercritical carbon dioxide has not been elucidated and it is not clear whether the germination step is involved. ZHANG et al. (2006b,c) investigated the effect of this process on *B. atrophaeus* and after treatment at 40°C, 27.5 MPa detected no significant release of dipicolinic acid, thus indicating that germination was not triggered. However, in this experiment the spores were lyophilized and inoculated into paper, so the conclusions might not be applicable to spore suspensions. Moreover (FURUKAWA et al. 2004) found that high-pressure gaseous carbon dioxide treatment at 35°C, 65 bar for 120 min initiated the germination of B. *coagulans* and *B. licheniformis* spores. The effect was confirmed by phase-contrast microscopy.

The aim of this study was to analyse the process of the germination of the spores of two strains of AAT, initiated by an innovative food preservation technique-supercritical carbon dioxide (SCCD)-and to estimate the relationship between the release of DPA and the germination of AAT spores after SCCD treatment.

Material and Methods

The International Federation of Fruit Juice Producers' method (2004/2007) was used to isolate the AAT strains TO-169/06 and TO-117/02 from Polish concentrated apple juice. These strains were chosen from among eight wild strains tested in our previous study (SKĄPSKA et al. 2012, SOKOŁOWSKA et al. 2008, SOKOŁOWSKA et al. 2012A). The TO-117/02 was highly resistant to external factors whereas the TO-169/06 strain was the sensitive one.

Spores were produced based on a method described by SOKOŁOWSKA et al. (2012B) and were then suspended in apple juice (11.2°Bx, pH 3.4) or in McIlvain buffer solutions 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 and approximately 9 log cfu/mL for determining the release of dipicolinic acid.

Samples of AAT spores were treated with supercritical carbon dioxide using apparatus for the extraction with supercritical fluids Speed SFE®, Applied

Separations, USA. The volume of the treatment chamber was 10 mL, with working pressure of up to 69 MPa and temperatures of up to 120°C.

Seven-millilitre samples tubes were exposed to supercritical carbon dioxide at pressures of 10, 30, 60 MPa at temperatures of 35, 50, 75°C for 30 min. The temperature was measured in the chamber. The assays were performed using two independent samples.

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 and after SCCD treatment, followed by heat treatment at 80°C for 10 min (VERCAMMEN et al. 2012, SOKOŁOWSKA et al. 2015). The results were expressed as log (cfu/mL).

The quantification of the DPA concentration in the samples was performed using the HPLC method (WARTH 1979). 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. Elution was with 1.5% *tert*-amyl alcohol in 0.2 M potassium phosphate, pH 1.8, at 25°C. 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 (REINEKE et al. 2013).

An analysis of the variance and Duncan's multiple-range test, using StatSoft® Statistica 7.1, was used to test the significance of the differences ($p<0.05$). The assays were performed using two independent samples. 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)* and coefficient of correlation (r).

Results and Discussion

The effect of pressure and temperature on the release of DPA and germination of the spores are presented in Figures 1–2. Two strains of *A. acidoterrestris*, treated with pressures of 10, 30 and 60 MPa at temperatures of 35, 50 and 75°C were used in this study.

The results indicate that the germination of AAT TO-169/06 spores in apple juice depended on the pressure and temperature. It was observed that pressure of 10 MPa applied at 50°C was not efficient for spore germination, which was 0.56 log under these conditions. Better results were achieved at 75°C when 1.5 log of germinated spores was observed. When the apple juice was treated with pressure of 30 MPa at 50°C and 75°C, the germination of the spores was 0.75 log and 1.8 log respectively. At 35°C germination was significantly less than at 50°C and after 30 min at 60 MPa achieved only 0.42 log in apple juice

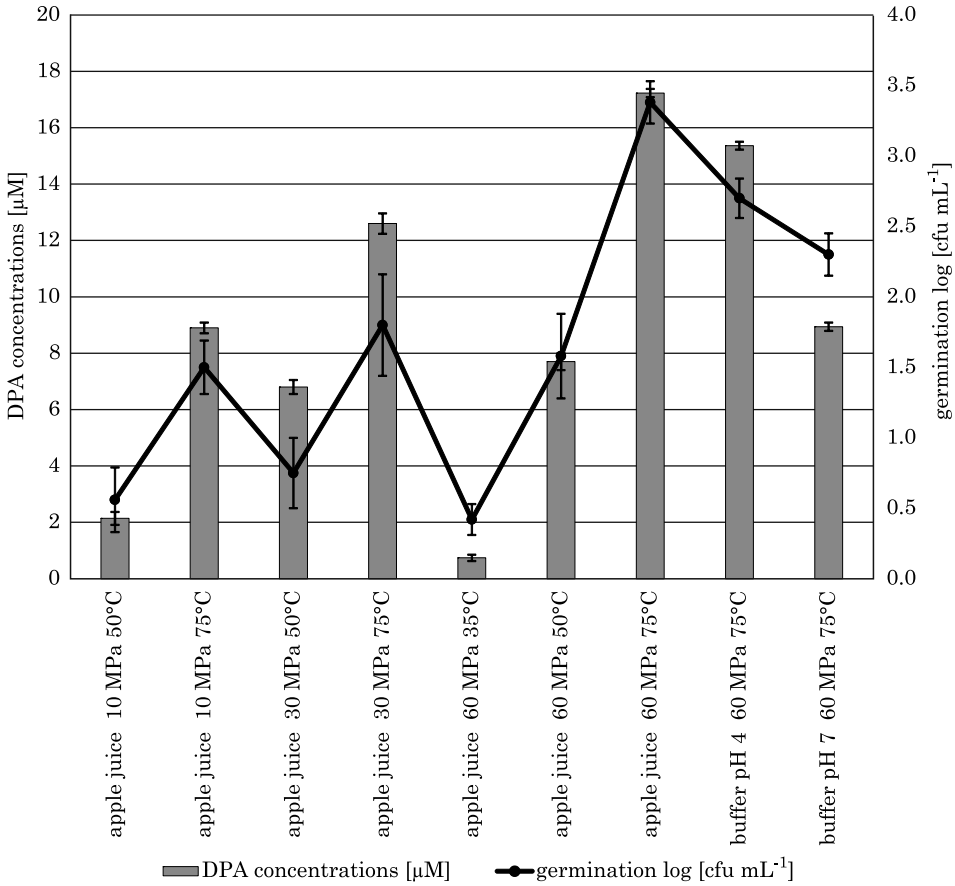

Fig. 1. Germination and DPA released from AAT TO-169/06 spores after 30 min of SCCD treatment

($p < 0.05$). When the temperature was increased to 50°C germination achieved 1.58 log ($p < 0.05$). The largest number of germinated spores-3.38 log-was observed during the experiment carried out using the highest pressure and temperature values, 60 MPa and 75°C ($p < 0.05$) – Figure 1.

To study the effect of pH on the germination of AAT TO-169/06 spores, a temperature of 75°C and pressure of 60 MPa were selected. The results of the process conducted in low (4.0) and neutral (7.0) pH buffer and real food-apple juice (pH 3.4)-were compared (Figure 1). Germination in the pH 4.0 buffer achieved 2.7 log and under the same conditions, however in the pH 7.0 buffer only 2.3 log of spores germinated ($p < 0.05$).

The results achieved in this part of our study show that low pH and the nutrients present in commercial apple juice can promote the germination of AAT spores during SCCD treatment. The same phenomenon was observed in

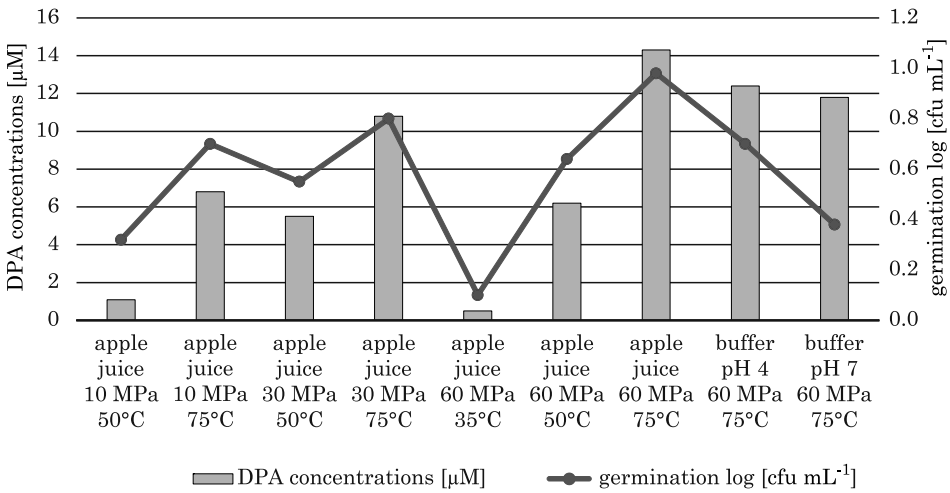

Fig. 2. Germination and DPA released from AAT TO-117/02 spores after 30 min of SCCD treatment

apple juice during treatment of these spores using high hydrostatic pressure (PORĘBSKA et al. 2015a,b, SOKOŁOWSKA et al. 2013, 2015) and in tomato juice (VERCAMMEN et al. 2012).

The total amount of DPA present in AAT TO-169/06 spores (released during sterilization) was 50.3 μM and 42.7 μM for the TO-117/02 strain (data not shown). The amount of DPA released from the spores after SCCD processing was strongly affected by the pressure and temperature and corresponded with the degree of germination of the spore population (Figures 1–2). When the processes were carried out at 50°C in apple juice, the highest amount of released DPA – 7.7 fM (15.30% of total DPA) was observed at 60 MPa for spores of the AAT TO-169/06 strain (Figure 1). When lower pressures, 10 and 30 MPa were used, the amounts of DPA released were slightly lower and reached 2.14 μM and 6.8 μM respectively. Temperature strongly stimulated DPA release, and it achieved 0.74 μM at 35°C and increased to 17.23 μM at 75°C (34.25% of the total DPA) when the process was conducted at 60 MPa. The effect of pH on the release of DPA was also observed. An acidic environment stimulated the release of DPA as well as germination. The DPA released at pH 4.0 achieved 15.36 μM, but only 8.94 μM at pH 7.0.

The same experiments were conducted with the second strain of AAT TO-117/02, giving similar results with regard to the spore germination and DPA release trends, however this strain turned out to be far more resistant to SCCD treatment (Figure 2). At 35°C only 0.1 log spores germinated in apple juice after 30 min at 60 MPa. Treatment at 50°C slightly supported germination in apple juice, and resulted in 0.32, 0.55 and 0.64 log of germinated spores after

processing at 10, 30 and 60 MPa ($p < 0.05$). The highest germination – 0.98 log-was achieved in apple juice when 60 MPa was used at 75°C. The effect of pH on germination was also observed for the TO-117/02 strain spores. In pH 4.0 buffer, germination achieved 0.8 log when 60 MPa at 75°C was used. A neutral pH inhibited germination, and only 0.38 log of spores germinated under the same conditions (Figure 2). Similar results were observed by SOKOŁOWSKA et al. (2015) in apple juice.

Similarly to the observations made for the previous strain, the amount of DPA released from TO-117/02 was proportional to the number of germinated spores. During 30 min of SCCD treatment with 60 MPa at 50°C, the amount of DPA released was 6.2 μM (14.52% of the total DPA). The temperature affected the DPA release process. The amount of DPA released in apple juice at 35°C after treatment at 60 MPa was 0.49 μM DPA and increased to 14.3 μM at 75°C (33.49% of the total DPA). The acidic environments also stimulated the release of DPA from the TO-117/02 spores, as well as germination. At pH 4.0 the amount of DPA released achieved 12.4 μM, and 6.2 μM at pH 7.0 (Figure 2).

The data obtained on the release of DPA corresponded with the level of both AAT strain TO169/06 and TO-117/02 spore germination. Similar results were obtained by other authors for *Bacillus subtilis* spores (REINEKE et al. 2013).

The relationship between the release of DPA after SCCD treatment and the pressure-induced germination of *A. acidoterrestris* spores is presented in Figure 3. A strong positive correlation ($R^2 = 0.863$, $r = 0.928$) was achieved.

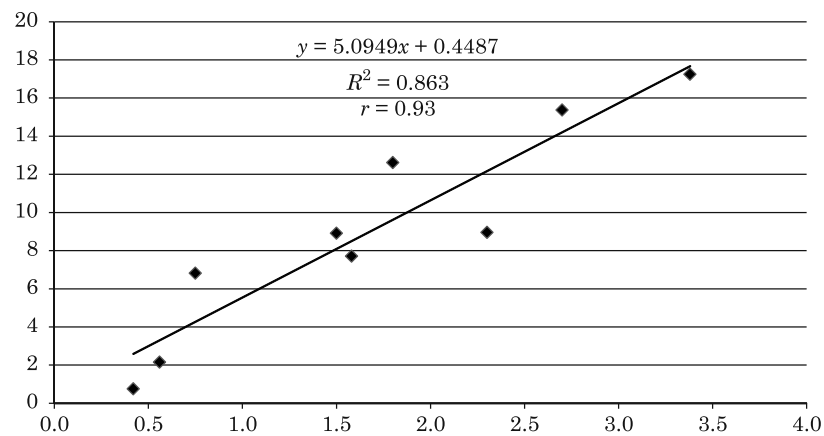

Fig. 3. DPA released from the spore suspensions vs the number of germinated spores of *A. acidoterrestris* after SCCD treatment

The significant variations in the DPA released from the population observed in the present study for different AAT strains are consistent with the results of a study by MARGOSCH et al. (2004) who also reported significant differences in the DPA levels between the populations of spores of different *Bacillus* species and *Clostridium* species (FRANCIS et al. 2015). These levels could also vary between the individual spores in a spore population, perhaps due to cellular heterogeneity (HUANG et al. 2007, LUU et al. 2014).

To summarize, the results obtained once again confirm that the resistance of AAT to pressure and elevated temperatures is strongly strain-dependent. The phenomenon of differentiation of the strains within the AAT species was also shown by BEVILACQUA et al. (2007). These processes may be associated with the complex spore structure and the related existence of a number of mechanisms of gene expression that govern the germination of spores. This is the first study which confirms the release of DPA during SCCD induced germination of AAT spores.

Conclusions

SCCD can induce the germination of AAT spores. Some of the process parameters, mainly temperature and low pH, strongly affected spore germination. The ability of spores to germinate under SCCD depended on the strain. The nutrients present in apple juice probably promoted the germination of AAT spores after pressurization using SCCD. The process of DPA release from the spores depended on the strain, pressure and temperature used. The amount of DPA released had a strong positive correlation to the amount of germinated AAT spores.

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