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Regular paper

Analysis of the germination proteins in *Alicyclobacillus acidoterrestris* spores subjected to external factors

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The presence of *Alicyclobacillus acidoterrestris*, a thermoacidophilic and spore-forming bacterium, in pasteurized acidic juices poses a serious problem for the processing industry. Therefore, the use of other more effective techniques, such as high hydrostatic pressure (HHP) and supercritical carbon dioxide (SCCD), is considered for preserving juices in order to inactivate these bacteria, while reducing the loss of nutrients and sensory quality of juices. On the other hand, HHP and SCCD when combined with a moderately elevated temperature can induce germination of bacterial spores, making them more vulnerable to inactivation. The spore germination can be also induced by nutrients, such as L-alanine or a mixture of asparagine, glucose, fructose and potassium ions (AGFK). The aim of this work was to determine whether applying activating agents: HHP, SCCD and nutrient germinants (L-alanine and the AGFK mixture), could influence the number of spores which start to germinate and how this affects the proteins involved in the spore germination. SDS-PAGE was used to resolve proteins isolated from the *A. acidoterrestris* spores. The results that were obtained indicate that the germination of *A. acidoterrestris* spores treated with HHP, SCCD and nutrient germinants reflect the number of spores which start to germinate. The SDS-PAGE data indicated changes in the level of selected proteins occurring when subjected to the germination activating factors as well as noticeable differences in those proteins' molecular weights.

Key words: *Alicyclobacillus acidoterrestris*, spore germination, germination proteins, SDS-PAGE

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Abbreviations: *A. acidoterrestri, Alicyclobacillus acidoterrestri;* AGFK, asparagine, glucose, fructose and potassium ions mixture; DPA, dipicolinic acid; HHP, high hydrostatic pressure; SCCD, supercritical carbon dioxide

INTRODUCTION

Alicyclobacillus acidoterrestris is a Gram-positive, spore-forming and thermoacidophilic bacterium which survives a typical pasteurization process, causing spoilage of juices by producing compounds associated with a disinfectant-like odour: guaiacol, 2,6-dibromophenol, 2,6-dichlorophenol and 2,6-dibromophenol (Orr *et al*., 2000).

The spores of *A. acidoterrestris* are extremely resistant to a variety of environmental stresses. The structure and chemical composition of the spores differs considerably from those of vegetative cells because of the multilayer structure surrounding the spore core, which consists of the exosporium, spore coat, outer membrane, cortex and cell germ wall, and the inner membrane. The spore core contains the DNA, RNA, ribosomes and most of the enzymes. The core also contains high levels of a small molecule, the dipicolinic acid (DPA), which exists as a 1:1 chelate with divalent cations, predominantly Ca^{2+}. Such structure largely accounts for the unique spore resistance to the environmental stress (Setlow *et al*., 2014b; Bassi *et al*., 2016).

Conditions within the core are strongly linked to the spore's resistance properties which are involved in protecting the spore's DNA from damage. There are two principal methods of minimizing the effect of DNA damage to the spore: firstly, by preventing the DNA damage to occur, and secondly by ensuring the detoxification and repair of any DNA damage during spore germination. Small acid-soluble proteins (SASP), which are found exclusively in spores, are synthesized late into sporulation and only in the developing spore, and are degraded early during germination, providing a vital source of free amino acids for the outgrowing spore. They contain a large percentage of hydrophobic amino acids and bind directly to and saturate the spore DNA, therefore providing an important component of spore resistance against external treatment which targets spore DNA from many types of damage (Moeller *et al*., 2014).

The spores of *A. acidoterrestris* have the ability to survive under typical conditions used for pasteurization, which also enables them to germinate and grow. Therefore, the use of other more effective techniques such as high hydrostatic pressure (HHP) and supercritical carbon dioxide (SCCD) are considered for preserving juices in order to inactivate these bacteria, while reducing the loss of nutrients and sensory quality of the juices. To enhance the effectiveness of the process, it is recommended that germination be induced and the spores transformed into a more susceptible vegetative form. The data indicate that in the case of *A. acidoterrestris,* HHP (Porębska *et al*., 2015a; 2015b; Sokołowska *et al*., 2015; Vercammen *et al*., 2012) as well as SCCD (Porębska *et al*., 2016a; 2016b), combined with a moderately elevated temperature, may be a useful technique for both, the germination and inactivation of *A. acidoterrestris* spores in apple juice and buffers. The germination of *A. acidoterrestris* spores can be also induced by various nutrients: amino acids, purine nucleosides, sugars (Lovdal *et al*., 2012), L-alanine (Paredes-Sabja *et al*., 2011; Kuwana *et al*., 2013; Cruz-Mora *et al*., 2015), ions and combinations of these, and a mixture of asparagine, glucose, fructose and potassium ions (AGFK) (Gosh *et al*., 2012; Stewart *et al*., 2012).

Spore germination of *Bacillus* species (Setlow 2014a) and *Clostridium* species (Paredes-Sabja *et al*., 2014; Francis

et al., 2015) is an excellent model system to study the molecular mechanisms underlying the pressure and nutritional control of growth and development. The binding of specific chemical nutrients to spore cognate receptors (GRs) located in the inner membrane triggers the germination process that leads to resumption of metabolism in spore outgrowth. A major signal that triggers spore germination is the presence of specific nutrients called germinants in the spore's environment. These nutrient germinants are typically amino acids, purine nucleosides or sugars which are recognized in a stereospecific manner by GRs.

The spores of *A. acidoterrestris* have characteristic properties and consist of complex structures including various types of proteins. The spore germination mechanism could be triggered by L-alanine, which acts through the GerA receptor, or a mixture of L-asparagine, fructose, glucose and potassium (AGFK) through the GerB and GerK receptors (Paredes-Sabja *et al*., 2011; Paidhungat *et al*., 2002). However, high hydrostatic pressure promoted germination at 100–400 MPa and also triggered germination through the GerA, GerB and GerK receptors, but a higher pressure, up to 600 MPa, retarded this process (Setlow *et al*., 2003a; Paidhungat *et al*., 2002; Paredes-Sabja *et al*., 2011; Nguyen Thi Minh *et al*., 2010; Luu *et al*., 2015).

As noted above, in addition to nutrients, spores can be germinated by using HHP of 100 to 800 MPa. At an HHP of 100 to 300 MPa, germination is caused by GR activation. However an HHP of 500–800 MPa, causes germination by the release of the Ca-DPA depot from the spores and may act on specific Ca-DPA channels in the inner membrane of the spore (Koong *et al*., 2014; Setlow, 2014b; Sarker *et al*., 2015; Wang *et al*., 2015; Porębska *et al*., 2015b).

The exposure of spores to relatively low HHP levels (100–400 MPa) and moderate temperatures (20–75ºC) stimulated spore germination and triggered the GRs of *B. subtilis* during the germination of individual spores. Under moderate pressure, the individual GRs displayed differing levels of sensitivity to pressure (Setlow *et al*., 2003B, Zhang *et al*., 2014).

The aim of this work was to determine whether applying activating agents: HHP, SCCD and nutrient germinants (L-alanine and AGFK mixture) could influence the number of spores which start to germinate and how this affects the proteins involved in the spore germination.

MATERIALS AND METHODS

Tested organism. The *A. acidoterrestris* strains TO-169/06 and TO-117/02 used in this work were isolated from a 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 previously (Skąpska *et al*., 2012). TO-117/02 was a strain highly resistant to temperature, HHP and SCCD and TO-169/06 was a more sensitive one (Porębska *et al*., 2015a; 2015b; 2016a; 2016b).

Spore production. Spores were produced based on the method described by Sokołowska and coworkera (2012) and 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, at approximately 6 log cfu/mL.

High hydrostatic 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 the 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 s. The pressurization times reported do not include the come-up and come-down times.

Thirteen milliliter samples, in polyethylene tubes (Sarstedt), were exposed to HHP treatment at 300 MPa and a temperature of 50°C for 15 min. The highest germination of *A. acidoterrestris* spores and release of DPA were achieved using these process parameters in previous studies (Porębska *et al*., 2015b). The assays were performed using two independent samples. Unpressurized samples were used as controls. After treatment, the samples were removed from the chamber and placed immediately on ice. They were then stored at 4°C until further analysis.

Supercritical carbon dioxide treatment. Samples of *A. acidoterrestris* spores were subjected to supercritical carbon dioxide using Applied Separations Spe-ed SFE supercritical fluid extraction apparatus. The volume of the treatment chamber was 10 mL, the maximum pressure 69 MPa, and the maximum temperature 120°C. Seven milliliter samples were exposed in glass tubes to supercritical CO_2 at 60 MPa, 75°C for 30 min. The highest germination of *A. acidoterrestris* spores and release of DPA were achieved in previous studies by using these process parameters (Porębska *et al*., 2016b). After processing, the samples were stored at 4°C until further analysis. The assay was performed with two independent samples. Unprocessed samples were used as controls.

Treatment with nutrient germinants. L-alanine (50 mM) or AGFK mixture (50 mM) were added to the spore suspension samples which were afterwards subjected to a temperature of 80°C/10 min and incubated at 45°C (Bevilacqua *et al*., 2014), up until the moment of a significant decrease in OD, indicating germination of the spores (Porębska *et al*., 2015a; 2016a).

Preparation of samples for SDS-PAGE. After exposure to the activating factors, the *A. acidoterrestris* spore suspension at approximately 6 log cfu/mL was centrifuged at 17 000×*g* for 10 min at 4°C. The spores were washed once with 10 mM sodium phosphate buffer (pH 7.2). To remove cellular debris and vegetative cells, the pellets were suspended in 0.1 ml of a lysozyme buffer (10 mM sodium phosphate, pH 7.2; 1% lysozyme; complete protease inhibitor tablets from Roche) and incubated at room temperature for 10 min. The pellets were then washed repeatedly with a buffer (10 mM sodium phosphate, pH 7.2; 0.5 M NaCl) at room temperature and then incubated with SDS and 2-mercaptoethanol to extract all germination proteins. The samples were stored at –20°C prior to analysis (Kuwana *et al*., 2002; Takamatsu *et al*., 2000; Lai *et al*., 2003; Sreshty *et al*., 2011, Thompson *et al*., 2011).

SDS-PAGE electrophoresis. Sodium dodecyl sulphate polyacrylamide gel electrophoresis (SDS-PAGE) was carried out with a Bio-Rad Mini PROTEAN®Tetra Cell electrophoresis system. SDS-PAGE was conducted to determine the number and size of the proteins of the germinated spores after being subjected to external factors.

Spore proteins were solubilized in 0.1 ml loading buffer (Tris-HCl, pH 6.8; 4% SDS; 10% 2-mercaptoethanol; 10% glycerol; 0.05% bromo-phenol blue) and boiled for 5 min. The proteins were separated on 14% SDS-PAGE and visualized by using Coomassie brilliant blue R-250

dyes. The stained gel was washed in 10% acetic acid and then analyzed (Takamatsu *et al.*, 2000). Each sample was examined three times.

RESULTS AND DISCUSSION

The results obtained in our previous studies, which examined, among other things, the influence of the temperature, time and pressure of HHP and SCCD treatments and incubation with L-alanine and AGFK mixture, on the degree of inactivation and germination of the *A. acidoterrestris* spores by using optical density methods (Porębska *et al.*, 2015a; 2016a; 2016c), and the DPA release during pressure-induced germination of *A. acidoterrestris* spores (Porębska *et al.*, 2015b; 2016b), enabled selection of the optimal temperature and pressure conditions for research in this work. In this work, we wanted to show changes in the level of proteins during spore germination of two strains of *A. acidoterrestris:* TO-117/02 and TO-169/06, when subjected to certain external factors.

Lanes 1–3 before SCCD processing, lanes 4–7 after SCCD processing

The results of the analysis of the changes in the level of proteins before and after treatment with the activating agents are shown in Figs 1–5. Proteins isolated from the *A. acidoterrestris* spores were tested using SDS-PAGE, and the profiles obtained were compared with the protein marker before and after being subjected to the external factors.

In order to estimate the levels of proteins released from the germinated *A. acidoterrestris* 169/06 spores, SDS-PAGE was used, where such proteins may be visualized with Coomassie blue staining and with some percussion showed their molecular mass, before and after SCCD treatment at 60 MPa, 75°C (Fig. 1).

Lanes 1–4 after HHP processing, lanes 7–8 before the HHP processing, lanes 5–6 were left empty

The levels of proteins released from the germinated *A. acidoterrestris* TO-117/02 spores, before and after HHP treatment at 300 MPa, 75°C are shown in Fig. 2.

Lanes 1–4 after HHP processing, lanes 5–6 before HHP processing

In order to estimate the levels of proteins released from the germinated *A. acidoterrestris* 169/06 spores, they were visualized using Comassie blue-stained SDS-PAGE before and after HHP treatment at 300 MPa, 75°C (Fig. 3).

Lanes 1–3 after incubation with L-alanine, lanes 4–6 before incubation with L-alanine

Figure 4 displays the levels of proteins involved in the germination of spores of *A. acidoterrestris* 169/06 before and after incubation with L-alanine.

Lanes 1–6 after incubation with the AGFK mixture, lanes 7–9 before incubation with the AGFK mixture

The levels of proteins in the total lysates of *A. acidoterrestris* TO-169/06 spores (lanes 4–6 and 8–9) and TO-117/02 (lanes 1–3 and 7) involved in the germination, before and after incubation with the AGFK mixture are shown in Fig. 5.

Comparing the lanes with samples taken before and after the action of activating factors, it is clear that a significant amount of proteins was released from the germinated treated spores, whereas no significant amount of proteins was released from the untreated, ungerminated spores.

Lower levels of proteins were observed in the case of *A. acidoterrestris* TO-117/02, indicating a weaker germination process. This phenomenon, and the difference between treated and untreated spores and differentiation between the strains was also observed by Setlow and coworkers (2015).

The levels of the proteins found in the germinated spores of *A. acidoterrestris* subjected to the external fac-

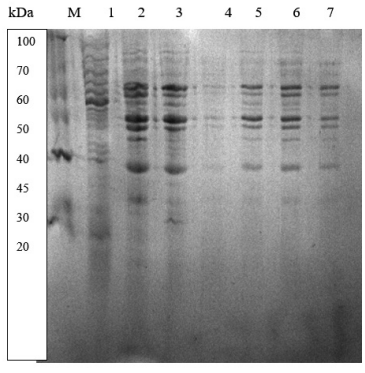

Figure 1. The levels of proteins involved in the spore germination of *Alicyclobacillus acidoterrestris* 169/06 (lanes 2–3 and 5–7) and 117/02 (lanes 1 and 4) after SCCD at 60 MPa, 75°C.

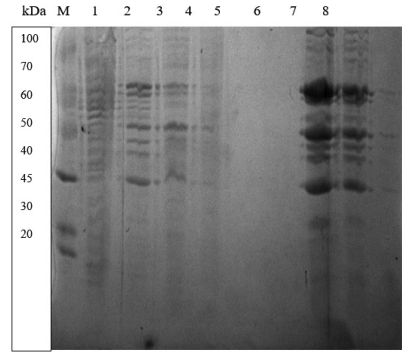

Figure 2. The levels of proteins involved in the germination of spores of *Alicyclobacillus acidoterrestris* 117/02 after HHP at 300 MPa, 75°C.

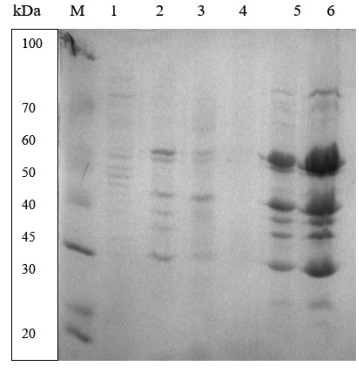

Figure 3. The levels of proteins involved in the germination of spores of *Alicyclobacillus acidoterrestris* 169/06 after HHP at 300 MPa, 75°C

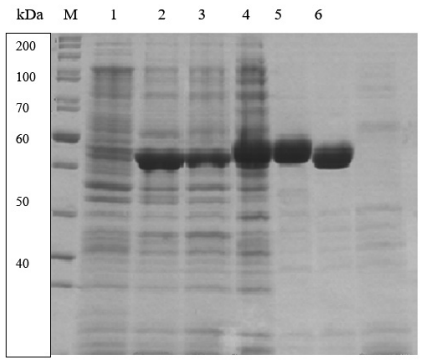

Figure 4. The levels of proteins involved in the germination of spores of *Alicyclobacillus acidoterrestris* 169/06 after incubation with L-alanine.

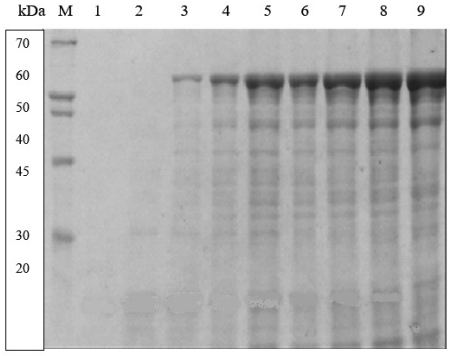

Figure 5. The levels of proteins in the total lysates of spores of *Alicyclobacillus acidoterrestris* TO-169/06 (lanes 4–6 and 8–9) and TO-117/02 (lanes 1–3 and 7) before and after incubation with the AGFK mixture.

tors are rather stable because there is no synthesis and degradation. The proteins which are involved in germination are not necessarily released from the spore, as for example the germination receptors. This proves that their level may change in the course of germination only in the direction of lowering their amount.

These results may be a prelude to expanding our current knowledge about the complex multifactorial process of sporulation and spore germination, and could help in analysis of a wider mechanism of gene expression involved in these processes in the future.

Changes in the protein level may be related to the existence of a number of mechanisms that govern the germination of spores. Bacterial spores contain unique high- and low-molecular-mass proteins. Some of these proteins contribute directly or indirectly to the unique characteristics of the spores, such as dormancy, a high degree of resistance and, in particular, a unique cell morphology. Therefore, a comprehensive analysis of the protein composition would provide useful basic information and facilitate our understanding of the germination process at the molecular level (Setlow 2014a; 2014b). Protein sequencing would be required in subsequent experiments to exactly determine the type of proteins involved in the germination process.

The changes in the levels of proteins in germinated spores are related to the DPA release by the GR-independent germinants (Bevilacqua *et al*., 2015; Wang *et al*., 2015; Porębska *et al*., 2015b; 2016b; 2016c) which was also confirmed when monitoring spore germination by measuring optical density at an early stage of spore germination (Porębska *et al*., 2015a).

The developments in new technologies, combined with advances in understanding the mechanisms of bacterial spore resistance and germination, justify the need to summarize the current knowledge of these molecular mechanisms to help identify novel strategies to inactivate bacterial spores using innovative technology (Troiano *et al*., 2015; Stewart & Setlow, 2013). Meanwhile, our knowledge on the mechanism of *A. acidoterrestris* germination is still very vague and limited. Selecting a group of proteins involved in the germination process of *A. acidoterrestris* spores by determining their level before and after exposure to an activating agent is important for understanding this complex phenomenon.

Until now, no studies have been reported on the analysis of germination proteins of *A. acidoterrestris* spores subjected to HHP, SCCD and nutrient germinants.

CONCLUSION

The results obtained in this study indicate that germination of *A. acidoterrestris* spores subjected to HHP and SCCD for different time periods and to nutrient germinants, reflect the number of spores which start to germinate and thus the proteins are more accessible for extraction, but overall the amount of proteins in the spore remains the same, independent of the way of breaking the dormancy state. Moreover, these results indicate that the external factors' influence level the amount of released proteins by the spores, visualized by SDS-PAGE gels.

The results obtained indicate that changes in the germination process of spores are visible at molecular level and that the level of proteins from untreated ungerminated spores was smaller than the amount of proteins which was released from the treated, germinated spores. These results may be a prelude to expanding our current knowledge about the complex multifactorial processes of sporulation and spore germination, and in the future help in analysis of the mechanism regulating gene e*xpression* involved in these processes.

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