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Sensitivity of *Bacillus weihenstephanensis* to acidic changes of the medium is not dependant on physiological state

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ABSTRACT

This study aims to quantify the effect of salt and acid preliminary exposure on acid resistance of vegetative cells of *Bacillus weihenstephanensis*. The psychrotolerant strain KBAB4 was cultured until the mid-exponentially phase (i) in BHI, (ii) in BHI supplemented with 2.5% salt or (iii) in BHI acidified at pH 5.5 with HCl. The growing cells were subsequently inactivated in lethal acid conditions ranging from 4.45 to 4.70. Based on statistical criteria, a primary mixed-Weibull model was used to fit the acid inactivation kinetics. The acid resistance was enhanced for acid-adapted cells and decreased for salt-adapted cells. The secondary modelling of the bacterial resistance allowed the quantification of the change in pH leading to a ten folds variation of the bacterial resistance, *i.e.* cells sensitivity (z_{pH}). This sensitivity was not significantly affected whatever the preliminary mild exposure and the presence of sub-populations with different acid resistances. These results highlighted that pre-incubation conditions influence bacterial acid resistance without affecting the sensitivity to acidic modifications, with a 10 fold reduction of *Bacillus* acid resistance observed for a reduction of 0.37 pH unit. Quantification of such adaptive stress response might be instrumental in quantitative risk assessment more particularly in food formulation, particularly for low-acid minimally processed foods.

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1. Introduction

Spore forming bacteria cause major problems in the food industry since they may be involved in both food poisoning and food spoilage, raising major safety and economical issues. The *Bacillus cereus* group consists of six closely related species: *Bacillus anthracis*, *Bacillus thuringiensis*, *Bacillus mycoides*, *Bacillus pseudomycoides*, *Bacillus weihenstephanensis* and *B. cereus* (*sensu stricto*). *B. cereus* and *B. anthracis* are known as human pathogens, *B. thuringiensis* is used as biopesticide, *B. mycoides* and, *B. pseudomycoides* are characterised by rhizoidal formations when grown on agar medium. The *B. weihenstephanensis* specie includes strains which can growth under chilled temperature (Lechner et al., 1998) leading major problem in ready-to-eat or ready-to-cook foods stored at chilled temperatures, also known as refrigerated processed foods of extended durability (REPFEDs).

The heat resistance of *Bacillus* spore was extensively studied and is used for FSO establishment (Membre et al., 2006). The influence of sporulation environment of *Bacillus* (Baril et al., 2011a,b, 2012; Garcia et al., 2010; Gonzalez et al., 1999; Planchon et al., 2011) is known to have a great impact on spore resistance properties and spore formation abilities. Recently, Baril et al. (2012) reported that sporulation boundaries of *B. weihenstephanensis* were included within the range of temperatures, pH and water activities supporting growth. A decrease in spore heat resistance and sporulation rate was observed for spores of *B. weihenstephanensis* produced for non optimal growth conditions. For instance, the time to get one spore per ml was tenfold longer when sporulation occurred at 10 °C rather than 30 °C (Baril et al., 2011a). Since a low sporulation rate and low resistance of spores was observed under sublethal conditions, *Bacillus* vegetative cells resistance appears as key issue for food industry particularly for minimally processed food through the food process and storage.

To ensure the microbial food safety and stability, the food industry uses combination of hurdles, mild preservation factors (Leistner and Gorris, 1995). A commonly used hurdle is a low pH which allows the decrease in growth rate but also the inactivation of pathogens or spoilage microorganisms (Coroller et al., 2006; Greenacre et al., 2003; Ita and Hutkins, 1991). For low-acid minimally processed foods, the main microbiological hazard is the

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presence of psychrotrophic, sporulated bacteria (Valero et al., 2000) among which B. weihenstephanensis is representative. Although B. weihenstephanensis spoilage ability which limit shelf life product and generate economic losses (Abee and Wouters, 1999; Andersen Borge et al., 2001; Priest, 1993) is well established, its pathogenicity remains uncertain. Indeed some B. weihenstephanensis strains, among which the KBAB4 strain, may be cytotoxic (Lapidus et al., 2008; Hoton et al., 2009; Réjasse et al., 2012; Stenfors et al., 2002). Thus, in this study the B. weihenstephanensis KBAB4 strain was used as bacterial model.

The aim was to investigate the acid resistance of vegetative cells with (adapted cells) and without (non-adapted cells) preliminary exposure to mild stress conditions. Since physiological state may greatly influence the bacterial resistance of vegetative cells, adaptation to mild saline and acid stress conditions were tested. To further apprehend the impact of lethal pH conditions on the acid resistance, the sensitivity was also determined.

2. Materials and methods

2.1. Bacterial strain pre-incubation and adaptation conditions

The psychrotolerant B. weihenstephanensis KBAB4 strain, kindly provided by the Institut National de la Recherche Agronomique (INRA, Avignon, France), was used throughout this study. The strain was stored at −80 °C in brain heart infusion (BHI, Biokar Diagnostic, Beauvais, France) supplemented with 50% (v/v) glycerol. Firstly, bacteria (1 ml of glycerol stock) were grown in 100 ml of BHI broth incubated at 30 °C for 8 h under shaking conditions (100 rpm) and an aliquot (1 ml) was transferred into a second flask of 100 ml BHI broth incubated for 15 h in the same conditions. For non-adapted cells (control), a portion (0.1 ml) of the pre-culture was transferred in a flask containing 100 ml of BHI broth and incubated at 30 °C with shaking at 100 rpm, until the cells reached the mid-exponentially phase ($OD_{600\ nm}$ values of 0.20 ± 0.02 meaning approximately 10^7 CFU ml^{-1}). In order to test different physiological state on the bacterial resistance behaviour, preliminary mild stress exposure was used. For mild stress adapted cells, the transfer was done in BHI supplemented with HCl (pH 5.5) or NaCl (2.5% w/v) and incubated at 30 °C under shaking conditions (100 rpm) until a $OD_{600\ nm}$ values of 0.15 ± 0.03 corresponding to around 10^7 CFU ml^{-1}. Bacterial enumeration was done on Nutrient Agar (Biokar Diagnostic) using an SPIRAL plater (AES Chemunex, Combourg, France) and serial dilutions using Tryptone Salt (Oxoid, Dardilly, France). Plates were subsequently incubated for 16–24 h at 30 °C.

2.2. Acid inactivation

Adapted and non-adapted mid-exponentially cells were subsequently submitted to 6 acid lethal conditions with a pH ranging from 4.40 to 4.7. At appropriate time from 5 min to 6 h, samples were performed to acquire inactivation kinetics. The survivor enumeration was done according the ISO 7218 (2007). Survivors were quantified on Nutrient Agar (Biokar Diagnostic) using an SPIRAL plater (AES Chemunex) and serial dilutions in Tryptone Salt (Oxoid). More than 20 CFU per petri dishes were counted leading to a detection limit equal to 400 CFU ml^{-1}. Plates were subsequently incubated for 16–24 h at 30 °C. Acid inactivation kinetics were performed in three biological replicates from independent frozen cells batches. To ensure the absence of spores in both pre-incubation and inactivation media, aliquots of 2 ml were submitted to heat treatment at 70 °C for 5 min (Baril et al., 2011b) and potential germinating spores were then quantified on nutrient agar (Biokar Diagnostic) medium at 30 °C after 24 h incubation.

2.3. Estimation of the bacterial resistance

Three primary mathematical models were used to fit the inactivation kinetics.

(i) The first-order model was as follow

$$\log_{10}N(t) = \log_{10}N_0 - \frac{t}{D} \quad (1)$$

where $N(t)$ is the concentration of survivors expressed in $\log_{10}$ CFU ml^{-1} at time 't'; N_0 is the inoculum concentration in $\log_{10}$ CFU ml^{-1}; t is the time of exposure to stress (in h) and D is the time necessary to obtain the logarithm decrease of the bacterial population.

(ii) The Weibull model was as follows:

$$\log_{10}N(t) = \log_{10}N_0 - \left(\frac{t}{\delta}\right)^p \quad (2)$$

where δ is the first decimal reduction time (h) and p is a shape parameter.

(iii) The biphasic mixed-Weibull model, which assumed the existence of two sub-populations, one more sensitive and one more resistant sub-population (Coroller et al., 2006), was determined with the following equation:

$$N(t) = \frac{N_0}{1+10^{\alpha}}\left[10^{-\left(\frac{t}{\delta_1}\right)^p+\alpha} + 10^{-\left(\frac{t}{\delta_2}\right)^p}\right] \quad (3)$$

Similarly, p and δ parameters represent the shape parameter and the first decimal reduction time, respectively. Index 1 refers to the more sensitive sub-population while index 2 refers to the more resistant sub-population. α corresponds to $\log_{10}(N_{01}/N_{02})$ and is function of the proportion of each sub-population in the inoculum N_0. For instance, when $\alpha = 2$, this means that 1 inoculated cell belongs to sub-population 2, while 100 cells belongs to the sub-population 1; and when $\alpha = 3$, this means that 1 inoculated cell belongs to the sub-population 2, while 1000 belongs to the sub-population 1.

2.4. Primary model fitting performances

The three models were used to fit the inactivation kinetics obtained for three replicates, for each experimental condition, using the minimization of the sum of square error. The minimum values were computed with a non linear fitting module (LSQCURVEFIT, MATLAB 6.5.1, Optimization Toll box, The Mathworks, Massachusetts, USA). To evaluate the model fitting performances and to select the most adequate model the r^2, the MSE, the AIC_C and the F test were calculated as indicated below. The regression coefficient r^2 is the proportion of the total variation of the data explained by the model. The value shall range between 0 and 1. The higher the value is, the better the fitting of the model is (den Besten et al., 2006). Regarding the mean square error of the model value (MSE_{model}), the lower the value is, the better the model is to describe the experimental data (den Besten et al., 2006). The corrected Akaike Information Criterion (AIC_C) (Hurvich and Tsai, 1995), allows comparison of models by taking both the goodness of fit and the parsimony into account. The lower the AIC_C value is, the best the model is. The F test was used to decide whether the fitting performance of a model was statistically accepted. The f value was

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