Innovative Food Science and Emerging Technologies 34 (2016) 250–258

Contents lists available at ScienceDirect

Innovative Food Science and Emerging Technologies

journal homepage: www.elsevier.com/locate/ifset

Inhibition of nutrient- and high pressure-induced germination of *Bacillus cereus* spores by plant essential oils

Jorinde Corthouts, Chris W. Michiels *

Laboratory of Food Microbiology, Leuven Food Science and Nutrition Research Center (LFoRCe), Department of Microbial and Molecular Systems (M^2S), KU Leuven, Kasteelpark Arenberg 22, B-3001 Heverlee, Belgium

ARTICLE INFO

Article history:
Received 18 December 2015
Received in revised form 3 February 2016
Accepted 24 February 2016
Available online 6 March 2016

Keywords:
Essential oils
High pressure
Bacillus cereus spores
Germination
Hurdle technology

ABSTRACT

The efficiency of high-pressure (HP) treatment to eliminate vegetative bacterial cells is synergistically increased by many natural antimicrobials, but the effects on spores are poorly described. Here we report the effect of eleven plant essential oils on the nutrient- and HP-induced germination of spores of a group VI psychrotolerant *Bacillus cereus* strain. Ten oils partially inhibited nutrient-induced germination. These oils also inhibited HP-induced germination, but some inhibited only germination at moderate (200 MPa) pressure and others only at very high (600 MPa) pressure. Inhibition of spore germination by essential oils may have an adverse effect on the effectiveness of spore inactivation by HP at moderate temperatures, and this should be taken into account when designing combined processes. Essential oil from carrot seed did not inhibit nutrient or HP germination although it showed growth inhibitory properties, and essential oils with these properties may therefore open interesting perspectives in combination treatments with HP.

Industrial relevance: HP treatment is an alternative processing technique that preserves a better balance of food quality and microbiological safety as compared to thermal processing. While most vegetative bacteria are efficiently inactivated by HP, inactivation of spores is inefficient. At moderate temperature, spore inactivation proceeds in a two-step process in which spores first germinate and are subsequently inactivated. The combination with natural antimicrobials is a promising approach to enhance the efficiency of HP processing because it exerts a synergistic effect on inactivation of vegetative bacteria. However, the current work is one of the first to document the effect of essential oils on the HP-induced germination of spores.

1. Introduction

Bacillus cereus is a Gram-positive, spore-forming, facultatively aerobic bacterium that is ubiquitous in nature and therefore present in many agricultural commodities. Consequently, the organism is also frequently isolated from a wide range of foods, including vegetables, starchy foods, meat, and dairy products. Growth of *B. cereus* in foods can cause spoilage, but also an emetic and a diarrheal type of foodborne illness. The emetic disease is caused by cereulide, a highly heat-, acid-, and protease-resistant toxin that is preformed in the food. It is mostly associated with starchy foods and causes emesis within 1–6 h after consumption. The diarrheal disease, on the other hand, is caused by outgrowth and production of heat labile enterotoxins by the pathogen in the small intestine 8–16 h after the consumption of contaminated food. The diarrheal strains produce multiple enterotoxins, but the contribution of each of these to the disease is not yet clear. The symptoms include abdominal pain and watery diarrhea and mainly protein-rich foods are incriminated (Ehling-Schulz, Fricker, & Scherer, 2004; Granum & Lund, 1997; Stenfors Arnesen, Fagerlund, & Granum, 2008).

Together with six genetically related species, *B. cereus* strains that cause foodborne disease make up a species complex designated as *B. cereus sensu lato*. The species within this complex have been historically defined based on practically relevant phenotypes such as their ability to cause disease, the type of disease, and the host, physiology and morphology. However, this species delineation does not entirely coincide with a more recently proposed classification in seven phylogenetic groups based on molecular, phenotypic, and descriptive data. Strains of *B. cereus sensu stricto*, which cause the above described emetic and diarrheal disease, are dispersed over phylogenetic groups II, III, IV, and V. Groups II and VI comprise psychrotolerant strains able to grow at temperatures down to 7 °C (II) or even lower (VI) (Guinebretière et al., 2008). Psychrotrophic strains are an increasing concern in minimally processed refrigerated foods (Daelman et al., 2013), although their spores are generally less heat resistant than those of mesophilic strains (Luu-Thi, Khadka, & Michiels, 2014), and although their importance in the etiology of *B. cereus* foodborne disease is not yet clear (Ceuppens, Boon, & Uyttendaele, 2013; Guinebretière et al., 2010).

The increasing demand for high-quality, natural, and fresh-like foods that are free of additives, has stimulated the exploration of alternative processing techniques that preserve a better balance of food quality and microbiological safety as compared to thermal processing. A

* Corresponding author. Tel.: +32 16 321578; fax: +32 16 321960.
E-mail address: Chris.michiels@biw.kuleuven.be (C.W. Michiels).

http://dx.doi.org/10.1016/j.ifset.2016.02.011
1466-8564/

technology that has been successfully adopted by the food industry is high-pressure (HP) processing, and the ability of HP treatment to inactivate spoilage and pathogenic micro-organisms with only limited effect on food texture, color, and nutritional value has been amply documented. However, bacterial spores are only efficiently inactivated by HP treatments at process temperatures of 90–120 °C (Ahn, Balasubramaniam, & Yousef, 2007; Luu-Thi, Grauwet, Vervoort, Hendrickx, & Michiels, 2014; Ramaswamy, Shao, & Zhu, 2010; Reddy, Tetzloff, Solomon, & Larkin, 2006; Zhu, Naim, Marcotte, Ramaswamy, & Shao, 2008). Combined HP high-temperature processes have been elaborated that allow the production of shelf-stable low acid foods, but the thermal component of these processes inevitably causes some quality deterioration. At moderate or low temperature, HP inactivation of spores is inefficient and is considered to be a two-step process in which spores first germinate and are subsequently inactivated (Gould & Sale, 1970). In *Bacillus subtilis*, HP-induced germination follows different routes depending on the pressure applied. Moderately high-pressure (MHP) (50–300 MPa), triggers germination via activation of the germinant receptors present in the spore membrane, even in the absence of their cognate germinant molecules. Very high pressure (VHP) (400–800 MPa), on the other hand, causes DPA release from the spore core via a mechanism that is not yet understood, but that is independent of the germinant receptors. DPA then triggers events further down in the germination process, like the activation of a cortex hydrolase. The completion of *B. subtilis* spore germination and the onset of outgrowth of the germinated spores upon VHP treatment is retarded compared to MHP-treated spores, and VHP-germinated spores therefore retain resistance to some lethal agents or treatments for a longer time after the treatment (Black et al., 2007a; Reineke et al., 2012; Setlow, 2003; Wei et al., 2010; Wuytack & Michiels, 2001; Wuytack, Soons, Poschet, & Michiels, 2000). Whether distinct MHP and VHP spore germination pathways also exist in other bacilli, including *B. cereus*, is not known.

The HP inactivation of vegetative bacteria, including highly pressure-resistant strains of enterohemorrhagic *Escherichia coli* and other pathogens, can be increased by combining HP treatment with natural antimicrobials, and this is an interesting avenue to address the increasing demand for natural alternatives to the traditional food preservatives. Plant essential oils have received particular attention because of their strongly synergistic enhancement of inactivation by HP. This synergy is of interest because it allows to reduce essential oil concentrations in specific applications, and thus to reduce the off-flavors that they may impart (Espina, Garcia-Gonzalo, Laglaoui, Mackey, & Pagan, 2013; Feyaerts, Rogiers, Corthouts, & Michiels, 2015; Karatzas, Kets, Smid, & Bennik, 2001; Ogawa, Matsuzaki, & Isshiki, 1998; Pina-Pérez, Silva-Angulo, Muguerza-Marquínez, Aliaga, & López, 2009; Pulido, del Arbol, Burgos, & Galvez, 2012; Somolinos, García, Pagán, & Mackey, 2008). Up until now, the effects of natural preservatives in combination with HP treatment at moderate temperatures (≤ 40 °C) on bacterial spores are poorly documented. The addition of olive powder resulted in no extra inactivation for *B. cereus* spores (Marco et al., 2011), while nisin enhanced the inactivation of spores from *Alicyclobacillus acidoterrestris*, *B. subtilis*, and *B. cereus* (Black et al., 2008; López-Pedemonte, Roig-Sagués, Trujillo, Capellas, & Guamis, 2003; Sokolowska et al., 2012). Interestingly, Black et al. (2008) found that nisin enhanced HP-induced spore germination in milk. However, we have not found similar studies with essential oils. It has been shown that specific organic molecules like organic acids, fatty acids, and alcohols inhibit nutrient-induced spore germination (Ababouch, Bouqartacha, & Busta, 1994; Cortezzo, Setlow, & Setlow, 2004; Trujillo & Laible, 1970; van Melis, Almeida, Kort, Nierop Groot, & Abee, 2012; Yasuda-Yasaki, Namiki-Kanie, & Hachisuka, 1978), while some others like calcium–dipicolinate or alkyl amines induce germination in absence of nutrient germinants (Paidhungat, Ragkousi, & Setlow, 2001; Rode & Foster, 1961). Therefore, the possibility that essential oils may affect spore germination by HP should be considered, and such effects should be taken in account when designing combined HP–essential oil processes.

The present study therefore aims to investigate the effects of a wide variety of essential oils on the spores of a group VI psychrotolerant *B. cereus* strain. First, the minimum inhibitory concentration (MIC) for both vegetative cells and spores is determined for 67 essential oils. Then, the effects of 11 selected essential oils on nutrient, MHP and VHP spore germination are investigated.

2. Materials and methods

2.1. Bacterial strain, culture conditions, and spore production

Experiments were conducted with strain *B. cereus* INRA TZ415 (INRA C1), belonging to the psychrotolerant phylogenetic group VI (kindly provided by M.H. Guinebretière, INRA, Avignon, France). Stationary phase cultures were obtained by aerobic growth with shaking (200 rpm) for 22 h at 30 °C in Brain Heart Infusion (BHI; Oxoid, Hampshire, U.K.) broth. For spore production, a singly colony from BHI agar was first inoculated in 4 ml BHI broth. After growing for 48 h at 30 °C and 200 rpm, the culture was tenfold diluted in sterile deionized water, and 100 μl of this suspension was surface-plated on Nutrient Agar (NA No.2; Oxoid). Plates were examined microscopically until more than 95% of the cells consisted of phase bright spores, usually after 7 days of incubation at 30 °C. Spores were then harvested from the agar surface in 1.5 ml deionized water, washed three times in deionized water, collected by centrifugation at 4000×*g* for 10 min and finally suspended in deionized water. Spore suspensions were stored at 4 °C and used within 1 month. Microscopic examination and plate counts of heated (70 °C for 20 min) and unheated suspensions indicated that >96% of the cells were heat-resistant spores.

2.2. Essential oils

Essential oils were purchased from Anthémis Aromatherapie (Oosterstreek, The Netherlands). The composition of each oil as indicated by the supplier is listed in the supplementary data (Table S1). A stabilized stock emulsion of 10% (v/v) of each essential oil was made in deionized water containing 0.2% (w/v) bacteriological agar (LabM, Lancashire, U.K.) and further dilutions were made just before use in BHI broth. Sorbic acid (Fluka, Buchs, Switzerland) was also used as inhibitor of spore germination at concentrations of 0.01–0.11% (w/v). In this case, the pH of the BHI broth was adjusted to 6.5 with HCl.

2.3. MIC determination by broth dilution method

Vegetative cells from a stationary phase culture or spores from a spore stock were collected by centrifugation at 4000 × *g* for 5 min and washed in 10 mM potassium phosphate buffer (pH 7.0). Subsequently, the vegetative cells were diluted 1/1000 in fresh BHI broth to approximately 10^6 cfu/ml. Spore suspensions were first heat activated at 70 °C for 20 min and then diluted in fresh BHI broth to approximately 10^5 cfu/ml. Hunderd μl portions of these suspensions were then transferred to a 96-well microtiterplate, and 100 μl of diluted essential oil solutions were added to achieve final concentrations of 0.05, 0.10, 0.50, and 1.00% (v/v). The optical density at 600 nm (OD_{600}) of the cell suspensions was measured at the start of the experiment and again after 48 h of incubation at 30 °C. This temperature was chosen because it is common to determine MIC values at (near-)optimal growth temperature. Blanks (BHI broth + essential oil), positive (BHI broth + cell/spore suspension) and negative controls (BHI broth) were included. The MIC value was defined as the lowest concentration required for complete inhibition of growth after 48 h (OD_{600} increase after 48 h < 0.1), and calculated as a mean value from three independent experiments.