CHARACTERIZATION OF SUPERDORMANT SPORES OF BACILLUS CEREUS AND BACILLUS WEIHENSTEPHANENSIS

by

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A thesis submitted to the Faculty of the University of Delaware in partial fulfillment of the requirements for the degree of Master of Science in Food Science

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by

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ABSTRACT

Superdormant spores are those which germinate extremely slowly compared to the rest of the spore population. Although the exact cause of spore superdormancy is not known, evidence suggests that superdormant spores have a fewer nutrient germinant receptors compared to dormant spores. The recent development of a method to isolate superdormant spores in a laboratory setting has made it possible to study the phenomenon of spore superdormancy. It is important to investigate the causes of spore superdormancy and how we can reduce the number of these spores in foods. Differences in the isolation of superdormant and heterogeneous spore populations of psychrotolerant strains of *Bacillus cereus* and *Bacillus weihenstephanensis* and one mesophilic strain of *B. cereus* were studied.

The effects of sporulation temperature on the isolation of superdormant spores using various concentrations of individual nutrient germinants were investigated. The average recovery of superdormant spores was 17.79 % with the greatest average recovery with 250 µM L-asparagine and the lowest with 10 mM L-alanine. Spores of psychrotolerant strains showed the same germination rates as the mesophilic strain (p = 0.7001). Overall superdormant spore recovery was not significantly different with spores initially sporulated at 30°C compared to those initially sporulated at 37°C (p=0.4976).

Aqueous ozone has been demonstrated to have high potential for the inactivation of bacterial spores in foods. The effects of 190 ppm and 6.25 ppm of ozone on the recovery of superdormant spores compared to ozone-treated
heterogeneous spore populations of all three strains was also studied. The percent recovery of spores for all strains and spore type was significantly reduced after 20 min of ozone treatment (p = 0.0001). Superdormant spores were identified to be approximately 20 % more resistant to ozone treatment than heterogeneous spore populations (p = 0.0234). In addition, psychrotolerant strains were significantly more resistant to ozone treatment than the mesophilic strain. A trend for the percent recovery of spores to increase above the control (above 100 % recovery) occurred commonly for superdormant spores exposed to lower levels of ozone for shorter-time intervals.

The potential of an ozone-high hydrostatic pressure (HHP) hurdle technology model was explored. Superdormant spores that were or were not pretreated with 190-ppm aqueous ozone for 10 min were subsequently treated at 500 MPa and 40°C for 10 min. The recovery of ozone-pretreated superdormant spores subsequently treated with HHP was not lower than the recovery of spores that were not pretreated with ozone (p = 0.7380). There was no significant difference in the percent recovery of superdormant spores after ozone and HHP treatment between psychrotolerant and mesophilic strains (p = 0.4865).

The results of this study help to further characterize superdormant spores and the cause of spore superdormancy. It is important to understand spore superdormancy in order to better control superdormant spores in the food industry. Hurdle technologies, including the proposed ozone-HHP technology, have great implications for the control of dormant and superdormant spores in foods and should continue to be explored.
Chapter 1

INTRODUCTION

It is estimated that there are an average of 63,623 illnesses and 20 hospitalizations caused by \textit{B. cereus} every year in the United States (Scallan et al., 2011). \textit{Bacillus cereus} is a motile gram-positive sporeforming bacterium found throughout nature but most commonly in soil and on plants (Priest et al., 2004; Valero et al., 2003). The emetic toxin of \textit{B. cereus} is heat-stable, can be produced in food, and remains stable after cooking or heating.

Over the past two decades there has been a significant increase in demand by consumers for convenient food products of high quality, which are commonly referred to as ready-to-eat, cooked or chilled, or refrigerated processed foods of extended durability (RPFEDs) (Nissen et al., 2002). Mild processing techniques can allow sporeforming bacteria such as \textit{Bacillus} and \textit{Clostridium} species to survive in the food product (Samapundo et al., 2010). Pathogenic psychrotolerant \textit{Bacillus} species may not only have the ability to germinate and grow at refrigeration temperatures (Valero et al., 2003; Samapundo et al., 2010) but may also have the ability to produce toxin in foods. Evolving species, such as psychrotolerant strains of \textit{B. cereus}, are of even greater concern because they have adapted to growth at reduced temperatures. A new species named \textit{Bacillus weihenstephanensis} was proposed to accommodate the psychrotolerant strains of \textit{B. cereus} (Letchner et al., 1998) that are able to grow optimally at temperatures below 10°C.
Bacillus species are sporeformers and no other life form is as difficult to eliminate as bacterial endospores. Sporeformers can serve as spoilage agents, as well as vectors for disease. Spores of Bacillus species are of considerable concern in the food industry, due to their common occurrence and extreme resistances to current food processing methods including heat, pressure, acidification, desiccation, and chemical disinfectants (Setlow, 2003; Coleman et al., 2007; De Vries et al., 2004). Spore formation is triggered by nutrient depletion whereby a vegetative cell enters dormancy, but is still able to respond to various agents in its environment including temperature, pH, and the presence of nutrients (Setlow, 2003; Wei et al., 2010).

Superdormant spores are those which germinate at a much slower rate compared to the general spore population. A simple method for the isolation of superdormant spores, developed by Ghosh and Setlow (2009a) for spores of B. subtilis, B. megaterium, and B. cereus, has enabled the study of superdormant spores in a laboratory setting. This method, called buoyant density centrifugation, separates dormant spores from germinating spores and debris. It is currently hypothesized that the cause of superdormancy is due to a lack or lack of expression of germinant receptors on the spore’s inner membrane (Ghosh and Setlow, 2009a,b; Wei et al., 2010, Zhang et al., 2010). It is extremely important to determine the factors which influence the level of spore superdormancy in order to more effectively control the incidence of bacterial spore contamination in the food industry.

Nonthermal processing technologies including ozone and high hydrostatic pressure have implications for the inactivation of spores while maintaining the sensory qualities of fresh food products in order to meet consumer demands. Aqueous ozone has a higher potential than most oxidizing agents to kill spores (Menzel, 1971; Kim et
al., 2003) and has the potential to improve more costly regimens, such as HHP, to inactivate intact spores in food (Young and Setlow, 2004). HHP technology also has a high potential for reducing bacterial spores in foods; however, the incomplete germination of spores often observed with HHP is currently being investigated by food processors. It may be possible to more effectively control the incidence of bacterial spore contamination in fresh foods using hurdle technologies including the combined use of ozone and HHP.
Chapter 2

LITERATURE REVIEW

2.1 Bacillus cereus and its endospores

*Bacillus cereus* is a motile gram-positive sporeforming bacterium that is a well-established foodborne pathogen (Kotiranta *et al.*, 2000; De Vries *et al.*, 2004; Chorin *et al.*, 1997). It is found throughout nature but is most commonly isolated from soil and plants (Priest *et al.*, 2004; Valero *et al.*, 2003). Foodborne illnesses caused by *B. cereus* are directly related to the production of two toxin types: An emetic-type enterotoxin and a group of several diarrheogenic-type enterotoxins (Chorin *et al.*, 1997; De Vries *et al.*, 2004). The enterotoxins cause different gastrointestinal illnesses including diarrhea and emesis (Lucking *et al.*, 2009). The emetic-type toxin, also known as cereulide, is a thermostable cyclic peptide (Lucking *et al.*, 2009; De Vries *et al.*, 2004). The enterotoxins responsible for the diarrheogenic symptoms caused by *B. cereus* are hemolysin (Hbl), non-hemolytic enterotoxin (Nhe), and cytotoxin (CytK) (Lucking *et al.*, 2009; Ehling-Schulz *et al.*, 2005). The cell wall of vegetative *B. cereus* is also covered by proteins (called the S-layer) that plays a role in cell adhesion and attributes to virulence of the organism (Kotiranta *et al.*, 2000).

Since the emetic toxin of *B. cereus* is heat-stable, it can remain stable after cooking or heating. Cereulide production does not occur until stationary growth phase,
and therefore, high counts of vegetative cells or spores able to germinate in foods are required for cereulide intoxication to occur (Thorsen et al., 2009).

Cereulide toxin is absorbed from the gut into the bloodstream and induces emetic-like symptoms including nausea and vomiting through stimulation of the vagus nerve (Jääskeläinen et al., 2003). Ingestion of approximately ≤ 8 µg kg⁻¹ body weight of cereulide toxin within a food product is required to cause illness in humans (Jääskeläinen et al., 2003). Cereulide toxin affects the mitochondria by acting as a potassium ion channel-former (Mikkola et al., 1999; Lucking et al., 2009; De Vries et al., 2004) and causes apoptosis of human natural killer (NK) cells (Paananen et al., 2002). Mesophilic strains of B. cereus can only produce cereulide at temperatures above 10 to 15°C (Thorsen et al., 2009). This can explain why the toxin is mostly associated with foods that are improperly cooled and stored, such as rice and pasta.

The diarrheogenic toxin is heat-labile and can be destroyed by food processing. This toxin is produced during the exponential growth phase and can cause intoxication when present in raw or minimally processed foods that do not require heating (Fermanian et al., 1994). Since spores of B. cereus are capable of surviving heat treatment and the acidic environment of the stomach, diarrheal-like symptoms can occur when spores of B. cereus are consumed in a raw or unprocessed food product and enter the small intestines where spores can germinate and multiply enabling the production of the diarrheogenic toxin. Depending on the amount of bacteria present in the food product, sometimes both sets of symptoms (emesis and diarrhea) can develop. This phenomenon is known as two-bucket disease such as that occurs with Staphylococcus aureus intoxication. In either case, symptoms from either B. cereus toxin should resolve within twenty-four to forty-eight hours from onset.
The foods most frequently associated with *B. cereus* intoxication include milk, vegetables, rice, potatoes, grains, cereals (including batters, mixes and breading), spices and various sauces (Doona and Feeherry, 2007). *Bacillus cereus* is not nutritionally fastidious, which is why *B. cereus* can replicate in soil and the low-nutrient foods including rice and pasta (Kotiranta *et al*., 2000). The reservoir for *B. cereus* is in the soil, where transmission of the organism can occur through various vectors (Figure 1) (Abee *et al*., 2011). Transmission of *B. cereus* to humans can occur through contact with any of these vectors; however, the most common vector is through food. As with many self-limiting foodborne gastrointestinal illnesses, people experiencing *B. cereus* intoxication usually do not seek medical attention due to the generally short duration of the illness and non-fatal symptoms. Even if medical attention is given, the illness is not reportable (Kotiranta *et al*., 2000). Lack of testing, reporting, and surveillance of the illness has led to underestimation in the actual incidence of foodborne illness caused by *B. cereus*. 
The Bacillus cereus group is comprised of six species: B. cereus, B. anthracis, B. thuringiensis, B. mycoides, B. pseudomycoiides, and B. weihenstephanensis. The members of the B. cereus group are nearly impossible to distinguish phenotypically from each other (Kotiranta et al., 2000). It has even been suggested through genetic evidence that members of the B. cereus group may actually represent one single species (Auger et al., 2009; Priest et al., 2004; Ehling-Schulz et al., 2005). Several epidemiological studies have been performed comparing genetic sequences of a variety of environmental and food B. cereus isolates. The authors of these studies reported that psychrotolerant B. cereus isolates were more genetically similar to other psychrotolerant species, including B. mycoides, than to mesophilic B.
cereus species which were identified to be more genetically similar to *B. thuringiensis* (Schraft *et al.*, 1996; Daffonchio *et al.*, 2000; Sorokin *et al.*, 2006). Therefore, it was suggested that the taxonomy of the *B. cereus* group be revised. In 1998, a new species named *Bacillus weihenstephanensis* was proposed to accommodate the psychrotolerant strains of *B. cereus* (Letchner *et al.*, 1998).

2.2 *Bacillus weihenstephanensis*

*B. weihenstephanensis* is differentiated from *B. cereus* by its ability to grow aerobically at 7°C in liquid culture and the absence of ability to grow at 43°C (Letchner *et al.*, 1998). Genetically, *B. weihenstephenensis* can be differentiated by the presence of the 16S rDNA signature sequence 1003-TCTAGAGATAGA and the signature sequence of the major cold-shock gene *cspA*, 4-ACAGTT (Letchner *et al.*, 1998). Other research shows that not all strains of psychrotolerant *B. cereus* can be classified as *B. weihenstephanensis* and that there is an intermediate form between the two species (Stenfors *et al.*, 2001). These intermediate forms produce both mesophilic and psychrotolerant genetic (PCR) products (Stenfors *et al.*, 2001). For example, in a study conducted by Stenfors *et al.* (2001) *B. cereus* strains identified as mesophilic by PCR demonstrated the ability to grow at 6°C and other strains containing the *cspA* signature sequence were able to grow at 43°C. Specific guidelines need to be established in order for researchers to be able to distinguish mesophilic and psychrotolerant species of *B. cereus* and *B. weihenstephanensis*.

In order to assess whether *B. weihenstephanensis* may serve as a hazard in refrigerated food products, it must first be determined whether this species has the ability to produce toxin, and if so, at what temperatures cereulide toxin can be
produced. This is especially important since this toxin is heat stable and cannot be destroyed during cooking and food processing. One *B. weihenstephanensis* strain isolated from whole liquid egg product was able to produce cytotoxin in whole liquid egg product at 6, 8, and 10°C, but not at 4°C; however, the isolate did not contain the *cesB* gene, which codes for cereulide production (Baron *et al.*, 2007). Environmental isolates of *B. weihenstephanensis* were able to produce the emetic toxin at temperatures as low as 8°C in food; however, toxin was not produced at levels great enough to cause illness (Thorsen *et al.*, 2006), approximately ≤ 8 µg kg⁻¹ body weight (Jääskeläinen *et al.*, 2003). Strains of *B. weihenstephanensis* have been demonstrated to contain the gene responsible for cereulide production (*cesB*) (Thorsen *et al.*, 2006; 2009. Although *B. weihenstephanensis* has not been demonstrated to be able to produce cereulide at recommended refrigeration temperatures (4°C), temperature abuse can often occur during food shipping, distribution, and storage. Because *B. weihenstephanensis* has demonstrated the ability to grow at 6°C and produce toxin at 8°C, this bacteria should be identified as a potential hazard for refrigerated foods which are commonly subject to temperature abuse.

Because of their potential to grow in refrigerated food products, their ability to produce toxins, and their implications in foodborne outbreaks, psychrotolerant species of *B. cereus* including *B. weihenstephanensis* have been of high concern in the food industry (Baron *et al.*, 2007). *Bacillus weihenstephanensis* is a causative agent of spoilage in white liquid egg products (Baron *et al.*, 2007). The *B. weihenstephanensis* strain isolated from the spoiled whole liquid egg product also demonstrated the ability to stick and form biofilms on processing equipment commonly used in egg breaking factories including stainless steel, model hydrophilic
materials (glass), and model hydrophobic materials (polytetrafluoroethylene) (Baron et al., 2007).

2.3 Foodborne illness estimates, outbreaks, and recalls

It is estimated that there are an average of 63,623 illnesses and 20 hospitalizations caused by *B. cereus* every year in the United States (Scallan et al., 2011). Foodborne illness caused by *B. cereus* is often underreported; however, recalls, illnesses, and even deaths caused by the organism have been documented within the past few years. According to the Food and Drug Administration (FDA, 2009), on December 3, 2009 a voluntary recall was conducted on all Unilever Slim-Fast™ ready-to-drink (RTD) products in cans, due to the possibility of contamination with *B. cereus*. Sonoma Beverage recalled their Metromint Flavor Water™ on December 4, 2007 due to possible *B. cereus* contamination (Santa Cruz Health Services Agency, 2007). According to *The Sydney Morning Herald*, an 81-year-old man died on January 12, 2007 after eating contaminated asparagus sauce prepared at a local restaurant (Kennedy, 2008). The article reported that the sauce was left out for more than four hours after being refrigerated, and contained 9.8 log CFU/g of *B. cereus*.

2.4 Bacterial spores in the food industry

No other life form is as difficult to eliminate as bacterial endospores. Spores are known for their extreme resistances to heat, pressure, extremes of pH, disinfectant chemicals, irradiation, desiccation, infectious agents, and just about any stress agent or conditions imaginable, including survival over extremely long time periods. Spores themselves are generally of no concern unless they are able to
germinate within the food or after consumption. Therefore, understanding the germination and outgrowth of spores is of fundamental importance.

Spores of *Bacillus* species are of considerable concern in the food industry, due to their common occurrence and extreme resistances (Setlow, 2003; Coleman *et al.*, 2007; De Vries *et al.*, 2004). Spore formation is triggered by nutrient depletion whereby a vegetative cell is transformed into a dormant spore in which it is able to respond to various agents in its environment (Setlow, 2003; Wei *et al.*, 2010). When the environment becomes favorable again with available nutrients, spores are able to transform back to the vegetative state (or growing cell). Such germinants have to bind to spore germinant receptors and subsequently trigger germination. These nutrient germinants include certain amino acids, sugars, and purine nucleosides (Setlow, 2003). There are also several non-nutrient germinant, including dodecylamine (DPA), pressure, specific peptidoglycan fragments, and bryostatin, an activator of serine/threonine protein kinases (Wei *et al.*, 2010).

The spore structure, from the outer to inner layers, consists of the exosporium, the spore coat, the outer membrane, the cortex, the germ cell wall, the inner spore membrane, and the core(Figure 2). The resistance properties of bacterial spores are directly linked to the spore’s dehydrated core and surrounding protective layers (Abee *et al.*, 2011). When a spore comes into contact with a particular germinant the spore can commit to germination; by a currently unknown mechanism (Setlow, 2003). After spore activation, which most commonly occurs through sub-lethal heat shock (Setlow, 2003), and exposure to a germinant, spores become committed to germination and will continue to germinate even if environmental conditions become less ideal or germinants are removed (Setlow, 2003).
Figure 2. **Bacterial spore anatomy.** Including the components of the spore germination apparatus. (Adapted from Setlow, 2003 and Black *et al.*, 2007).

Spore germination takes place without detectable energy metabolism in two stages (Setlow, 2003)(Figure 3). After spore activation, protons and monovalent cations are released from the core of the spore and elevate spore pH. This change in pH is essential for the function of spore enzymes and metabolism. The spore core’s depot of pyridine-2, 6-dicarboxylic acid (dipicolinic acid or DPA) and other divalent cations are also subsequently released from the core. DPA that has been released is replaced by water thus increasing core hydration. In the final germination steps, the
spore’s peptidoglycan cortex is hydrolyzed, and core begins to swell due to further water uptake and expansion of the germ cell wall. After sufficient hydration of the spore’s core, mobility and enzymatic action returns to the cell. After metabolism returns, spore outgrowth occurs and the spore is converted back into a growing cell.

![Diagram of spore germination stages](image)

**Figure 3.** Events in germination triggered by nutrients. (Adapted from Black *et al.*, 2007).

Spores can survive for long periods of time in food products, particularly in foodstuffs where nutrient content is low or nonexistent (Coleman *et al.*, 2007). When these spores germinate, foodborne illness can occur (Setlow, 2003). It would be
ideal to trigger germination of spores present in the food product prior or during any preservation treatment since spores are much less resistant and more susceptible to inactivation after they have germinated (Ghosh and Setlow, 2009a). Although this strategy seems simple, germination rates vary, and a small percentage of spores commonly germinate extremely slowly or not at all after exposure to germinants (Ghosh and Setlow, 2009a; Wei et al., 2010). Such spores are known as superdormants.

### 2.5 Superdormant spores

Until recently studies on bacterial spores have primarily focused on populations and have neglected spores that either fail to germinate or germinate extremely slowly (Ghosh and Setlow, 2009a). The development of a simple method for isolation of superdormant spores was developed by Ghosh and Setlow (2009a) for spores of *B. subtilis, B. megaterium*, and *B. cereus*. This method, called buoyant density centrifugation, separates dormant spores from germinating spores and debris. After using this type of centrifugation, dormant spores pellet and germinated spores float (Ghosh and Setlow, 2009a). Through multiple cycles of heat shock, germination, and buoyant density centrifugation, the majority of spores will have germinated leaving spores that have either failed to germinate or take longer to germinate than the remaining spore population.

It appears that the physiological state for superdormancy is similar for all *Bacillus* species (Ghosh and Setlow, 2009a). Recent studies provide evidence that suggest that one reason for superdormancy is a reduced level of germinant receptors (Ghosh and Setlow, 2009a; b; Wei et al., 2010, Zhang et al., 2010). Because
superdormant spores are not genetically different from the remaining spore population, there is no current method to determine whether a spore is superdormant. Simply, a spore that fails to germinate or germinates much more slowly compared to the spore population from which it was isolated can be classified as superdormant.

It has been demonstrated that sublethal heat treatment prior to germination decreases the yield of superdormant spores; however, superdormant spores still show a higher temperature optimum for heat activation than the remainder of the spore population (Ghosh and Setlow, 2009a). It also appears that superdormant spores have greater wet-heat resistances and lower core water contents (Ghosh et al., 2009). Superdormant spores germinate poorly in the presence of nutrient germinants as compared to other germinants, such as dodecylamine or calcium dipicolinic acid (Ca-DPA). This is not surprising since germination by dodecylamine or Ca-DPA does not require nutrient-binding by receptors nor does it require prior heat activation (Ghosh and Setlow, 2009a; Wei et al., 2010).

In agreement with the findings of Ghosh and Setlow (2009a), Zheng et al. (2010) found that a number of factors increase the rate of spore germination, including heat activation and an increased level of germinant receptors. It is suspected that different germinant receptors within an individual spore interact through aggregation that could potentially amplify signals from large numbers of germinant receptors (Ghosh and Setlow, 2009a). The lack of these nutrient receptors may inhibit the amplification of this germination signal and may explain why higher yields of superdormant spores are observed with Bacillus strains that lack one or more germinant receptor (Ghosh and Setlow, 2009a). In order to understand why spore germination is determined by the level of germinant receptors on an individual spore,
it must first be determined how ligand binding to germinant receptors triggers spore germination (Ghosh and Setlow, 2009). It may be possible that a low level of GerD receptors on a spore can contribute to spore superdormancy as reported by Ghosh and Setlow (2009) who reported the rate of germination by nutrients is increased in a spore population containing higher numbers of GerD receptors. Heterogeneity in a spore population, resulting in varying rates of germination among individual spores, may also be due to adaptation of a particular bacterial species. Spores that germinate more slowly or at a reduced rate than the majority of the population, are more likely to survive environmental changes where the majority of germinating spores are inactivated, thus increasing the likelihood of survival for the entire population (Ghosh and Setlow, 2009a).

Hydrostatic pressure inactivation studies on *B. cereus* and *B. subtilis* by Wei et al., (2010), using relatively low and high pressure magnitudes, demonstrated almost identical results for both species. Both germinated normally at 150 and 500 MPa (Wei et al., 2010). It was not surprising that spores germinated after exposure to 500 MPa since spore inactivation at this pressure level does not affect nutrient germinant receptors and does not require heat activation (Wei et al., 2010); however, it was surprising that superdormant spores germinated at 150 MPa since spore germination at this pressure magnitude requires activation of nutrient receptors (Wei et al., 2010). Further studies on pressure inactivation of superdormant spores are essential for high pressure processing to be more efficiently utilized by the food industry. Superdormant spores are no doubt a significant contributing factor in the incomplete sterilization of low-acid foods using high hydrostatic pressure processing. Wei et al., (2010) also found that superdormant spores of *Bacillus* species were able to
germinate normally when exposed to peptidoglycan fragments and bryostatin. This was expected since germination initiation by these two agents appears to be triggered by eukaryotic-like membrane-bound ser/thr protein kinase domains (Shah et al., 2008) and is not a result of interaction with germinant receptors.

2.6 Minimally processed foods and psychrotolerant pathogens

The increase in demand by consumers for convenient food products of high quality, which are commonly referred to as ready-to-eat, cooked, or chilled foods, has led to the increased development of food products known as refrigerated processed foods of extended durability (RPFEDs) (Nissen et al., 2002). The presence of oxygen in the modified atmosphere packaging (MAP) of minimally processed foods can also increase the ability of spores of pathogenic psychrotolerant *Bacillus* species to germinate and grow at refrigeration temperatures (Valero et al., 2003; Samapundo et al., 2010). Such products may require minimal heat processing which will enable the sensorial qualities of the food product to be preserved, but such mild processing techniques can allow sporeforming bacteria such as *Bacillus* and *Clostridium* species to survive in the food product (Samapundo et al., 2010). Processing methods that rely less on thermal technologies to reduce bacterial spore contamination in these types of foods are needed to produce safe products that will be acceptable to the consumer.

2.7 Low-temperature storage of spores

For mesophilic sporeforming species, lower temperatures are generally known to prevent spores from germinating. That is why in laboratory settings, spores are often stored at refrigeration temperatures to prevent them from germination,
however, in the case of psychrotolerant sporeforming species, reduced temperatures may allow for spore germination, outgrowth, and perhaps even cell multiplication. This is why emerging psychrotolerant sporeforming species, including *B. weihenstephanensis*, which can potentially germinate and grow in foods stored in the refrigerator are of concern. In the case of RPFEDs, which are often not heated or processed prior to eating, bacterial spore contamination is of great concern for consumers and producers.

### 2.7.1 Low-temperature storage of superdormant spores

A storage study completed by Ghosh and Setlow (2009b) reported that superdormancy is maintained at -80°C compared to -20 and 4°C. The authors concluded that superdormancy is not permanent and suggested at least two factors caused superdormancy. One possibility is the lack of receptors on superdormant spores (Ghosh and Setlow, 2009b). The second is the activation status of the class of superdormant spores over periods of time (Ghosh and Setlow, 2009b). The authors theorized that superdormant spores in the initial dormant spore population have more stringent requirements for heat activation than the population as a whole (Ghosh and Setlow, 2009b).

### 2.7.2 Temperature of sporulation

Spores of mesophilic *B. cereus* isolates were previously shown to germinate more easily in 10 mM-L-alanine when sporulated at 37°C as compared to those sporulated at 20°C (Raso *et al.*, 1998); however, Gounina-Allouane *et al.* (2008) found the opposite with psychrotolerant *B. cereus* spores produced at 15 and 20°C.
which were more easily germinated than spores produced at 37°C. Several previous studies demonstrated similar patterns with psychrotolerant species as spores of *B. subtilis* and psychrotolerant *C. botulinum* type E were more easily germinated with nutrients when sporulation occurred at lower temperatures (Cortezzo and Setlow, 2005; Evans *et al.*, 1997). These results may indicate that spores produced at higher temperatures are more resistant to germination and can yield spore populations containing higher numbers of superdormant spores.

Heat activation of spores prior to germination increased spore germination and decreased overall yields of superdormant spores (Ghosh and Setlow, 2009a; Wei *et al.*, 2010; Garcia *et al.*, 2010); however, the mechanism by which heat activation affects germination is not well understood. Further investigation on the relationship of sporulation temperature on spore germination and superdormancy is needed. Understanding of these mechanisms may also lead to better understanding of spore germination and outgrowth in refrigerated food products.

In addition to spore germination, sporulation temperature has also been shown to affect spore size. In a study by Garcia *et al.*, (2010), spores of *B. weihenstephanensis* sporulated at 12, 20, and 30°C and were evaluated by SEM (scanning electron microscopy). Spores obtained at 12°C and 30°C were of similar size at approximately 1.5 µm, while spores obtained at 20°C were slightly larger at approximately 1.8 µm (Garcia *et al.*, 2010). Sporulation temperature may also affect the degree by which spores are germinated or inactivated through food processing methods. As much as 99% of *B. weihenstephanensis* spore populations produced at 30°C germinated using 150 MPa of pressure whereas only 50 and 15% germinated at 12 and 20°C, respectively, at the same pressure (Garcia *et al.*, 2010). Spore
populations sporulated at all three temperatures germinated similarly with the application of 500 MPa with only a 35% germination rate.

2.8 Ozone inactivation of superdormant spores

The first commercial use of ozone as a disinfectant was in 1906 in a municipal water supply treatment in Nice, France (Rice et al., 1981). The FDA (Food and Drug Administration) approved ozone a GRAS (generally recognized as safe) status for the use in bottled water in 1982 (Guzel-Seydim et al., 2004). It was not until 2001 that ozone was approved for disinfecting and sanitizing of food and food processing environments in gaseous and aqueous form (FDA, 2001) when used in accordance with GMPs (good manufacturing practices). With increasing consumer demand for fresh food products, the development of nonthermal food processing technologies has also increased to satisfy consumer demand. Such technologies include the use of ozone and other oxidative agents including the use of pulsed electric fields (PEF), irradiation, high intensity light pulses, and high hydrostatic pressure (San Martin et al., 2002). Use of nonthermal technologies such as ozone may help to conserve the sensory qualities of food products that are normally degraded or destroyed by processing technologies that involve heat.

Ozone, a triatomic oxygen (O3) molecule, is a naturally occurring form of oxygen that exists as a bluish gas with a pungent odor. In nature, ozone is formed via ultraviolet irradiation from the sun and via lightening discharge (Karaca and Velioglu, 2007). Commercially, the corona discharge method is used to generate ozone which uses high tension and low tension electrodes that discharge electrons into a narrow discharge gap separated by a dielectric medium which dissociates oxygen molecules to
form ozone molecules (Karaca and Velioglu, 2007). Ozone is partially soluble in water and is effective at killing a range of microorganisms through the oxidation of cellular membranes of vegetative cells (Langlais et al., 1991). It has an oxidizing potential of 2.07 mV, which is considerably higher than that of other oxidants used in the food industry (Karaca and Velioglu, 2007). Ozone decomposes in solution to produce hydroperoxyl (·HO₂), hydroxyl (·OH), and superoxide (·O₂) radicals (Hoigne and Bader, 1975). The reactivity of ozone is due to these free radicals (Hirneisen et al., 2010) that are able to inactivate bacteria, fungi and their spores, viruses and protozoa (Hirneisen et al., 2010).

2.8.1 Current applications of ozone in the food industry

The antimicrobial effectiveness of ozone has been shown to be much higher than that of chlorine and to affect a broader spectrum of microorganisms than chlorine and other disinfectants (Hirneisen et al., 2010). Ozone can also degrade mycotoxins and pesticides present in foods (Karaca and Velioglu, 2007). In addition, there is little concern of residual ozone in treated food products due to the rapid decomposition of ozone into oxygen (Graham, 1997) and ozone is currently certified for use on organic foods.

Ozone is applicable in the food industry to treat process water, as a fruit and vegetable wash, in fruit and vegetable storage, and in recycled water (Hirneisen et al., 2010). Aqueous ozone has been used to decontaminate apples, strawberries, and in juices such as apple cider and orange juice (Hirneisen et al., 2010); however, the efficacy of aqueous ozone is largely dependent upon the presence of organic residues, pH, and temperature of the aqueous medium (Hoigne and Bader, 1985; Karaca and
Velioglu, 2007). In general, ozone is more effective at lower temperatures, pH 5.0, and higher humidity (Karaca and Velioglu, 2007; Kuscu and Pazir, 2004).

There are some issues with ozone that make its use as a disinfectant in foods less appealing. Ozone is a very reactive molecule that has the ability to inactivate a broad range of microorganisms however; it also reacts with nearly all organic and inorganic compounds (Karaca and Velioglu, 2007). Therefore, the higher the amounts of organic matter present in a food product, the lower the effectiveness of ozone. Ozone may also cause slight deleterious effects on the quality and physiology of food products such as losses in sensory quality including enzymatic browning, antioxidants, vitamins, and minerals (Karaca and Velioglu, 2007). Exposure of humans and animals to high levels of ozone can also have detrimental effects on health, which causes concern for workers in processing plants. In the US, OSHA (Federal Occupational Safety and Health Administration) limits exposure to ozone to a 0.1-ppm threshold for continuous exposure for an 8-h period and 0.3 ppm for a 15-min period (Suslow, 2004).

2.8.2 Ozone inactivation of bacterial spores

Spores have been shown to be inactivated by several oxidizing agents including chlorine dioxide, hydrogen peroxide, organic hydroperoxides, ozone, and sodium hypochlorite. Aqueous ozone has a higher potential than most oxidizing agents to inactivate spores (Menzel, 1971; Kim et al., 2003). Studies involving the inactivation of spores by oxidizing agents suggest that inactivation is a result of oxidative damage to the spore’s inner membrane.
Young and Setlow (2004) found that when treated with ozone, spores of *B. subtilis* were more easily inactivated when spores were uncoated prior to ozone treatment, that spores did not germinate with nutrient germinants or Ca2+ DPA after ozone treatment, and germination of spores with ozone did not cause release of DPA from the spore’s inner core (Young and Setlow, 2004). The authors concluded that spores are not inactivated with ozone by DNA damage and that the major resistance factor of spores to ozone is the spore coat (Young and Setlow, 2004).

Studies performed by Cortezzo *et al.* (2004) confirmed that ozone causes damage to the spore’s inner membrane because ozone treated spores of *B. subtilis* were more easily penetrated by methylamine and germinated faster with dodecylamine. Since the inner membrane of the spore is known to be a barrier to methylamine, this study demonstrated that the inner membrane was damaged by its inability to prevent methylamine from leaking through the barrier into the spore. Damage to the spore’s inner membrane via oxidization can have several effects including inability to germinate, spore death after germination, or cell lysis (Cortezzo *et al.*, 2004). More interestingly, the authors found that spore survivors of ozone treatment exhibited increased sensitivity to inactivation by a normally minimal heat treatment. Spores treated with ozone were also more sensitive to NaCl in plating media than nontreated spores. Since heat treatment and NaCl treatment are not lethal to spores under normal conditions, these findings further confirm that ozone causes damage to the spore’s inner membrane, making the spores more sensitive to these treatments. The authors hypothesize that ozone treatment causes damage to key proteins in the spore’s inner membrane although more research needs to be performed to determine what these proteins are (Cortezzo *et al.*, 2004).
2.9 **High hydrostatic pressure**

The use of pressure in food processing was first used by Hite in 1899 observed that pressure treatment of milk could increase its shelf-life (San Martin *et al.*, 2002). Over a century later, research in the application of pressure in the food processing industry still occurs. With consumer demands for fresh food products on the rise, the demand for nonthermal processing techniques that will not damage sensory qualities of food products while reducing microbial contamination are also needed. High hydrostatic pressure is currently one of the nonthermal processing methods utilized by the food industry. HHP-processed foods were first introduced to the Japanese market in 1990 and are slowly being introduced into other countries (San Martin *et al.*, 2002). Currently HHP is used to commercially process guacamole, pre-sliced deli meats, juices, and oysters. Researchers and producers are interested in expanding the application of HHP to a wider variety of foods.

2.9.1 **HHP inactivation of vegetative cells**

Vegetative cells are inactivated by HHP through a variety of mechanisms. One hypothesis is that a pressure induced decrease in cell volume can lead to cell leakage and death (San Martin *et al.*, 2002); however, the mechanism of inactivation of cells by HHP can be dependent on the type of pressure applied (cyclic or continuous), temperature, treatment time, strain, cell shape, gram stain type, growth stage, and treatment medium (San Martin *et al.*, 2002). HHP of vegetative bacterial cells is generally more effective at higher temperatures unless the bacterial species contains the heat shock protein (Hsp), in which case heat would cause a baroresistant effect (Iwahashi *et al.*, 1996). HHP applied in cyclic phases rather than continuously
also tends to be more effective at inactivating bacterial species. Rod shaped cells are more sensitive to HHP than cocci (Ludwig and Schreck, 1996). The presence of various ions in the medium may or may not induce baroresistance or sensitivity, depending on the microorganism. In the presence of low water activity (aw) and large amounts of sorbitol and glycerol, a baroprotective effect on the inactivation of microorganisms can take place (Hayert et al., 1996). HHP treatment has also been hypothesized to cause cleavage of the cell’s DNA and may be a cause of cell death (Chilton et al., 1996). The resistance of microorganisms to HHP is largely dependent upon the species and strain of the microorganism and is extremely variable (Raso et al., 1998), but most vegetative cells of bacteria and yeast are generally inactivated at pressures around 300-400 MPa at ambient temperature (Knorr, 1995).

2.9.2 HHP inactivation of spores

One of the current disadvantages of HHP is its inability to inactivate spores by pressure alone without altering the sensory qualities of the product (San Martin et al., 2002; Black et al., 2007). Complete inactivation of spores remains a top priority for high-pressure food processors. It is important to understand the physiology of spores, especially those that pertain to spore inactivation by HHP (Black et al., 2007).

It is currently hypothesized that cell death via HHP is caused by pressure-induced combined with temperature-induced spore germination in which spores lose their resistance properties and are inactivated due to increased sensitivity to pressure (Black et al., 2007). This process is more specifically known as electrostriction where a pressure-induced decrease in water volume of the cell causes a local collapse of the
bulk water structure of the cell, thus beginning the germination process (San Martin et al., 2002). While spores are generally resistant to pressures as high as 1,200 MPa, low-pressure treatments from 50 to 300 MPa can induce spore germination at higher temperatures (San Martin et al., 2002; Black et al., 2007). Lower pressures can trigger spore germination through activation of the nutrient receptors on the inner membrane of dormant (Wuytack et al., 2000; Paidhungat et al., 2002; Black et al., 2005) and superdormant spores (Wei et al., 2010). SASP (small acid-soluble protein) degradation, which normally accompanies nutrient-induced germination, has also been observed to take place in spores treated with moderate pressures but not high pressures (Wuytack et al., 1998). Extremely high pressures trigger spores, dormant or superdormant, to germinate causing release of Ca 2+-DPA to release from the spores core (Black et al., 2007). Spores germinated with high pressures are able to complete germination but go through outgrowth much more slowly than spores treated at lower pressures (Wuytack et al., 1998).

There are several other factors that can affect the germination of spores with HHP. As with vegetative cells, spore germination and inactivation with HHP is also more effective when applied in cycles (Hayakawa et al., 1994; Palou et al., 1998). Sporulation temperature has also been demonstrated to influence the HHP inactivation of spores of B. cereus (Raso et al., 1998). In a study by Raso et al., (1998), spores that were initially sporulated at 37°C were more significantly germinated and inactivated with HHP compared to spores that were initially sporulated at 20°C. Wuytack et al., (1998) demonstrated that B. subtilis spores exposed to HHP at >200 MPa were more sensitive to pressure, UV light, and hydrogen peroxide compared to spores not pretreated with pressure.
2.9.3  HHP inactivation of superdormant spores

Since spore germination with moderate pressures is triggered by activation of nutrient receptors, spores with an increased number of germinant receptors will be more easily inactivated by moderate pressures (Wutack et al., 2000, Paidhungat et al., 2002; Black et al., 2005; Pelczar et al., 2007). Therefore, it would be expected that superdormant spores, which have a decreased number of germinant receptors, would germinate poorly with low or moderate pressures.

In a study by Wei et al., (2010), superdormant spores of *B. subtilis* and *B. cereus* that were not exposed to heat activation germinated normally, or similarly to the rest of the spore population, at a pressure of 500 MPa. According to the authors, this result was expected since spore germination at this level of pressure does not require activation of nutrient germinant receptors. More interestingly, superdormant spores germinated normally at 150 MPa, which was surprising to the authors because spore germination induced at this level of pressure requires activation of the nutrient germinant receptors, and spore germination induced by this level of pressure is increased by prior heat activation (Wei et al., 2010). The findings of this study were significant in that the factors responsible for spore germination by pressure were not identical to those responsible for spore superdormancy or nutrient germination (Wei et al., 2010). It is an important finding for the food industry that superdormant spores are not the cause of incomplete germination of *Bacillus* species by HHP. It is important to determine the cause of incomplete germination and inactivation of spores by HHP so that this nonthermal processing technology can be more widely utilized by the food industry.
2.10 Use of hurdle technology to reduce the number of spores in foods

Hurdle technology involves the combination of processing technologies to establish hurdles for microbial growth and/or survival (Leistner, 1995). There are some hurdles, or technologies, that are considered high hurdles and some that are considered low (Leistner and Gorris, 1995). Due to synergistic effects of treatments, a combination of low hurdles may be as successful as the application of a single high hurdle (Leistner and Gorris, 1995). The use of combined milder processing techniques may not only challenge the survival of bacterial spores within the product, similarly to more intense individual processing techniques, but may also help preserve the sensory qualities of the food product. However, low hurdles might not be sufficient for making nutrient rich foods safe (Leistner and Gorris, 1995) (Figure 4b and combination of high hurdles may be required.)
Figure 4. **Example of hurdle effect used in food processing.** Figure (a.) shows an example of a hurdle technology using mild hurdles of similar intensity that large numbers of microorganisms present in nutrient rich foods would normally be able to overcome thus creating an unstable product. Figure (b.) shows an example of a hurdle technology using higher hurdles of different intensities that would be more challenging for microorganisms to overcome. Symbols have the following meaning: F, heating; T°, temperature; aw, water activity; Eh, low redox potential; pres., preservatives; pH, acidification; V, vitamins; N, nutrients. (Adapted from Leistner and Gorris, 1995).
Chapter 3

MATERIALS AND METHODS

3.1 Bacterial strains

The psychrotolerant strains, *Bacillus cereus* 6A16 and *Bacillus weihenstephanensis* 6A23, were obtained from Dr. Daniel Zeigler at the Ohio State University’s *Bacillus* Genetic Stock Center. Mesophilic *Bacillus cereus* T was obtained from H.O. Halvorson (Table 1).

<table>
<thead>
<tr>
<th>Strain</th>
<th>Growth temperature phenotype</th>
<th>Isolated from:</th>
<th>References</th>
</tr>
</thead>
<tbody>
<tr>
<td><em>B. cereus</em> 6A16</td>
<td>psychrotolerant</td>
<td>infant cereal product</td>
<td>Duc <em>et al.</em>, 2005</td>
</tr>
<tr>
<td><em>B. weihenstephanensis</em> 6A23</td>
<td>psychrotolerant</td>
<td>Milk</td>
<td>Letchner <em>et al.</em>, 1998</td>
</tr>
<tr>
<td><em>B. cereus</em> T</td>
<td>mesophilic</td>
<td>unknown</td>
<td>Wei <em>et al.</em>, 2010</td>
</tr>
</tbody>
</table>

*B. cereus* 6A16 was isolated from an infant cereal product that was implicated to be the cause of projectile vomiting in two infants in a study conducted by Duc *et al.* (2005). This strain was identified as psychrotolerant and did not have the ability to produce the emetic toxin in this study. It was therefore determined that the
cause of foodborne illness in each infant of the outbreak was due to bacterial load and not cereulide intoxication.

*B. weihenstephanensis* 6A23 was isolated from milk in an environmental study conducted by Letchner et al., (1998) in which the creation of the species *B. weihenstephanensis* was first proposed. This strain has the ability to grow at temperatures below 10°C and to produce hemolytic, nonhemolytic, and cytotoxic enterotoxins, but not the emetic toxin.

### 3.2 Spore preparation

Spore crops of each strain were generated by inoculating 5 ml of log phase culture into 1,000 ml of casein-casein yeast medium (CCYM) (Appendix 1) (Stewart *et al.*, 1981) and incubated with shaking at 200 rpm for 5 – 7 days at either 30 or 37°C. For sporulation temperature studies, two spore crops were produced (sporulated) for each strain at 30 and at 37°C, respectively. Following incubation, spores were harvested by centrifugation and washed three to four times with 5 ml of cold (4°C) sterile water with repeated centrifugation at 9,000 x g for 20 min until > 90 % free spores were visible by phase contrast microscopy, and resuspended in cold sterile water (4°C). During phase contrast microscopy dormant spores are phase bright, or refractile, while germinating spores and vegetative cells are phase dark. Percentages were determined by counting of spores using a haemocytometer under a phase contrast microscope. During each washing step, the liquid layer above the spore pellet containing germinated spores, vegetative cells, and debris was discarded. All spore crops were stored at -20°C until use.
3.3 Isolation of superdormant spores

Superdormant spore crops were produced according to the buoyant density centrifugation method described by Ghosh and Setlow (2009a). Dormant spore samples of *B. cereus* and *B. weihenstephanensis* initially sporulated at either 30 or 37°C were adjusted to an optical density O.D. 600 of ~1.0 (10^9 spores/ml). Spore samples were heat activated at 65°C for 30 min and cooled at 4°C for 20 min. Samples were inoculated into a variety of high and low concentrations of individual nutrient germinants including 1 mM and 25µM inosine, 10 and 25 mM L-alanine, 5 and 10 mM L-valine, and 6 mM and 250 µM L-asparagine. Spores were incubated with individual germinants at 37°C for ~2h until spore outgrowth began as determined by phase contrast microscopy. Spores were recovered by centrifugation two to three times at 9,000 x g for 20 min. The spore pellet was washed after each centrifugation step with cold sterile water to ensure that the spore sample was > 98% free of germinating spores, vegetative cells, and debris as was determined by phase contrast microscopy. Spore pellets were then resuspended in 500 µl of 20% Nycodenz (Axis-Shield, Oslo, Norway) Aliquots of the spore suspension (100 µl) were layered on a total volume of 1 ml of 50% Nycodenz and centrifuged for 45 min at 13,000 x g. Under these conditions dormant spores pellet and germinated spores float. The final spore pellet was washed with cold sterile water and recentrifuged at 9,000 x g for 20 min 2-3 times to remove the Nycodenz solution. The cycle of heat activation, germination, and buoyant density centrifugation was repeated once. The remaining spore sample was considered a superdormant spore population.
3.4 Measuring the effects of sporulation temperature on superdormant spore isolation

Initial dormant spore crops were produced as described in section 2.4. Spore crops for each *B. cereus* 6A16, *B. weihenstephanensis* 6A23, and *B. cereus* T were produced at 30 and 37°C, respectively, in order to determine how each sporulation temperature would affect the recovery of superdormant spores with each germinant. Prior to superdormant spore isolation, 100 µl of control samples was taken in order to determine initial concentrations of samples prior to germination. For each of the three strains, superdormant spores were produced from each initial spore crop with each individual germinant. Germinants in 25 mM KPO4 buffer were 1 mM inosine, 25µM inosine, 25 mM L-alanine, 10 mM L-alanine, 10 mM L-valine, 5 mM L-valine, 6 mM L-asparagine, or 250µM L-asparagine. Superdormant spores were isolated using the above germinants and buoyant density centrifugation as described above.

The number of superdormant spores recovered from either 30 or 37°C sporulated spore crops for each strain with each nutrient germinant listed above was determined by standard plate count. Control and treatment samples were serially diluted in TSB and 100µL of each sample was pour-plated in TSA and incubated for 24-48 h at 37°C. Enumeration was determined by SPC using TSA due to its ability to germinate large numbers of spores (Ghosh and Setlow, 2009). SPCs did not increase after incubation longer than 48 h (data not shown). The recovery of superdormant spores was calculated as the percentage of superdormant spores recovered from the number of spores in the control sample where $N_0 =$ the number of spores in the control sample, $N =$ the number of superdormant spores recovered from the control, and $(N/N_0)$ multiplied by 100 = the percentage of superdormant spores recovered.
Superdormant spore recovery values generated from crops initially sporulated at 30°C were compared to those initially sporulated at 37°C.

3.5 Ozone generator specifications

Two ozone generators were used in this study. The Golden Buffalo ozone generator (Golden Buffalo, Orange, CA) produces ozone at a continuous output of 6.25ppm. It was confirmed that this output could be maintained for the duration of the experiments (20 min) using an ozone test kit (Hach, Loveland, CO). The Mongoose ozone generator (Marhoc, Inc., Alvin, TX) produces ozone at a continuous output of 190ppm, which was also confirmed to last the duration of the experiment time using the ozone test kit. Ozone was delivered into a 250-ml glass flask containing 45-48 ml of sterile water and 2-5 ml of spore suspension through nonreactive tubing. An air stone was attached to the end of the tubing for aeration of ozone gas through the water.

3.6 Sample preparation for ozone studies

Superdormant spores of *B. cereus* 6A16, *B. weihenstephanensis* 6A23, and *B. cereus* T were produced for this study using buoyant density centrifugation and using 5 mM inosine in 25 mM KPO₄ buffer (pH 7.4) as the isolation germinant. This germinant was selected due to its use in other studies on superdormant spores of *B. cereus* species (Wei et al., 2010). Superdormant spores were stored in 1-ml samples at an O.D.₆₀₀~1.0 at -20°C until use.
3.7 **Ozone inactivation of superdormant spores with ozone**

For ozone inactivation studies 2 ml of spores were inoculated into 48 ml of sterile water in a 250-ml flask. Bubbling gaseous ozone was delivered into the flask from the ozone generator through nonreactive polyethylene tubing. Two different ozone generators were used with ozone delivery rates of 6.25 and 190 ppm. Samples were exposed to aqueous ozone for up to 20 min with 1-ml samples taken at 0, 30, 1, 5, 10, and 20 min. After ozone treatment 2 ml of 10% sodium thiosulfate was added to quench any remaining ozone. Samples were centrifuged two to three times at 9,000 x g for 20 min and resuspended in cold (4°C) sterile water to remove sodium thiosulfate until superdormant spore samples were > 98% free of germinating cells, vegetative cells, and debris as determined by phase contrast microscopy. Spore samples were serially diluted in TSB and pour-plated in TSA. Enumeration was performed using standard plate counts and values were calculated as the percentage of superdormant spores recovered at each time sample from the control where \( N_0 \) = the number of spores present in the 0 min time sample, \( N \) = the number of spores present in the treatment time samples, and \( \frac{N}{N_0} \) multiplied by 100 = the percentage of spores recovered.

3.8 **Pressure unit specifications**

High pressure experiments of spores were conducted using a PT-1 Research System pressure unit (Avure Technologies, Kent, WA). This unit can apply pressures of up to 600 Megapascals (MPa) or 100,000 pounds per square inch (psi) to volumes of up to 50 ml. Samples for pressure treatment were immersed in water at a controlled temperature inside of the 50-ml pressure chamber. The system applies
pressure to the samples in the pressure chamber of up to 600 MPa by converting air pressure into an intermediate hydraulic oil pressure is then converted by pumping water into the pressure chamber. Pressure build-up time was approximately 15 sec to reach a level of 500 MPa, and depressurization time was consistently < 5 sec. The PT-1 pressure unit is controlled by a PC-driven acquisition and control system running through DaisyLab 7.0 (National Instruments, Austin, TX). DaisyLab is a program that allows simple programming of control and data acquisition functions.

3.9 Sample preparation for high pressure processing studies

Superdormant spores for all strains were isolated with the same methods used for ozone inactivation experiments using buoyant density centrifugation and 5 mM inosine in 25 mM KPO₄ buffer as the nutrient germinant. Superdormant spore suspensions of 5 ml were inoculated into 45 ml of sterile water in a 250-ml flask and treated with 190-ppm ozone for 10 min. Ozone was quenched immediately following ozone treatment with 2 ml of 10 % sodium thiosulfate. Samples were centrifuged two to three times at 9,000 x g for 20 min and resuspended in cold (4°C) sterile water to wash away sodium thiosulfate until superdormant spore samples were > 98% free of germinating cells, vegetative cells, and debris as determined by phase contrast microscopy. Superdormant spore samples without ozone pretreatment were also used in this study. Spore pellets were removed from all samples and resuspended in 1 ml of 50 mM Tris-HCl buffer (pH 7.4) prior to pressure treatments.

Samples of ozone treated superdormant spores were measured out into 1-ml volumes at an O.D.₆₀₀ ~ 1.0 in Tris-HCl buffer and stored at -20°C until use. Samples were aseptically transferred to sterile pouches (VWR International, Mississauga,
Canada) and heat sealed with as much air expelled as possible. Each sample pouch was inserted into another outer pouch and heat sealed. Control samples in volumes of 100µl were taken from treatment samples prior to pressure treatment. Control samples were serially diluted in TSA and pour-plated in TSA to determine the initial concentration of spores.

3.10 Inactivation of ozone-treated superdormant spores using high pressure processing: A potential hurdle technology

Superdormant spore samples without ozone pretreatment and superdormant spore samples with 10 min of 190-ppm ozone treatment were used in this study to determine if ozone pretreatment could increase the efficacy of high pressure on the inactivation of superdormant spores of B. cereus species. All samples samples were pressure treated at 500 MPa for 10 min. Samples were aseptically removed from pouches to 1.5-ml microcentrifuge tubes and centrifuged two to three times at 9,000 x g for 20 min and resuspended in cold (4°C) sterile water until superdormant spore samples were > 98% free of germinating cells, vegetative cells, and debris as determined by phase contrast microscopy. It may be possible that some spores are lost in this washing step, although the amount of lost spores is likely to be very few. Samples were serially diluted in TSB and pour plated in TSA. Enumeration was performed using standard plate counts and values were calculated as the percentage of superdormant spores recovered after each pressure treatment from the control where N0 = the number of spores present in the nonpressure-treated sample, N = the number of spores present in the treated samples, and (N/N0) multiplied by 100 = the percentage of spores recovered from the control after pressure treatment.
3.11 Reproducibility and Statistical Analysis

All experiments were performed in triplicate. Averages for each data set were calculated and illustrated in the figures provided. Relationships and significant differences between variables were calculated using ANOVA (analysis of variance) single factor ($\alpha = 0.05$). For some experiments linear regression ($\alpha = 0.05$) was used to further test the relationship of the independent variable with dependent variables in the experiment. Correlation matrices $(x,y)$ were generated to determine common trends in the data set despite the significance of the relationship as determined by ANOVA and linear regression. All statistical analyses were performed using JMP 8.0.1 (SAS Institute Inc., 2009).

All values in this study are reported as the percent recovery of spores. This value includes the percentage of spores that have remained intact, according to standard plate count, from the number of intact spores in the control sample. Because it was not determined whether the unrecovered spores were germinated or inactivated during spore isolation, ozone, or HHP treatments, we cannot report these values as percent germination or percent inactivation.
Chapter 4

RESULTS

4.1 The effects of sporulation temperature on the isolation of superdormant spores using single nutrient germinants

The objective of this study was to determine if the sporulation temperature of a spore population has any effect on spore superdormancy in psychrotolerant strains compared to mesophilic strains. The recovery of superdormant spores with each germinant varied from strain to strain (Figure 5; Table 2). For *B. cereus* 6A16 the lowest overall recovery of superdormant spores was from an initial sporulation temperature of 30°C and germination with 10 mM L-alanine (0.24 %). The highest recovery was produced with an initial sporulation temperature of 37°C and germination with 5 mM L-valine (55.68 %). Similarly to *B. cereus* 6A16 the lowest overall recovery of *B. weihenstephanensis* 6A23 superdormant spores was with an initial sporulation temperature of 30°C and germination with 10 mM L-alanine (0.63 %); however, the highest recovery was with an initial sporulation temperature of 37°C and germination with 250 µM L-asparagine (83.65%). For *B. cereus* T, the lowest overall recovery of superdormant spores was with 5 mM L-valine at a sporulation temperature of 37°C (3.19 %). The highest overall recovery for *B. cereus* T was with 10 mM L-valine at a sporulation temperature of 37°C (64.71 %). High and low percent recovery values for each strain can be viewed in Table 2.
The average recovery of superdormant spores was 17.79% with the greatest average recovery from 250 µM L-asparagine and the lowest from 10 mM L-alanine (Figure 6). ANOVA statistical analyses showed that there was no significant difference in the % recovery of spores between species (p = 0.7001). Meaning that spores of psychrotolerant strains *B. cereus* 6A16 and *B. weihenstephanensis* 6A23 were no more or less resistant to nutrient germinants than the mesophilic strain *B. cereus* T. The only significant difference in the percent recovery of superdormant spores was between germinants 10 mM L-alanine and 250 µM L-asparagine (p = 0.0308). Overall superdormant spore recovery was higher with spores initially sporulated at 30°C however; this observation was not statistically significant (p = 0.4976).
Figure 5. Average percent recovery of superdormant spores of each strain sporulated at either 30 or 37°C with each nutrient germinant. BC = *B. cereus* 6A16, BW = *Bacillus weihenstephanensis* 6A23, and BT = *B. cereus* T. The numbers 30 and 37 next to abbreviations represent sporulation temperature.
Table 2. **Average spore recovery.** The average percent recovery of superdormant spores sporulated at either 30 or 37°C after isolation with various nutrient germinants. Values listed in **bold** indicate either the highest or lowest recovery percentage for each species.

<table>
<thead>
<tr>
<th>Germinant</th>
<th>37°C</th>
<th>30°C</th>
<th>37°C</th>
<th>30°C</th>
<th>37°C</th>
<th>30°C</th>
</tr>
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<tr>
<td>1mM inosine</td>
<td>11.75</td>
<td>18.17</td>
<td>1.78</td>
<td>3.01</td>
<td>6.84</td>
<td>16.01</td>
</tr>
<tr>
<td>250µM inosine</td>
<td>21.35</td>
<td>17.61</td>
<td>5.85</td>
<td>19.12</td>
<td>5.81</td>
<td>64.44</td>
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<tr>
<td>25mM L-alanine</td>
<td>1.63</td>
<td>0.46</td>
<td>3.06</td>
<td>23.48</td>
<td>3.79</td>
<td>23.07</td>
</tr>
<tr>
<td>10mM L-alanine</td>
<td>3.55</td>
<td>0.24</td>
<td>0.91</td>
<td>0.63</td>
<td>4.37</td>
<td>22.81</td>
</tr>
<tr>
<td>10mM L-valine</td>
<td>9.26</td>
<td>8.3</td>
<td>8.08</td>
<td>23.91</td>
<td>4.48</td>
<td><strong>64.71</strong></td>
</tr>
<tr>
<td>5mM L-valine</td>
<td><strong>55.68</strong></td>
<td>8.78</td>
<td>9.4</td>
<td>19.21</td>
<td><strong>3.19</strong></td>
<td>31.01</td>
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<td>6 mM L-asparagine</td>
<td>45.85</td>
<td>11.81</td>
<td>55.1</td>
<td>8.17</td>
<td>7.16</td>
<td>12.95</td>
</tr>
<tr>
<td>250µM L-asparagine</td>
<td>18.68</td>
<td>13.94</td>
<td><strong>83.65</strong></td>
<td>1.41</td>
<td>7.7</td>
<td>61.98</td>
</tr>
</tbody>
</table>
4.2 Inactivation of superdormant spores compared to heterogeneous spore populations using 190- and 6.25-ppm aqueous ozone

The effects of aqueous ozone on the inactivation of superdormant spores and heterogeneous spore populations of *B. cereus* 6A16, *B. weihenstephanensis* 6A23, and *B. cereus* T with two different ozone strengths including 6.25 and 190 ppm were identified in this study. At an ozone delivery rate of 6.25 ppm the lowest percentage of spore recovery was for *B. cereus* T heterogeneous spore populations (3.42 %) (Figure 7a) and the highest percentage was for superdormant spores of *B. weihenstephanensis* (102.06 %) (Figure 7b). At an ozone delivery rate of 190 ppm the lowest percentage of spore recovery was for *B. cereus* 6A16 heterogeneous spore populations (Figure 8a)
(0.95 %) and the highest percentage was for heterogeneous spore populations of *B. cereus* T (12.41%) (Figure 8b). An average of a 1.41-, 1.36-, and 2.08-log reductions could be achieved with 190-ppm ozone for 20 min for *B. cereus* 6A16, *B. weihenstephanensis* 6A23, and *B. cereus* T, respectively.

According to ANOVA one way analysis there was no significant difference between the percent recovery of ozone-treated heterogeneous spore populations and superdormant spores for any of the three strains used in this study. The p values for the relationship between the percent recovery and strain type for *B. weihenstephanensis* 6A23, *B. cereus* 6A16, and *B. cereus* T were 0.3602, 0.0943, and 0.2135, respectively. In terms of ozone treatment time, for *B. weihenstephanensis* and *B. cereus* 6A16 an ozone treatment time of 10 min was needed to observe a significant drop in the percent recovery of spores (p = 0.0191). *B. cereus* T was slightly more sensitive to ozone where an ozone treatment time of only 5 min was necessary to achieve a significant decrease in the percent recovery of spores (p = 0.0041). For the psychrotolerant species, *B. weihenstephanensis* and *B. cereus* 6A16, there was a significant difference in the percent recovery of 190-ppm ozone-treated spores versus 6.25-ppm ozone-treated spores (p = 0.0032). For the mesophilic strain, *B. cereus* T, there was no significant difference in the percent recovery of spores for either ozone delivery rate.

A regression analysis was performed to further analyze the relationships of the variables in this experiment. These statistics were able to determine that the percent recovery of spores, for any strain, any spore type, and at either ozone delivery rate, was significantly reduced after 20 min of ozone treatment (p = 0.0001). According to the parameter estimates, the percent recovery of spores decreased
approximately 2.96% after each time interval of ozone treatment. According to ANOVA analysis, the difference in the percent recovery between superdormant spores and heterogeneous spore populations was not significant; however, according to the regression analysis superdormant spores were identified to be overall more resistant to ozone treatment than heterogeneous populations (p = 0.0234). According to the parameter estimates, the percent recovery of ozone-treated spores from heterogeneous populations is approximately 20.47% less than that of superdormant spores. Other than this statistic, ANOVA and linear regression results provided similar results. According to the parameter estimates of the regression analysis, the percent recovery of psychrotolerant spores treated with 190-ppm ozone is approximately 31.93% less than spores treated with 6.25-ppm ozone.
Figure 7a. The percent recovery of heterogeneous spore populations with an ozone delivery rate of 6.25 ppm. BW = *B. weihenstephanensis* 6A23, BC = *B. cereus* 6A16, BT = *B. cereus* T.
Figure 7b. The percent recovery of superdormant spores with an ozone delivery rate of 6.25 ppm. BW = B. weihenstephanensis 6A23, BC = B. cereus 6A16, BT = B. cereus T.
Figure 8a. The percent recovery of heterogeneous spore populations with an ozone delivery rate of 190 ppm. BW = *B. weihenstephanensis* 6A23, BC = *B. cereus* 6A16, BT = *B. cereus* T.
4.3 **Inactivation of ozone-treated spores using high hydrostatic pressure**

Superdormant spores of *B. cereus* 6A16, *B. weihenstephanensis*, and *B. cereus* T that were pretreated with or without 10 min of 190-ppm aqueous ozone were treated at 500 MPa for 10 min at 40°C. This study was performed to determine if ozone combined with HHP would be an effective hurdle technology to inactivate superdormant spores and to determine if ozone-treated spores were more susceptible to HHP than spores not pretreated with ozone.

For superdormant spores of *B. weihenstephanensis*, the percent recovery of spores was decreased for spores that were pretreated with ozone as expected. The average percent recovery of superdormant spores of *B. weihenstephanensis* that were
not pretreated with ozone was 72.25 % where the recovery for spores that were pretreated with ozone was reduced to 40.53 %. Surprisingly, the percent recovery of spores for *B. cereus* 6A16 and *B. cereus* T, that were pretreated with ozone were actually higher than spores not treated with ozone (Figure 9). The average percent recovery of superdormant spores of *B. cereus* 6A16 that were not pretreated with ozone was 33.75 % where the recovery for spores that were pretreated with ozone was increased to 48.79 %. In addition, the average percent recovery of superdormant spores of *B. cereus* T that were not pretreated with ozone was 9.75 % where the recovery for spores that were pretreated with ozone was increased to 46.68 %. After ozone treatment, subsequent HHP was demonstrated 0.59-, 0.47-, and 0.49-log reductions for superdormant spores of *B. cereus* 6A16, *B. weihenstephanensis* 6A23, and *B. cereus* T, respectively.

According to ANOVA analysis, superdormant spores pretreated with 190-ppm ozone for 10 min were not more susceptible to inactivation at 500 MPa for 10 min than spores that were not pretreated with ozone (p = 0.7380). Statistical analysis also confirmed that there was no significant difference in the percent recovery of superdormant spores after ozone and HHP treatment between strains (p = 0.4865) meaning that no strain was more sensitive or resistant to the treatment hurdles than the other. Regression analysis confirmed these results.
Figure 9. The recovery of superdormant spores after treatment with 500 MPa for 10 min at 40°C with or without pretreatment with 190-ppm aqueous ozone for 10 min. BC = B. cereus 6A16, BW = B. weihenstephanensis 6A23, and BT = B. cereus T.
Chapter 5

DISCUSSION

5.1 Effect of sporulation temperature on the isolation of superdormant spores using single nutrient germinants

It is important to note that superdormancy is a relative term. It is clear that the level of superdormant spores in a given spore population is dependent upon the germinant used to isolate them. In previous studies, nutrients including inosine and L-alanine were demonstrated to induce spore germination to a much higher degree than L-valine and L-asparagine (Ghosh and Setlow, 2009). Unexpectedly, in this study it appears that lower levels of L-alanine induced spore germination to a greater degree compared to germination with higher levels of L-alanine. This can most likely be explained by the generation of D-alanine by alanine racemase, which is produced when spores come into contact with L-alanine (Chen et al., 2006). It is possible that the exposure of spores to higher levels of L-alanine (25 mM) led to an inhibitory effect caused by D-alanine generated by alanine racemase preventing those spores from germinating as well as they did when exposed to 10 mM L-alanine. A similar pattern was demonstrated by germination with L-valine where higher yields of superdormant spores were isolated by 5 mM L-valine compared to 10 mM L-valine. Perhaps a similar inhibitory effect is occurring in which D-valine generation inhibited germination.

Sporulation temperature has been previously demonstrated to affect spore characteristics including germination and inactivation by wet heat and HHP (Raso et
According to Raso et al. (1998) spores of *B. cereus* ATCC14579 that were sporulated at 37°C were more resistant to germination with wet heat than those sporulated at 20 or 30°C. Conversely, the authors found that spores sporulated at 20°C were more resistant to germination and inactivation by HHP. In a study performed by Gounina-Allouane et al. (2008), spores of the same strain (*B. cereus* ATCC14579) sporulated at lower temperatures were more easily germinated in response to L-alanine and inosine than spores that were sporulated at 37°C. It is thus easy to hypothesize that sporulation temperature plays a role in the way spores are susceptible to germination.

In these particular studies, spores of *B. cereus* that were initially sporulated at higher temperatures (37°C) were more resistant to germination by heat and with nutrient germinants. Spores germinated at lower temperatures (20°C) were more resistant to HHP. Because spores that were initially sporulated at 37°C were more resistant to germination by nutrient germinants, it is easy to hypothesize that the isolation of superdormant spores would be influenced by sporulation temperature. Because superdormant spores are hypothesized to have a lack of or a lack of the expression of nutrient germinant receptors on the spore’s inner membrane, we can then hypothesize from the latter studies that spores sporulated at higher temperatures (37°C) would be more likely to be superdormant.

In the current study sporulation temperature did not affect the level of isolation of superdormants with various nutrient germinants including L-alanine and inosine. Statistical analysis showed that there was a slight correlation between sporulation temperature and the percentage of superdormant spores recovered \((x, y = -0.0545)\) indicating the trend for yields of superdormant spores to be lower for spore
populations isolated at 30°C which confirms the hypothesis presented; however, statistical analyses revealed that this association was not significant. We can therefore not confirm that sporulation temperature has an effect on spore superdormancy.

More studies need to be conducted with nutrient germination of superdormant spores to determine factors that contribute to superdormancy. It would also be beneficial to repeat the experiments performed in this study with spore crops sporulated at higher and lower temperatures. The ability to predict the contributing factors to spore superdormancy would be of great value to the food industry. Once we are able to better understand the phenomenon which causes spore superdormancy, we may be better able to control the incidence of slow germination spores in the food supply.

5.2 Inactivation of superdormant spores compared to heterogeneous spore populations using 190- and 6.25-ppm aqueous ozone

It has been hypothesized that spore inactivation by aqueous ozone renders spores defective in germination due to damage on the spore’s inner membrane with inner membrane proteins being the target (Young and Setlow, 2004). Since the level of superdormancy in a particular spore is thought to also be determined by the level of germinant receptors located on the spore’s inner membrane, it could be hypothesized that the effects of aqueous ozone on superdormant spores may be different than that on nonsuperdormant spores. If the structure of the inner membrane of superdormant spores is in some way different than that of a nonsuperdormant spore, the response of these types of spores to ozone germination or inactivation could very well be different. This experiment was performed to determine if the recovery of superdormant spores treated with ozone was different from that if heterogeneous spore populations and to
additionally determine the potential of aqueous ozone on the inactivation of spores. This type of nonthermal processing technology would be especially beneficial for use in the food industry on products including fresh foods, RPFEDs, or other products where bacterial spore contamination is a problem.

In the current study, superdormant spores were slightly (~20%) more resistant to ozone at either 190 or 6.25 ppm than heterogeneous spore populations. This result indicates that the inner membrane structure of superdormant spores, which is apparently different than that of nonsuperdormant spores on the level of nutrient germinant receptors, may increase the resistance of spores to ozone. The conclusion that superdormant spores are more resistant to ozone than heterogeneous spore populations may indicate that ozone alone may not be an efficient technology to decrease the incidence of all spores within a food product, even at high ozone delivery rates of up to 190 ppm and for treatment times up to 20 min. Because these experiments were carried out in a medium with an extremely low organic load, recovery results should be expected to be much lower than they would be in a scaled up experiment using actual food products. It would be expected that results would be even less ideal if experiments were carried out in a medium with high organic load including food products. It may therefore be more efficient to introduce other hurdles involving other nonthermal processing technologies to effectively reduce the number of spores in a food product.

Interestingly, the recovery of ozone treated spores of psychrotolerant strains used in this study, including *B. cereus* 6A16 and *B. weihenstephanensis* 6A23, was significantly higher for spores treated with 190-ppm ozone than 6.25-ppm ozone. Ozone-treated spores of the mesophilic species, *B. cereus* T, were overall more
sensitive to ozone treatment with no significant difference in the recovery of spores with either ozone delivery rate. The observation that psychrotolerant spores require a higher level of ozone to effectively decrease the number of spores in a sample may indicate the need for the use of higher levels of ozone for refrigerated foods where psychrotolerant sporeformers, such as *B. weihenstephanensis*, are problematic. Foods that may be of particular concern for psychrotolerant sporeformers include RPFEDs and any other refrigerated foods.

It should also be noted that spore recovery after certain time intervals of ozone treatment increased above the control (0 min). Although no significant relationships between the variables and the incidence of the percent recovery of spores being above 100%, a correlation matrix \((x,y)\) was generated to determine which variables could possibly be influencing the incidence of the increased recovery. It was more common for the recovery of superdormant spores to be above 100% for spores of *B. weihenstephanensis* although this correlation was very weak \(x,y = -0.1652\). There was also a trend for the percent recovery of spores to be above 100% for spores treated with the lower ozone delivery rate \(x,y = -0.1491\). It was also more common for spore recovery to be over 100% with shorter ozone exposure times \(x,y = -0.1204\). Lastly, the increased spore recovery was more likely to occur for superdormant spores than for heterogeneous spore populations \(x,y = 0.1491\). In summary, the occurrence of increased spore recovery after ozone exposure above the control (100%) was more likely for superdormant spores of the *B. weihenstephanensis* strain treated with 6.25ppm at shorter exposure times between 0.5 and 10 min. Although these correlations are very weak, it is important to be aware of them for future research purposes.
It is important to address the question as to why an increase in the percent recovery of spores may be observed after ozone treatment at lower levels of ozone for shorter time periods. Looking at the percent recovery of spores, it is hard to determine in these studies whether recovery was to spore germination or inactivation. It could be possible that ozone exposure at lower levels for shorter time periods may cause spore stability, enabling them to germinate more easily in nutrient-rich laboratory medium, causing an increase in standard plate counts for these time intervals. After ozone treatment, spore samples were washed and centrifuged. The absence of vegetative cells and germinating spores was confirmed using phase contrast microscopy (data not shown). It is therefore, highly unlikely that the presence of vegetative cells and germinated spores are the cause of the increased plate counts. In this case, ozone exposure would trigger spore germination that would increase standard plate counts at earlier time intervals including 0, 0.5, 1, 5, and even 10 min. After ozone exposure of 10 min, the majority of superdormant spores are apparently germinated or inactivated during ozone treatment, which is why standard plate counts decreased at later time intervals. These trends can be clearly visualized in Figures 7 and 8. Further experiments need to be performed to determine the cause of increased plate counts at these time intervals with varying levels of ozone.

5.3 Effects of high hydrostatic pressure on spores pretreated with or without ozone

As previously stated, ozone used alone as a nonthermal processing technology may not effectively reduce the incidence of superdormant spores in refrigerated foods; however, ozone used as a hurdle in combination with other nonthermal processing technologies may have increased applications in the food
According to Cortezzo et al. (2004), mild treatment with oxidizing agents including aqueous ozone causes damage to the spore’s inner membrane that may be lethal under normal conditions. The authors of this study hypothesized that the damaged inner membrane of ozone treated spores may be less able to maintain integrity, particularly osmotic stress. Such osmotic stress could potentially be induced by high hydrostatic pressure. In this study superdormant spores that were pretreated and not pretreated with 190-ppm aqueous ozone for 10 min were subsequently treated with 500 MPa for 10 min at 40°C. The percent recovery of superdormant spores that were pretreated with ozone was compared to those of spores that were not pretreated with ozone. It was expected that the percent recovery of superdormant spores pretreated with ozone would be significantly decreased for spores pretreated with ozone.

Surprisingly, there was no significant difference in the percent recovery of spores exposed to HHP that were or were not pre-exposed to ozone. Even more surprisingly the percent recovery of spores pretreated with ozone was actually slightly higher than for spores not pretreated with ozone \((x, y = -0.0985)\), although this difference was not significant \((p = 0.6974)\). B. weihenstephanensis superdormant spores were more resistant to the ozone + HHP hurdle technology and B. cereus T superdormant spores were most sensitive, which was consistent with other experiments performed in this study although this difference was also not significantly different \((p = 0.3378)\).

The greatest reduction in the percent recovery of superdormant spores using ozone alone was achieved for spores of B. cereus T with a log reduction of 2.08 log CFU/ml. The greatest achieved additional log reduction spores that were treated with
HHP following ozone pretreatment was 0.59 log CFU/ml with spores of *B. cereus* 6A16. Therefore, the combined ozone-HHP hurdle technology could potentially achieve a 2.67 log CFU/ml reduction in the percent recovery of superdormant spores in a sample. Considering the number of spores present in a food product is usually low, the ability for ozone-HHP to reduce spores of *Bacillus* species by 2.67 log CFU/ml provides evidence that this technology has potential applications for the food industry.

It is once again very important to address the issue as to why the recovery of superdormant spores is not significantly reduced for spores pre-exposed to ozone. It could be possible, as explored in the previous ozone experiments discussed that ozone exposure combined with HHP could actually increase the sensitivity of the spores to germination in the nutrient-rich laboratory media causing increased plate counts. Because samples were centrifuged using buoyant density centrifugation following ozone and HHP experiments, it is highly unlikely that increased plate counts were due to the presence of vegetative or germinated spores. In this case the combined hurdle technology may be effective at inducing germinating superdormant spores, but not inactivating them. Higher pressures, treatment times, or higher treatment temperatures should be explored in combination with ozone pretreatment in order to determine if this is the case. It may also be beneficial to determine the effects of the reversed process of the hurdle technology, looking at the effects of aqueous ozone on the percent recovery of HHP pre-exposed superdormant spores.
REFERENCES


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### APPENDIX

1. **CCY Medium Recipe**

<table>
<thead>
<tr>
<th>Step 1: Nutrient Stock Preparation</th>
</tr>
</thead>
<tbody>
<tr>
<td>In 100ml distilled water combine the following:</td>
</tr>
<tr>
<td>1. 100 mg L-glutamine</td>
</tr>
<tr>
<td>2. 5 g Oxoid Acid Casein hydrolysate</td>
</tr>
<tr>
<td>3. 5 g Difco Bacto Casitone</td>
</tr>
<tr>
<td>4. 2 g Difco Bacto Yeast Extract</td>
</tr>
<tr>
<td>5. 10 g 3% w/v glycerol</td>
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</table>

<table>
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<th>Step 2: Buffer Preparation</th>
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<tr>
<td>In 1 L of distilled water combine the following:</td>
</tr>
<tr>
<td>1. 1.8 g KH₂PO₄</td>
</tr>
<tr>
<td>2. 4.52 g K₂HPO₄</td>
</tr>
<tr>
<td>3. 20 ml Nutrient Stock</td>
</tr>
<tr>
<td>Dispense 1 L amounts into 2 L flasks and autoclave</td>
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</table>

<table>
<thead>
<tr>
<th>Step 3: CCY Salt Solution Preparation</th>
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<tbody>
<tr>
<td>In 100ml of distilled water combine the following:</td>
</tr>
<tr>
<td>1. 0.68g ZnCl₂</td>
</tr>
<tr>
<td>2. 10.16g MgCl₂·6H₂O</td>
</tr>
<tr>
<td>3. 0.197g MnCl₂·4H₂O</td>
</tr>
<tr>
<td>4. 2.94g CaCl₂·2H₂O</td>
</tr>
<tr>
<td>5. 1.35g FeCl₃·6H₂O</td>
</tr>
<tr>
<td>6. Dilute pure HCl N=12N) to 0.1N N/10) HCl. Add 1 ml.</td>
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<table>
<thead>
<tr>
<th>Step 4: Addition of culture and sporulation</th>
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</thead>
<tbody>
<tr>
<td>1. Inoculate 5 ml of a log phase culture per 1L CCY Medium.</td>
</tr>
<tr>
<td>2. At the same time as the bacterial culture, add 2 ml of the salt solution</td>
</tr>
<tr>
<td>3. Incubate at 30°C - 37°C while shaking at 200 rpm for 5 - 7 days</td>
</tr>
<tr>
<td>4. Add 0.1µg/L lysozyme and continue shaking for 30 min</td>
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