Approaches for Enhancing Lethality of Bacterial Spores Treated by Pressure-Assisted Thermal Processing

Dissertation

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Abstract

Selected approaches for enhancing pressure-assisted thermal processing (PATP) lethality on bacterial spore inactivation were investigated using high pressure microbial kinetic testing equipment. *Bacillus amyloliquefaciens* TMW 2.479 spores were used as the test organisms. Efficacy of pressurization rate and double-pulse treatment in enhancing PATP lethality was examined. Pressurization rate influenced the PATP lethality as a function of pressure holding time. During short pressure holding times (≤ 2 min), PATP treatment with the slow pressurization rate (3.75 MPa/s) provided enhanced spore reduction over that of fast pressurization rate (18.06 MPa/s). Regardless of the pressurization rate, after 5-min treatment at 105°C-600 MPa, 6 log reduction of *B. amyloliquefaciens* spores were observed. Double-pulse treatment enhanced PATP lethality by approximately 2.4 to 4 log CFU/mL, in comparison to single pulse for a given pressure holding time.

The efficacy of combining organic acids (acetic, citric, and lactic; 100 mM, pH 5.0) with PATP treatment in enhancing *B. amyloliquefaciens* inactivation was studied. In combination with 2-min PATP treatment at 700 MPa-105°C, acetic and citric acids were found to provide synergistic effect on inactivating *B. amyloliquefaciens* spores than the spores suspended in lactic acid or deionized water. Organic acids in combination with PATP treatment also induced spore germination (33% to 80%) as a function of pressure

holding time and type of organic acid used. Combining organic acids with PATP also inhibited the growth and recovery of the remaining survivor population during 28-day storage at 32°C in a low-acid food model system (carrot puree, pH 5.0).

Biochemical changes in *B. amyloliquefaciens* spores grown in two different sporulation media (TSAYE and NAYE) as influenced by PATP, high pressure processing (HPP), and thermal processing (TP) were investigated using Fourier-transform infrared spectroscopy (FT-IR). FT-IR spectra of the bacterial spores were not only influenced by the processing conditions, but also by the sporulation media. PATP and TP treatments acted upon specific targets of calcium dipicolinate (CaDPA), which were identified by bands at 1381, 1415, and 1442 cm^{-1}. Changes in secondary protein bands were also observed during PATP and TP. Surviving spore populations of *B. amyloliquefaciens* after PATP and TP treatments could be estimated by cross-validated PLSR models using spectral region (900-1800 cm^{-1}). However, such a developed model based on single-pulse data could not be used for predicting double-pulse lethality especially during 2^{nd} pulse pressure holding time, possibly due to differences in respective spore inactivation mechanisms.

In conclusion, various physical- (pressurization rate, pulsing) and chemical- (organic acid) based approaches can be used for enhancing PATP lethality of highly pressure-thermal resistant *B. amyloliquefaciens* spores. Different processing (PATP, TP) conditions and sporulation media caused changes in FT-IR spectra especially in CaDPA

and secondary protein bands. Organic acids, citric and lactic acids in particular, in combination with PATP treatment enhanced PATP lethality and further inhibited spore recovery in the treated carrot puree during extended storage (up to 28 days) at 32°C.

Dedication

Dedicated to my beloved father, mother, and sister

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Fields of Study

Major Field: Food Science and Technology

Table of Contents

List of Tables

List of Figures

Figure Page

Chapter 1: Introduction

Due to the increased consumer demand for fresh-like, minimally processed, and additive-free food products, the food industry is interested in identifying alternative food technologies for preserving shelf-stable low-acid products that can ensure microbial safety without degrading product quality attributes. One of the key challenges during shelf-stable low-acid food processing is the inactivation of bacterial spores, which normally requires higher treatment intensity than those required for vegetative bacteria. Among the technologies being investigated, pressure-assisted thermal processing (PATP) generated lot of attention because of its potential to overcome the limitations of the conventional thermal-based preservation methods. The unique advantages of the technology include a rapid temperature increase in treated samples due to heat of compression during pressurization and expansion cooling upon depressurization. This reduces thermal impact on final product quality.

Milder form of pressure "pasteurization" treatment involves an application of pressures (300 to 600 MPa) at process temperatures < 45ºC. This effectively inactivates variety of vegetative pathogenic and spoilage bacteria, yeasts and molds, and viruses. Pressure pasteurization has been successfully utilized for preserving variety of commercial products (e.g., guacamole, salsa, juices, smoothies, deli meat, oysters, and cooked ham) that are distributed under refrigeration. Pressure pasteurized products are

available in North American, European, and Asian markets.

Bacterial spores cannot be inactivated by pressure treatment alone at or near ambient temperatures. Combination of pressure and moderate heat is required for bacterial spore inactivation. During PATP, the pre-heated food material is subjected to a combination of elevated pressure (500 to 700 MPa) and temperature (90 to 121°C) for a short duration (≤ 10 min). Various bacterial spores and thermophilic microorganisms are successfully inactivated by the simultaneous application of pressure and heat. Heat sensitive shelf-stable products such as soup, tea, meat entrée can be preserved by this technology. During February 2009, PATP has been approved by Food and Drug Administration (FDA) for commercial sterilization of mashed potato product. More studies on PATP spore lethality and process uniformity are necessary before PATP treated products can be successfully introduced into commercial market.

Like thermal processing, during PATP, *Clostridium botulinum* has been identified as the primary pathogenic target for sterilization. Since pathogenic spores cannot be utilized in the industrial environment, studies have identified *Bacillus amyloliquefaciens* as one of the more pressure-temperature resistant non-pathogenic spores that can be used as the indicator organisms.

In general, PATP lethality increases with increasing pressure, temperature, and holding time. In comparison to thermal sterilization, PATP treatment exposes the food products to limited thermal exposure (90 to 121°C) over a short duration. Yet, it is still desirable to further reduce the severity of the process to preserve fresh-like product attributes. Various approaches for enhancing PATP spore lethality are desired.

Similar to thermal processing, process come-up time can be an important factor that can influence PATP process lethality. Pressurization rate determines the process come-up time, which is primarily a function of the target pressure and the horsepower of the pump. Until now, there is limited information available on the role of pressurization rate and pressure pulsing on bacterial spore inactivation using PATP.

Use of antimicrobial compounds (i.e., nisin, sucrose esters) and acidification are some of the common chemical-based approaches used in thermal processing to reduce process severity and improve microbial stability. Very limited studies have been conducted to investigate the role of organic acids on enhancing bacterial spore inactivation during PATP.

While many mechanisms have been proposed on pressure effects for the inactivation of vegetative bacteria and bacterial spore, the knowledge of inactivation mechanisms for bacterial spores during combined pressure-thermal processing (especially at PATP conditions) are at infancy. Improved understanding of spore inactivation mechanism will facilitate the introduction of microbiologically safe, premium shelf-stable low-acid products.

Therefore, the objectives of this dissertation were:

1. To examine the effect of pressurization rate and pressure pulsing in enhancing spore inactivation during combined pressure-thermal treatment.
2. To investigate the efficacy of organic acids in combination with pressure-thermal treatment in enhancing bacterial spore inactivation and microbial stability.

3. To elucidate bacterial spore inactivation mechanisms occurring during thermal and pressure-assisted thermal processing.

Chapter 2: Bacterial spore inactivation by pressure-assisted thermal processing: Challenges in finding a suitable biological indicator for PATP process validation

2.1 Introduction

Canning has been the technology of choice for commercial sterilization of shelf-stable low-acid foods. Even though retorting has been proven safe and reliable, slow heating (and cooling) during the process results in significant temperature gradients within the processed product. The harsher thermal treatment often significantly deteriorates product quality and heat sensitive ingredients. To satisfy increased consumer demand for fresher, minimally processed food products, the food industry is interested in identifying alternative technologies that can ensure microbiological safety in the low-acid foods without compromising its quality. Among the alternative technologies, pressure-assisted thermal processing (PATP) provides a unique opportunity to overcome the limitations of conventional thermal processes. The technology has emerged as one of the viable alternatives for traditional retorting for producing commercially sterile shelf-stable low-acid foods at reduced temperatures.

Industrial applications for high pressure technology were initially developed in the chemical processing, ceramics, and metallurgical industries where it is used, for instance, in sheet metal forming and isostatic pressing of advanced materials. In 1899, Bert Hite observed the delay of microbial spoilage of milk by the application of high

pressure at 600 MPa and room temperature for 1 h (Hite, 1899). Hite and his colleagues continued their investigation on a variety of foods, especially fruits and vegetables (Hite et al., 1914). However, it took until 1980's for the technology to become commercially viable in the food industry. During 1980's, Japanese universities and industries pioneered the commercialization of value-added pressure pasteurized products such as jam, jelly, juice, etc. High-pressure pasteurization involves an application of elevated pressures (300 to 600 MPa) at process temperatures < 45ºC (Cheftel, 1995; Smelt, 1998; San Martín et al., 2002; Balasubramaniam, 2003; Lau and Turek, 2007). This effectively inactivates variety of vegetative pathogenic and spoilage bacteria, yeasts and molds, and viruses. During 2007, the production of HPP products is estimated around 150,000 tonne per year. Pressure pasteurization technology has been commercialized in North America (Canada, Mexico, USA), Europe (Germany, Italy, Spain, Portugal, UK), and Asia (including China, Japan, Korea). Guacamole, salsa, smoothies, deli meat, oysters, and cooked ham are examples of commercial products in the market (Saiz et al., 2008).

Bacterial spores are highly resistance to pressure treatments at ambient temperatures. To achieve commercial sterility of shelf-stable low-acid food products, inactivation of bacterial spores, especially *Clostridium botulinum* is required. Pressure-assisted thermal processing (PATP), also referred as pressure-assisted thermal sterilization (PATS) or high pressure-high temperature sterilization (HPHT), involves a combined application of elevated pressures (500 to 700 MPa) and temperatures (90 to 121ºC) for a short duration to a preheated food product (Ananta et al., 2001; Margosch et al., 2004a; Rajan et al., 2006a; Ahn et al., 2007). Temperature of the product is rapidly

increased during compression and the expansion cooling occurs upon depressurization (Ting et al., 2002). This limits the severity of the thermal effect encountered within a conventional retort. Combined pressure-heat effect successfully inactivates various bacterial spores.

2.2 Process engineering basics

2.2.1 General principles governing pressure-assisted thermal processing

Application of high pressures is governed by two key principles (Cheftel, 1995; Smelt, 1998). Le Chatelier's principle states that any phenomenon (phase transition, change in molecular configuration, and chemical reaction) accompanied by a decrease in volume will be enhanced by pressure (Mozhaev et al., 1994; Earnshaw et al., 1995; Smelt, 1998). Secondly, the isostatic principle states that pressure is instantaneously and uniformly transmitted independent of size and geometry of foods (Smelt, 1998).

During compression phase of the pressure treatment, product temperature increases due to heat of compression. Product temperature reverts back to its initial value upon depressurization. Temperature increase of food materials under pressure is dependent on factors such as target pressure, product compressibility, and initial temperature. The extent of this temperature change can be estimated using the following equation (Hoogland et al., 2001; Matser et al., 2004):

$$\frac{dT}{dP} = \frac{\alpha T}{\rho C_P} \tag{2.1}$$

where T is temperature (K), P is pressure (Pa), α is volumetric expansion coefficient (1/K), ρ is density (kg/m^3), and C_P is specific heat (J/kgK). Often, for most materials,

above properties under pressure are not readily available. This is the topic of current research. Heat of compression values for various food materials can be experimentally determined (Otero et al., 2000; Rasanayagam et al., 2003).

Most of the food material contains water. Temperature of water increases about 3ºC for every 100 MPa pressure increase at room temperature (25ºC). On the other hand, fats and oils have a heat of compression value of 8 to 9ºC per 100 MPa, proteins and carbohydrates have intermediate heat of compression values (Ting et al., 2002; Rasanayagam et al., 2003). Table 2.1 presents heat of compression of selected food materials. It is worth to note that while heat of compression values of water is dependent on initial temperature, for fats and oils the values are not influenced by initial sample temperature.

2.2.2 Typical pressure-assisted thermal processing

At present, PATP is essentially a batch process. The process starts with packaging the food in a flexible container. At least one interface of the package should be flexible so that it readily allows the pressure transmission (Balasubramaniam et al., 2004). Then, the container is pre-heated to a pre-processing temperature (T_1) so that the product can be processed at a process pressure (P) and a target process temperature (T_3) (Figure 2.1). Pre-heated samples are loaded into a high pressure chamber filled with a pressure-transmitting fluid. Water is the commonly used pressure transmitting fluid in the food industry. Glycol, silicone oil, sodium benzoate solution, ethanol solution, and castor oil are examples of pressure transmitting fluids used in laboratory model high pressure equipments (Balasubramaniam et al., 2004). The pressure transmitting fluid within the

pressure chamber is also approximately maintained at the same pre-processing temperature (T_1) as that of the product. The system is then pressurized when the sample temperature reaches a predetermined initial temperature (T_2). T_2 value can be estimated from the knowledge of target process temperature (T_3), process pressure (P), heat of compression values of the food material (CH), and the extent of heat exchange (ΔT_H) with the surroundings (Rajan et al., 2006a; Nguyen et al., 2007):

$$T_2 = T_3' - \left(CH \cdot \Delta P + \Delta T_H\right) \tag{2.2}$$

Minimized ΔT_H can be achieved by a trained equipment operator through a set of preliminary trials (Nguyen et al., 2007). It is important to use an appropriate thermal insulation to reduce heat loss between the sample and the surroundings (Balasubramaniam et al., 2004).

Pressure come-up time (t_2) is defined as the time required to increase the pressure of the sample from an atmospheric pressure to the target process pressure (Farkas and Hoover, 2000; Balasubramaniam et al., 2004). It is principally a function of the target process pressure and the pump horsepower. After the appropriate processing time (t_3-t_4), high pressure is released to the atmospheric pressure. Normally, the pressure holding time (t_3-t_4) does not include the pressure come-up time (t_2) or the depressurization time (t_5) (Figure 2.1). Then, the processed food material can be removed from the pressure chamber and immediately cooled. In general, the pressure and temperature histories of the process condition have been rarely reported in the literature. Therefore, it is undeniably difficult to compare results of microbial inactivation obtained from various laboratories. The failure to control the process temperature under pressure could be one

of the major reasons for the large variability in the reported microbial inactivation data from the literature (Balasubramaniam et al., 2004).

2.3 Microbial efficacy of pressure treatment on bacterial spores

2.3.1 Spore biology

One of the major objectives of any food processing is the inactivation of harmful pathogenic bacteria as well as organisms that cause economic spoilage in processed foods. Spore-forming bacteria such as *Alicyclobacillus*, *Bacillus*, *Clostridium*, *Desulfotomaculum*, and *Sporolactobacillus* spp. often cause food safety or spoilage problems (Setlow and Johnson, 2001). Specially, spores produced by *Bacillus* (aerobe and facultative anaerobe) and *Clostridium* (strict anaerobe) are the major concern for the production of shelf-stable low-acid foods since they are generally found in soil and have found to be resistant to food processing treatments (Gould, 1999). Spores are formed in response to unfavorable environmental conditions, likely when there is nutrient depletion. However, spores can germinate and outgrowth again when environment conditions become favorable. During the development of spores, several morphological and biochemical changes take place. The sporulation process typically involves seven stages. These include stage I (axial filament), stage II (asymmetric separation), stage III (engulfment), stage IV (cortex formation), stage V (coat formation), stage VI (spore maturation), and stage VII (release of mature spore) (Piggot, 2000; Setlow and Johnson, 2001). Vegetative cells are stage 0. Stage I starts with the presence of two nucleotides in an axial filament. During stage II, unequal cell division takes place, leading to the

formation of a smaller spore compartment, the forespore, separated from the larger mother cell compartment. Then, an endospore is formed through the engulfing of the forespore by the mother cell. As a result, there are two complete membranes (inner and outer forespore membrane) surrounding the forespore (stage III). A large peptidoglycan structure (cortex) is then formed between the inner and outer forespore membranes, while the germ cell wall is formed between the cortex and the inner forespore membrane (stage IV). Then, the forespore synthesizes glucose dehydrogenase and small, acid-soluble proteins (SASPs). Whereas the function of glucose dehydrogenase is unknown, SASPs is reported to be involved with spore resistance and DNA protection (Gould, 1999; Setlow and Johnson, 2001; Montville and Matthews, 2005). After the pH in the forespore decreases by 1 to 1.3 units (occurring during the late stage III), the dehydration process begins (Gould, 1999; Setlow and Johnson, 2001). In the stage IV to stage V transition, the proteinaceous coat layers are laid down outside the outer forespore membrane. These coats vary significantly from species to species. During stage VI, the deposit of divalent cations (Ca^{2+}, Mg^{2+}, and Mn^{2+}) and pyridine-2,6-dicarboxylic acid (dipicolinic acid; DPA) occurs. After the complete spore formation, the spore is released by the lysis of the mother cell (stage VII). Heat resistance of spores considerably increases during the later stages of the core dehydration and the cortex formation (Gould, 1999). Spore core contains complete genome, ribosomes, cytoplasmic enzymes, DPA, divalent cations, and SASPs in a relatively low water content environment (0.4 to 1 g of water per g of spore dry weight) (Setlow and Johnson, 2001; Montville and Matthews, 2005). The structure of a dormant spore is shown in Figure 2.2.

Thermal resistance of spores is generally due to low water content, mineralization of the core, and relative impermeability of the cortex (Gould, 1999; Gould, 2006). In addition, physical structure of spores (a thick layer of cortex and proteinaceous spore coats) is also known to protect spores from environmental stresses. Inner forespore membrane serves as a permeability barrier to hydrophilic molecules and to most molecules of >150 molecular weight (Setlow and Johnson, 2001). Moreover, SASPs bound with spore DNA, and DPA associated with divalent ions (mostly Ca^{2+}) are believed to involve in spore stability (Deeth and Datta, 2002). All of these unique characteristics make bacterial spores among the most resistant microorganisms to various environmental stresses such as heat, irradiation, desiccation, extreme pH, and toxic chemicals like ethanol or chloroform (Piggot, 2000; Setlow, 2003). Margosch et al. (2004a) stated that the ability of spores to retain DPA identified their resistance to the combined high pressure and temperature treatments. Spores can convert back to vegetative cells when they are exposed to germinants such as amino acids, sugars, or purine nucleosides (Setlow, 2003). Dormant spores are induced to the germination step by nutrients and non-nutrient agents. Nutrient germinants bind the receptors in the spore's inner membrane, which trigger the release of the spore core components like DPA and divalent cations. Water replaces the inner core components and triggers the hydrolysis of the spore's cortex by cortex-lytic enzymes such as CwlJ and SleB in *B. subtilis* (Setlow, 2003; Moir, 2006). After the cortex hydrolysis, the expansion of germ cell wall allows full hydration in spore core, continuation of spore metabolism, and macromolecule synthesis (Setlow and Johnson, 2001). Examples of non-nutrient

germinants include lysozymes, salts, high pressure, calcium dipicolinate (CaDPA), and cationic surfactants such as dodecylamine (Setlow, 2003). The release of CaDPA from one spore may stimulate the germination of other neighboring spores.

Once spores are mixed with germinants, the germination step begins and continues even after the germinants are removed. The first step is the release of hydrogen ion (H^+), mono- and di-valent cations from the spore core. Secondly, DPA is released. Dipicolinic acid accounts for about 10% of spore dry weight (Piggot, 2000). It is normally associated with divalent cations like Ca^{2+}. Thirdly, the spore is rehydrated leading to the decrease in spore moist-heat resistance. The fourth step is the hydrolysis of peptidoglycan spore cortex. Lastly, further water uptake causes swelling of the spore core and expansion of the germ cell wall. After germination, the germinated spore develops into a growing cell and eventually resumes normal cell function (Setlow, 2003).

2.3.2 Factors influencing spore inactivation

Unique structural and physiological characteristics of bacterial spores are responsible for the extreme resistance to environmental factors such as pressure and temperature. It has been long known that bacterial spores can survive pressures up to 1,200 MPa at ambient temperatures over extended holding times. There is no apparent correlation between pressure and thermal resistance of bacterial spores (Nakayama et al., 1996; Smelt et al., 2002; Margosch et al., 2004a). Researchers are currently investigating the combined pressure-thermal resistance of various bacterial spores (Table 2.2). Several intrinsic and extrinsic factors, and their interactions can influence spore inactivation during PATP. These include type of the microorganism, species and strains,

physicochemical conditions (e.g., sporulation temperature, media composition, mineral content), process parameters (e.g., pressure, temperature, and pressure holding time), mode of pressure application (e.g., static or pressure cycling), and food composition (e.g., pH, a_w, fat content, protein content). Depending upon pressure-temperature regime, presence or absence of spore germination has been reported. It is further interesting to note that possibly due to differences in equipment design characteristics and inadequate reporting of experimental methodologies, it is difficult to compare results from different laboratory groups. Approaches that may aid in meaningful comparison of results from various laboratories are provided by Balasubramaniam et al. (2004).

2.3.2.1 Sporulation conditions

Difference in sensitivity of bacterial spores among different strains is likely caused by the morphological and physiochemical characteristics of the spores during sporulation. The rigidity of spore cortex and coat as well as components in spore core can influence spore resistance (Wuytack and Michiels, 2001; Oomes and Brul, 2004). Very limited studies have investigated the effect of bacterial sporulation conditions such as temperature, sporulation media composition, pH on the combined pressure-heat resistance. Decreasing sporulation temperature in general is reported to increase pressure resistance of bacterial spores. For the same process conditions (690 MPa, 60ºC, 30 s), decreasing sporulation temperature from 37 to 20ºC, decreased *B. cereus* spore inactivation (from 7 to 4 log CFU/mL) (Raso et al., 1998a). Similarly, the type of mineral present in the spore can also influence pressure resistance. Igura et al., (2003) reported that pressure resistance of *B. subtilis* spores increased after demineralization with Ca^{2+} or

Mg^{2+}, whereas the resistance did not change when the spores were remineralized with Mn^{2+} or K^{+}. More studies are required to further understand the role of sporulation and bacterial spore growth conditions on the combined pressure-heat resistance of a variety of bacterial spores, especially at elevated process conditions.

2.3.2.2 Process conditions

In general, bacterial spores are inactivated as a function of pressure, process temperature, and pressure holding time (Rovere et al., 1996; Ananta et al., 2001; Margosch et al., 2004a, 2004b; Kouchma et al., 2005; Margosch et al., 2006; Patazca et al., 2006; Rajan et al., 2006a, 2006b; Ahn et al., 2007; Black et al., 2007b). Bacterial spores are often resistant to pressures above 1,000 MPa at ambient temperatures (Cheftel, 1995).

However, when combined with heat, spore inactivation in general increases with increasing pressure, temperature, and pressure holding time. For example, increasing process temperature from 35 to 55ºC, increased *C. botulinum* type E spores reduction by approximately 4 log units at 827 MPa after 5-min pressure holding time (Reddy et al., 1999). However, the synergy between heat and pressure diminished at elevated temperatures, and heat becomes the dominant contributor to the spore inactivation, especially at temperatures ≥ 121ºC (Rajan et al., 2006a; Ahn et al., 2007).

It is further interesting to note that different bacterial spores seem to exhibit different levels of the combined pressure-thermal resistance for the same pressure come-up time (Ahn et al., 2007). Margosch et al. (2004a) reported 1.5 log reduction in *C. botulinum* TMW 2.357, 3 log reduction in *C. thermosaccharolyticum*, > 5 log reduction

in *B. subtilis*, and < 0.5 log reduction in *B. amyloliquefaciens* after pressure come-up time at 600 MPa and 80ºC. Hayakawa et al. (1998) reported that rapid decompression (1.30 to 1.65 ms) after a treatment (200 MPa at 75ºC for 60 min) enhanced spore inactivation of *G. stearothermophilus* IFO 12550.

Pressure cycling (also known as pulsed pressurization, oscillatory pulses, or reciprocal pressurization) has been reported as an effective way to enhance microbial inactivation (Hayakawa et al., 1994; Palou et al., 1998; Furukawa et al., 2000; Meyer et al., 2000; San Martín et al., 2002; Furukawa et al., 2003). Spore inactivation during pressure pulsing is governed by the target pressure, process temperature, pressure holding time during each pulse, number of pulses, and time interval between pulses. Ahn and Balasubramaniam (2007a) reported that double- and triple-pulse pressurization were lethal in inactivating *C. sporogenes* spores, in comparison to equivalent static pressure treatments. Hayakawa et al. (1994) reported complete inactivation of 10^6 CFU/mL *G. stearothermophilus* spore population when 6 pulses (5 min each) at 600 MPa and 70°C were used. In the case of *B. subtilis* and *B. licheniformis* spores, the first pulse was applied at 800 MPa and 70ºC for 2 min, then followed by 800 MPa and 70ºC or 0.1 MPa and 70ºC (Margosch et al., 2004b). Comparable results were obtained from both treatments. This could be explained by the release of DPA during treatments. Once spores released > 90% of their DPA content, the inactivation was not influenced by pressure any further (Margosch et al., 2004b). Even though the enhanced spore inactivation was reported with the pulsed pressurization, the increased cost associated with possible equipment wear and tear should be considered.

2.3.2.3 Influence of food composition

Unlike buffer systems, food composition (such as fat, carbohydrate, and protein) can provide protective effect to bacterial spores (Raso et al., 1998b; Garriga et al., 2004; Solomon and Hoover, 2004). Very limited studies have investigated the influence of the food matrix on spore inactivation during pressure treatments. Ananta et al. (2001) reported that bacterial spores were protected by their surrounding matrix against lethal effects of pressure and heat. Milk with varying fat concentration (0, 2, and 4%) did not contribute to the protective effect on *B. cereus* spores, in comparison to those obtained from buffer solutions after treatment at 690 MPa-40ºC for 2 min (Raso et al., 1998b). Egg patties with complex ingredients did not provide protective effect on the inactivation of *G. stearothermophilus* spores when processed at 700 MPa and 105ºC (Rajan et al., 2006b). Similar observations were reported for the inactivation of *C. botulinum* nonproteolytic type B spores suspended in phosphate buffer and crabmeat (Reddy et al., 2006). More studies are needed to investigate the influence of various food constituents on PATP spore resistance.

2.3.2.4 Importance of pH and water activity on spore inactivation

pH is known to influence microbial inactivation under pressure (Raso et al., 1998b; Stewart et al., 2000; Ananta et al., 2001). Application of pressure can cause transient pH shift (Earnshaw et al., 1995; Smelt, 1998; Smelt et al., 2002). The ionic dissociation of water and several weak acids is enhanced under pressure, thereby shifting the pH of the solution towards the acidic side (Cheftel, 1995). The decrease in pH may promote protein denaturation and microbial inactivation. Upon depressurization, pH

likely shifts back close to its initial value. Due to the challenges associated with the development of in-situ pH sensors under pressure, the influence of pH on microbial inactivation during combined pressure-heat treatment largely remains unknown.

Many researchers observed that acidic pH enhanced spore inactivation during pressure treatments (Roberts and Hoover, 1996; Raso et al., 1998b; Knorr, 1999; Stewart et al., 2000) while some others found insignificant effect of pH on bacterial spore inactivation (Wuytack and Michiels, 2001). Various spore-forming bacteria cannot grow below pH of 3.7 and spores are typically more resistant to pH slightly above their optimum (Murakami et al., 1998). Inactivation of *B. coagulans* spores was higher at pH 4 than at pH 7 under 400 MPa and 40^{o}C (Roberts and Hoover, 1996). Stewart et al. (2000) reported that *C. sporogenes* spore inactivation was enhanced at pH 4.0, compared to the same treatment (404 MPa for 15 min) at pH 7.0. The enhanced spore inactivation under acidic environment might be due to the transformation of native spores into H-spores (Paredes-Sabja et al., 2007). Acidic pH delays the transport of divalent cations in bacterial spores, leading to heat sensitive H-spores (Wuytack and Michiels, 2001). Margosch et al. (2004a) reported that DPA release was enhanced when the pH was reduced to 4.0 or when the temperature was increased. The inactivation of spores by low pH is produced by a drastic change in the spore permeability barrier, which leads to the loss of DPA and a concomitant hydration of the core (Margosch et al., 2004a). Spore permeability barriers of the inner membrane are more sensitive to ions and protons with the application of high-pressure, high-temperature, and pressure-induced pH shift (Paredes-Sabja et al., 2007).

Water activity (a_w) also influences spore inactivation during pressure treatments. Pressure resistance of bacterial spores increases with the addition of sugar or salt due to the increased spore dehydration (Cheftel, 1995). Raso et al. (1998b) observed the increased resistance of bacterial spores to HPP at low a_w when a non-ionic solute (e.g., sucrose) was present in the suspending solutions. Specially, there was no inactivation obtained at a_w 0.92 after the treatment at 690 MPa and 40ºC for 2 min. The authors explained their observations by the osmotic dehydration of the spore protoplast by sucrose increasing the spore's pressure resistance. In vegetative microorganisms, low a_w protects proteins and whole organisms against heat and pressure (Oxen and Knorr, 1993; Smelt et al., 2002). However, the surviving injured cells after the treatment were more sensitive to a_w values suboptimal to their growth (Smelt, 1998; Smelt et al., 2002).

2.3.3 Proposed mechanistic approaches for pressure inactivation of bacterial spores

Although there have been many proposed mechanisms regarding the effects of pressure on vegetative bacteria inactivation (Cheftel, 1995; Lado and Yousef, 2002; Smelt et al., 2002) , pressure inactivation mechanisms for bacterial spores are not well established. Application of pressure can inactivate bacterial spores with or without germination depending on the pressure-temperature combination used (Ananta et al., 2001).

Depending upon the target pressure-temperature process conditions utilized, various researchers have proposed different mechanisms for spore inactivation (Wuytack et al., 1998; Paidhungat et al., 2002; Margosch et al., 2004a, 2004b, 2006; Black et al., 2007b; Subramanian et al., 2007). High pressure can trigger spore germination, and this

could be one of the reasons that high pressure treatments inactivate spores as germinated spores are more sensitive to inactivation treatments (Black et al., 2007b). Proposed approaches to inactivate spores by high pressure treatment include a two-stage strategy (Clouston and Wills, 1969; López-Pedemonte et al., 2003), pressure treatment at moderate temperatures (Mills et al., 1998; Paidhungat et al., 2002; Oh and Moon, 2003; Black et al., 2007b), "hit and wait" strategy (Margosch et al., 2004b), and pressure treatments at elevated temperatures or PATP (Ananta et al., 2001; Margosch et al., 2004b; Rajan et al., 2006a; Ahn et al., 2007).

The two-stage strategy involves the use of initial low pressure (60 to 100 MPa) and mild temperature treatment (< 30ºC) with an extended holding time followed by a shorter treatment at moderate pressures (> 300 MPa) and temperatures. During the first stage, low pressures and mild temperatures induce spore germination by activating the nutrient germinant receptors (Wuytack et al., 2000; Paidhungat et al., 2002). Once spores germinate, the outgrowing cells are less resistance to high pressure and subsequently killed during the second stage with moderate pressure treatments. The main limitation of this strategy is that a small fraction of spores remain ungerminated (Sale et al., 1970). If higher pressures (> 300 MPa) at mild temperatures (< 30ºC) were directly applied by missing the first stage, less spore inactivation would be obtained (Mills et al., 1998; Raso et al., 1998a).

Pressure treatments (500 to 600 MPa) applied at moderate temperatures (< 60ºC) enhanced spore inactivation (Mills et al., 1998). Under these conditions, DPA is released from the spore core through specific channels in the inner membrane or on the membrane

itself. This further triggers spore germination (Paidhungat et al., 2002; Black et al., 2007a). However, some spores will slowly complete the germination process due to the potential damaged spore germination system such as the cortex lytic enzyme system (Wuytack et al., 1998; Ananta et al., 2001; Paidhungat et al., 2002; Black et al., 2007b).

At elevated temperatures, Ahn and Balasubramaniam (2007b) reported the insignificant numbers of pressure-induced germinated *B. amyloliquefaciens* TMW 2.479 Fad 82 spores after 700 MPa-105ºC treatment. Increasing pressure holding time up to 5 min appeared to prolong the lag phase of the surviving spores. PATP-induced spore injury likely occurred rather than PATP-induced spore germination (Ahn and Balasubramaniam, 2007b). This is in agreement with an earlier study from Ananta et al. (2001).

The "hit and wait" strategy, proposed by Margosch et al. (2004a), also utilizes the two-stage mechanism for spore inactivation. First, an application of short pressure pulse (600 to 800 MPa) at high temperature (> 60ºC) releases at least 90% of the spore DPA content. Subsequently, the DPA-free spores are inactivated by heat upon pressure release (Margosch et al., 2004a, 2004b). DPA is released mainly by a physicochemical process producing sublethaly injured DPA-free spores. The success of the strategy depends upon the ability of the spores to retain DPA and the heat resistance of DPA-free spores. For example, *B subtilis* TMW 2.485 and *B. amyloliquefaciens* TMW 2.479 Fad 82 spores released 96% and 58% of DPA, respectively, after 2 min of the pressure treatment at 800 MPa and 80ºC (Margosch et al., 2004b). Furthermore, *C. botulinum* TMW 2.357, *B. amyloloquefaciens* TMW 2.479 Fad 82, and TMW 2.482 Fad 11/2 were highly resistance

to the combined pressure-heat treatment, due to their ability to retain DPA, possibly as a result of difference in spore composition or structure (Margosch et al., 2004a).

Subramanian et al. (2006, 2007) studied mechanisms of PATP spore inactivation using FT-IR spectroscopy. The authors reported the significant decrease in DPA absorption bands during the initial stage of a treatment at 700 MPa and 121ºC, whereas there was little or no change of DPA bands occurring during thermal processing at the same temperature. At 121ºC, the bands associated with secondary proteins were significantly changed during spore inactivation.

More mechanistic studies at the molecular level are needed for further strengthening the understanding of the fate of bacterial spores under the combined pressure-thermal conditions. Especially needed are more studies on spore physiology and insights on the pressure-temperature conditions over which spore germination and inactivation can occur. The extent and mechanisms of bacterial injury during high-pressure sterilization merit further investigation.

2.4 Modeling kinetics of spore inactivation

Microbial kinetic studies are essential to any new technology in food preservation since the accurate prediction of microbial inactivation is necessary (Mañas and Pagán, 2005). Any models used for prediction should be simple and built on parameters based on the physiological mechanism of inactivation (Smelt et al., 2002). While linear thermal inactivation kinetics have been historically used by the food industry over the years , both linear and non-linear kinetics models have been proposed for PATP. Unlike thermal

processing, pressure and temperature are the two major factors governing spore inactivation. Shoulders and tails are deviations commonly found in bacterial survivor curves leading to non-linearity relationship. The activation of dormant spores, the presence of cell clumping, and sublethal injury are causes of shoulders, whereas tails could arise from the occurrence of subpopulations with different resistance (Heldman and Newsome, 2003; Tay et al., 2003; Mañas and Pagán, 2005). In general, tails are detected after 4 to 5 log reduction (Smelt et al., 2002). This tailing behavior can also be found in thermal processing, but the effect is more pronounced in HPP and PATP (Patterson, 2005). Many researchers have discovered that typical pressure survivor curves have characteristic tailing with upward concavity (Chen and Hoover, 2004; Rajan et al., 2006a; Ahn et al., 2007). Researchers used various non-linear models including the n^{th}-order kinetics, the log-logistic, the Weibull distribution, and the biphasic model (Ananta et al., 2001; Ardia et al., 2003; Koutchma et al., 2005; Rajan et al., 2006a, 2006b; Ahn et al., 2007; Ahn and Balasubramaniam, 2007b) to describe PATP spore inactivation kinetics. It is important to note that depending upon the type of bacterial spore, certain level of log reduction can occur during the process come-up time. This should be considered when developing kinetic models (Rajan et al., 2006a, 2006b; Ahn et al., 2007). Most microbiological kinetics models are empirical in nature, and should not be extrapolated beyond the experimental investigation range (Heldman and Newsome, 2003). It is also desirable that the chosen kinetic model adequately describes the experimental data, and is not merely forced to fit the experimental data (Balasubramaniam et al., 2004).

2.5 Combining high pressure-temperature treatment with other hurdles

To achieve commercial sterility in low-acid foods and reduce severity of process condition, various hurdle concepts (e.g., salt, pH, a_w, antimicrobial compounds) can be employed. The use of natural antimicrobial compounds during the high pressure-temperature process is of interest to reduce the amount of food additives (Raso and Barbosa-Cánovas, 2003). The use of bacteriocins (e.g., nisin, pediocin) in combination with pressure to inactivate bacterial spores has been reported (Stewart et al., 2000; Kalchayanand et al., 2003; Black et al., 2008). A concentration of 500 IU/mL nisin and the treatment (500 MPa at 40ºC for 5 min, cycled twice) caused 5.7 to 5.9 log reduction when *B. subtilis* spores were suspended in phosphate buffer saline and reconstituted skim milk, respectively (Black et al., 2008). A combination of nisin, pediocin, and the treatment (345 MPa at 60ºC for 5 min) increased the storage time of roast beef inoculated with a mixture of four clostridial spores (*C. sporogenes*, *C. perfringens*, *C. tertium*, and *C. laramie*) to 84 days at 4ºC, compared to 42 days at 4ºC from pressure treatment alone without bacteriocins (Kalchayanand et al., 2003). In another study of Kalchayanand et al. (2004), the bacteriocin-based preservatives (3:7 ratio of pediocin and nisin) supplemented with lysozyme or Na-EDTA could be used in combined with pressure to inactivate germinated spores during post-pressurization storage. In model cheese inoculated with 10^6 CFU/g *B. cereus* spores, the presence of nisin (1.56 mg/L of milk) efficiently enhanced spore inactivation while lysozyme (22.4 mg/L of milk) did not provide any synergistic effect with pressure (López-Pedemonte et al., 2003).

A combination of citric acid (pH 4.0) with a treatment at 400 MPa and 70ºC for 30 min resulted in 6 log reduction of *B. coagulans* spores when the media containing 0.8 IU/mL nisin (Roberts and Hoover, 1996). Other food additives like sucrose laurate (an emulsifying agent) have also been reported to give an additional effect on spore inactivation when used with high pressure processing. In milk and beef, the presence of < 1% sucrose laurate with the treatment at 392 MPa and 45ºC for 10 to 15 min decreased *B. cereus* and *B. coagulans* spores by 3.0 to 5.5 log CFU/mL (Shearer et al., 2000). When 0.5% sucrose laurate was added to the agar medium used for recovery, it inhibited the germination and outgrowth of *C. sporogenes* PA 3679 spores (Stewart et al., 2000). For *B. subtilis* 168 spores suspended in McIlvaine citrate phosphate buffer (pH 6.0), a pressure treatment (404 MPa at 45ºC for 15 min) with 0.1% sucrose laurate could eliminate an initial inoculum of 10^6 CFU/mL.

Some researchers investigated the possibility of combing pressure treatment with irradiation for reducing process severity during inactivation of bacterial spores. Crawford et al., (1996) applied a mild dose of irradiation (2 to 6 kGy) to fresh chicken inoculated with *C. sporogenes* spores before pressurization at 689 MPa and 80°C for 20 min. Enhanced inactivation and increased storage time were observed, when compared to the results from each treatment alone. The positive effect of gamma irradiation prior to pressure treatments was also reported by Sale et al. (1970). The authors found the increased pressure sensitivity of *B. coagulans* surviving spores after the treatment. Simultaneous application of pressurization and irradiation was found to have an additive sporicidal effect (Gould and Jones, 1989). Systematic studies documenting the potential

synergistic (or antagonistic) effects of pressure, temperature, and other combination processes and their respective kinetic of microbial inactivation are very limited, and more of such studies are needed.

2.6 Validation

US Food, Drug, and Cosmetic Act (FD&C Act) is the basis by which FDA promulgates specific regulations. Similar to the thermal pasteurized counterpart, pressure pasteurized products are distributed under refrigerated conditions. Similarly, they are required to be processed under GMP conditions and processors must ensure relevant commodity specific regulations (e.g., juice HACCP, Pasteurized Milk Ordinance (PMO), Sea Food HACCP, etc.) be followed. The potential for temperature abuse during refrigerated storage and distribution has to be carefully evaluated and minimized. Processors must also work with equipment vendors to ensure that any part of the equipment, which may have incidental contact with the food, is made from approved materials.

At the moment, very limited literature is available on validation of pressure sterilized shelf-stable low-acid food products. Successful PATP validation will require a multidisciplinary approach integrating basic microbiological, physical, chemical, and engineering principles to demonstrate the uniformity and sterility of the process. Commercial sterility is defined in 21 CFR 113 as “a process, which renders a product free of pathogens and spoilage organisms under normal conditions of storage and distribution. Since temperature plays an important role during PATP lethality, FDA’s regulations

pertinent to "Thermally processed low-acid foods packaged in hermetically sealed containers" is likely applicable (Sizer et al., 2002). Sizer et al. (2002) discussed a number of options for validation of the process consisting of establishing the process as a thermal process and using kinetic approaches. For example, the process should deliver, at least, an equivalent spore log reduction as a process that delivers a 12 log reduction of *C. botulinum* spores which is the pertinent pathogen for low-acid canned foods. Nonpathogenic surrogate microorganisms used in thermal inactivation studies may not be suitable for the PATP because the relationship between pressure resistance and heat resistance of bacterial spores is not clear. Identification of appropriate spore-forming surrogates to be used as biological indicators during validation of PATP process conditions in a specific low-acid food matrix is necessary. Margosch et al. (2006) indicated that based on current limited knowledge, it may not be possible to identify a target indicator microorganism for pressure-processed low-acid food products. Moreover, very limited published information is available on the combined pressure-thermal resistance of various *C. botulinum* spores. More research is needed to compare PATP resistance of various pathogenic and non-pathogenic bacterial spores in various food matrices of industrial interest under controlled process conditions.

2.7 Conclusions and future directions

Currently, the high pressure processing at ambient temperature is not a viable option for processing shelf-stable low-acid canned foods. Pressure alone is not sufficient to inactivate spores; however, pressure in combination with moderate temperature seems

to be a promising approach for inactivating bacterial spores to produce safe, superior quality shelf-stable low-acid foods. Adequate attention should be paid to proper reporting of type of pressure equipment employed, process parameters, and microbiological methodologies used, so that data from various laboratories can be critically compared. Even though current research demonstrated that elevated pressure in combination with temperature can be effective in inactivating a variety of non-pathogenic bacterial spores, more studies are necessary to document the inactivation of various strains of *C. botulinum* spores. Kinetic models describing bacterial inactivation under combined pressure-thermal conditions in various food matrices are also needed for microbial process evaluation. Furthermore, the identification of suitable surrogate organisms, or organisms that are used in place of the pathogen as biological indicators of the adequacy of the lethal process**,** is desirable for process validation studies. More research is needed to evaluate process uniformity at elevated pressure-thermal conditions that can facilitate the successful introduction of shelf-stable low-acid foods. Combinations of non-thermal technologies with high pressure could further reduce the severity of the process pressure requirements.

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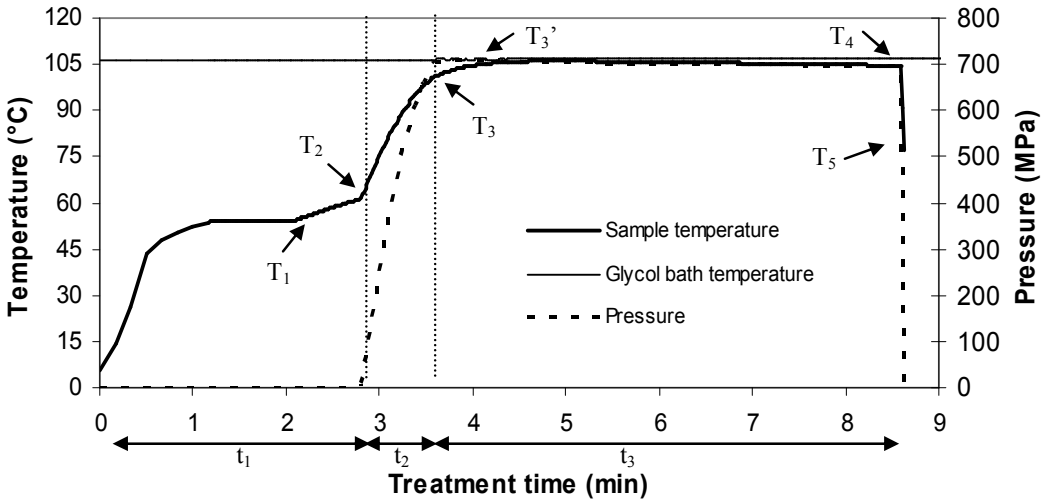

Figure 2.1. Pressure and temperature curves for samples subjected to PATP condition (700 MPa at 105°C for 5 min). Processing times include pre-process time (t_1), pressure come-up time (t_2), and pressure holding time (t_3). In this example, pressure vessel was maintained at the target process temperature.

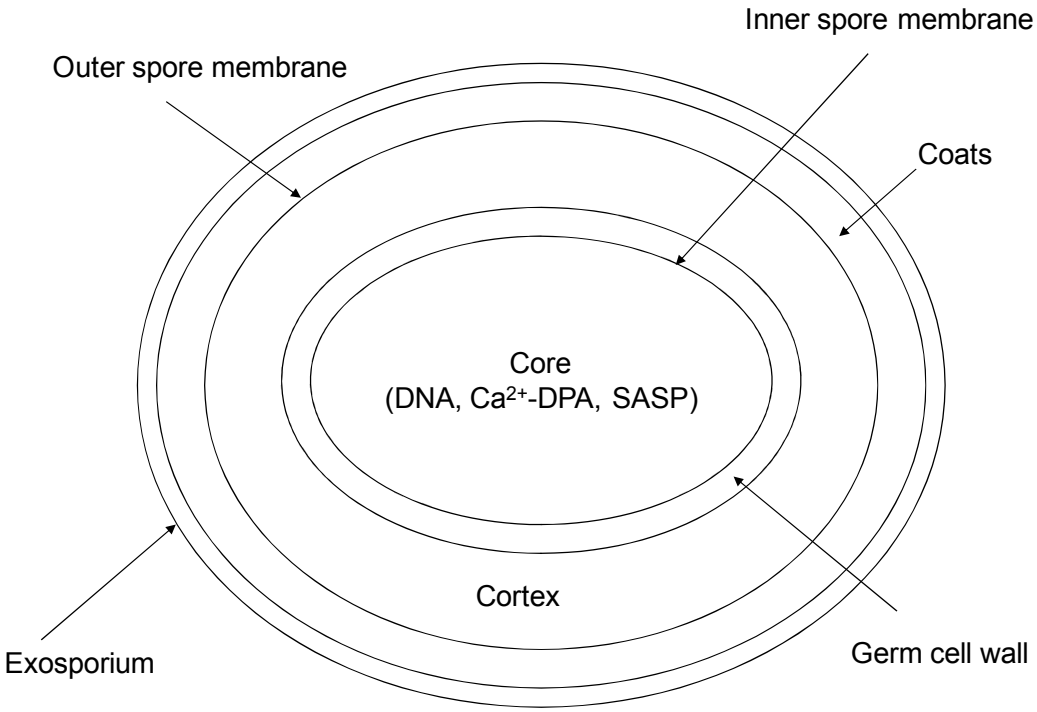

Figure 2.2. Structure of an entire dormant spore.

Substance	Initial product temperature (ºC)	Temperature increase (ºC) per 100 MPa
Water	20	2.8
	60	3.8
	80	4.4
Orange juice, tomato salsa, skim milk	25	2.6-3.0
Steel	20	0
Salmon	25	3.2
Chicken fat	25	4.5
Cheese (Gouda type)	20	3.4
Water/glycol mix (50:50)[a]	25	3.7-4.8
Beef fat	25	6.3
Milk fat	20	8.5
Olive oil [a]	25	6.3-8.7
Soybean oil [a]	25	6.2-9.1

[a] Substance exhibited decreasing temperature as pressure increased

Source: Ting et al., 2002; De Heij et al., 2003; Rasanayagam et al., 2003; Balasubramaniam et al., 2004.

Table 2.1. Temperature changes of selected substances due to compression heating.

Microorganisms	Initial inoculum	Suspending matrices	Process conditions (Pressure, P; temperature, T; holding time, t)	Other conditions (CUT and DT)[a]	Relevant Results	References
Alicyclobacillus acidoterrestris N1089	~$1x10^6$ CFU/mL	Tomato juice (pH 4.5, with addition of <1% sucrose laurate and <0.1% lauricidin)	392 MPa 25, 45, 50, 55ºC 10-15 min	CUT = 165 s DT = 40 s	3 log reduction after 392 MPa-45°C for 10 min with 0.005% sucrose laurate. No spore outgrowth when sucrose laurate conc. increased.	Shearer et al. (2000)
A. acidoterrestris DMS 2498	$4x10^8$ to $8x10^8$ CFU/mL	Orange juice	100-700 MPa 80, 85, 90, 95°C (initial temperature; IT) 0-50 min	CUT = 30 s DT = N/A	More than 7 log reduction after 700 MPa-80°C (IT) for 20 min. 6 log reduction was predicted by the n^{th}-order kinetic model with the treatment of 780 MPa-95°C (IT) for 30 s.	Ardia et al. (2003)
Bacillus amyloliquefaciens TMW 2.479 Fad 82	$2.0x10^6$ to $4.5x10^8$ CFU/g	Mashed carrots (pH 5.15)	800 MPa 80 ºC 0-16 min	CUT = 400 s DT = 400 s	2 log reduction after 800 MPa-80°C for 16 min. Among the spores tested, *B. amyloliquefaciens* TMW 2.479 exhibited higher pressure-heat resistance than *C. botulinum* TMW 2.357.	Margosch et al. (2004a)
B. amyloliquefaciens (7 strains)	$2.2x10^7$ to $9.6x10^8$ CFU/g	Mashed carrots (pH 5.15)	0.1-800 MPa 60-80 ºC 0-16 min	CUT = 400 s DT = 400 s	Strains of Fad 11/2, Fad 82, Fad 77, Fad 99, and Fad 108 formed highly pressure-resistant spores.	Margosch et al. (2004b)

Continued

Table 2.2. Bacterial spore inactivation in response to the combined effects of high pressures and moderate to high temperatures.
[a] Pressure come-up time (CUT) and depressirzation time (DT) is based on authors provided information or calculated from information provided by authors.

Table 2.2. continued

B. amyloliquefaciens TMW 2.479 Fad 82	$5.0x10^7$ to $4.5x10^8$ CFU/mL	Tris-His buffer (pH 4.0, 5.15 and 6.0)	600-1,400 MPa 70-120ºC 0-16 min	CUT < 20 s DT < 20 s	More than 4 log reduction after 1,200 MPa-120ºC for 2 min.	Margosch et al. (2006)
B. amyloliquefaciens Fad 82	~$1x10^8$ CFU/g	Egg patty mince (pH 7.25, a_w 0.99)	0.1-700 MPa 95-121 ºC 0-15 min	CUT = 42 s DT < 4 s	No or limited log reduction at 500 MPa, 95-105ºC during CUT. At 121ºC, temperature was the dominant factor contributing to spore inactivation than process pressure.	Rajan et al. (2006a)
B. amyloliquefaciens TMW 2.479 Fad 82, TMW 2.482 Fad 11/2, ATCC 23350, and ATCC 49763	$1.8x10^6$ CFU/mL	Deionized water	0.1-700 MPa 105, 121ºC 0-5 min	CUT = 35 s DT < 1 s	Strain ATCC 23350 and 53495 were inactivated to undetectable level at 121ºC during CUT (with or without 700 MPa). Pressure come-up time significantly impacted spore inactivation from different strains. Strain Fad 82 and Fad 11/2 were highly resistant to pressure-heat effects. More than 5 log reduction was obtained after 700 MPa-105°C for 2 min.	Ahn et al. (2007)
B. amyloliquefaciens TMW 2.479 Fad 82	~$1x10^2$ to $1x10^8$ CFU/mL	Deionized water	700 MPa 105°C 0-10 min	CUT = 35 s DT < 1 s	About 5.5 log reduction was obtained with high level of inoculum (~10^8 CFU/mL) after 3 min. The survivors were below the detectable level at lower level of inoculum (~10^2 and 10^4 CFU/mL).	Ahn et al. (2007c)

Continued

Table 2.2. continued

B. cereus ATCC 14579	~1x10^8 CFU/mL	McIlvaine citrate phosphate buffer (pH 3.5, 4.6, 6.7, and 7.8)	690 MPa 40°C 2 min	CUT = N/A DT = N/A	Maximum inactivation was obtained (5 to 6.5 log reduction) at pH 6 when *B. cereus* sporulated at 30 and 37°C. For those sporulated at 20°C, inactivation increased when pH was decreased.	Raso et al. (1998b)
B. cereus KCTC 1012	~1x10^8 to 1x10^9 CFU/mL	McIlvaine buffer (pH 4.5, 6.0, 7.0 and 8.0)	0.1-600 MPa 20, 40, 60ºC 15 min	CUT = N/A DT = N/A	Spores suspended in pH 4.5 were highly resistant to inactivation than those in pH 6.0, 7.0, and 8.0	Oh and Moon (2003)
B. cereus LMG6910	6x10^8 CFU/mL	UHT skim milk	100-600 MPa 30-60ºC 15, 30 min	CUT = 360 s DT < 1 s	More than 6 log reduction after 400 MPa-60°C for 30 min.	Opstal et al. (2004)
B. cereus NCFB 1771, NIZO LB1, NIZO LB5, and NIZO 578	~1x10^8 CFU/mL	Reconstituted skim milk (10%)	500 MPa 40ºC 5 min	CUT = 100 s DT = 22.5 s	Addition of 500 IU/mL nisin with twice cycles resulted in 5.9 log reduction of strain NIZO LB5.	Black et al. (2008)
B. coagulans 7050	~1x10^6 CFU/mL	Tomato juice (pH 4.5, <1% sucrose laurate and <0.1% lauricidin)	392 MPa 25, 45, 50, 55ºC 10-15 min	CUT = 165 s DT = 40 s	Almost 5 log reduction after 392 MPa-45ºC for 10 min with 1% sucrose laurate.	Shearer et al. (2000)
B. coagulans FRR B2626, FRR B2723, FRR B2735	~1x10^6 to 1x10^7 CFU/mL	Reconstituted skim milk (9.5%)	600 MPa 25, 75, 85, 95ºC (IT) 1 min	CUT = 120 s DT < 120 s	Initial temperatures (75-95ºC) did not enhance spore inactivation of strain FRR B2735. About 3 log reduction was obtained after 1 min at 600 MPa.	Scurrah et al. (2006)
B. licheniformis TMW 2.492	2.2x10^7 to 9.6x10^8 CFU/mL	Mashed carrots (pH 5.15)	0.1-800 MPa 60-80 ºC 0-16 min	CUT = 400 s DT = 400 s	Spores were inactivated to undetectable level at 80ºC and 600 or 800 MPa after 16 min.	Margosch et al. (2004b)

Continued

Table 2.2. continued

B. licheniformis (9 strains)	~1x10^6 to 1x10^7 CFU/mL	Reconstituted skim milk (9.5%)	600 MPa 25, 75, 85, 95ºC (IT) 1 min	CUT = 120 s DT < 120 s	More than 6.3 log reduction of *B. licheniformis* NZ 25 after 600 MPa for 1 min at IT 95ºC.	Scurrah et al. (2006)
B. sphaericus NZ 12, NZ 13, NZ 14, NZ 15	~1x10^6 to 1x10^7 CFU/mL	Reconstituted skim milk (9.5%)	600 MPa 25, 75, 85, 95ºC (IT) 1 min	CUT = 120 s DT < 120 s	*B. sphaericus* NZ 14 was the most resistant to the treatment (600 MPa, 1 min). While 1 log reduction was obtained at IT 75ºC, more than 5.3 log reduction was observed at IT 95ºC.	Scurrah et al. (2006)
B. sphaericus NZ 14	1.8x10^6 CFU/mL	Deionized water	0.1-700 MPa 105, 121ºC 0-5 min	CUT = 35 s DT < 1 s	About 3.7 log reduction was observed after CUT at 700 MPa-105ºC. Spores were inactivated to undetectable level for holding times greater than 2 min.	Ahn et al. (2007)
B. subtilis 168	~1x10^6 CFU/mL	McIlvaine citrate phosphate buffer (pH 4.0-7.0)	404MPa 25, 45, 70, 90ºC 15, 30 min Addition of 0.2-1.0 IU/mL nisin or 0.1-1.0% sucrose laurate	CUT = N/A DT = N/A	Spores suspended in buffers at pH 4.0, and 5.0 were inactivated to undetectable level after 404 MPa-70ºC for 30 min. Nisin reduced the outgrowth of spores, whereas sucrose laurate did not.	Stewart et al. (2000)
B. subtilis 168	~1x10^6 CFU/mL	2% milk fat (with addition of <1% sucrose laurate and <0.1% lauricidin)	392 MPa 25, 45, 50, 55ºC 10-15 min	CUT = 165 s DT = 40 s	5 log reduction after 392 MPa-25°C for 10 min with 0.1% sucrose laurate. 3 log reduction after 392 MPa-45°C for 10 min with 0.001% lauricidin.	Shearer et al. (2000)

Continued

Table 2.2. continued

B. subtilis (10 strains)	$2.2x10^7$ to $9.6x10^8$ CFU/g	Mashed carrots (pH 5.15)	0.1-800 MPa 60-80°C 0-64 min	CUT = 400 s DT = 400 s	For laboratory strains, more than 6 log reduction was observed after 800 MPa-70°C for 1 min From food-isolated strains, about 2 to 4 log reduction was observed after 800 MPa-70°C for 16 min	Margosch et al. (2004b)
Clostridium botulinum type A strains (BS-A, 62-A)	~$1.0x10^5$ CFU/mL	Sorensen phosphate buffer (0.067M, pH 7.0) and crabmeat	414-827 MPa 60, 65, 70, 75ºC 0-30 min	CUT = 145 s DT < 2 s	Crabmeat blend did not protect spores of *C. botulinum* against the treatment at 827 MPa-75ºC. About 2-3 log reduction was obtained from both matrices at this condition.	Reddy et al. (2003)
C. botulinum nonproteolytic type B strains (2-B, 17-B, KAP8-B, KAP9-B)	~$1.0x10^5$ CFU/mL	Sorensen phosphate buffer (0.067M, pH 7.0) and crabmeat	827 MPa 40-75ºC 0-30 min	CUT = 145 s DT < 2 s	More than 6 log reduction of strains 2-B, 17-B, and KAP9-B suspended in both matrices was obtained after 827 MPa-75ºC for 20 min.	Reddy et al. (2006)
C. botulinum type E strains (Alaska and Beluga)	~$1.0x10^5$ CFU/mL	Sorensen phosphate buffer (0.067M, pH 7.0)	414-827 MPa 25-60 ºC 0-10 min	CUT = 145 s DT < 2 s	Spores from both strains were inactivated to undetectable level after 827 MPa-55 ºC for 5 min.	Reddy et al. (1999)
C. botulinum (proteolytic: type A, B, F; nonproteolytic: type B)	$2.0x10^6$ to $4.5x10^8$ CFU/g	Mashed carrots (pH 5.15)	600 MPa 80ºC 0-64 min	CUT = 300 s DT = 300 s	Among 7 strains tested, *C. botulinum* TMW 2.357 was the most pressure resistant strain. About 1.2 log reduction was obtained after 600 MPa-80°C for 16 min.	Margosch et al. (2004a)

Continued

Table 2.2. continued

C. botulinum proteolytic type B strains (TMW 2.357, REB 89)	$5.0x10^7$ to $4.5x10^8$ CFU/mL	Tris-His buffer (pH 4.0, 5.15 and 6.0)	600-1,400 MPa 70-120ºC 0-16 min	CUT < 20 s DT < 20 s	Pressure-mediated spore protection was observed at 120ºC and 800, 1,000, or 1,200 MPa. Superdormant spores survived conditions up to 1,400 MPa and 120ºC.	Margosch et al. (2006)
C. sporogenes NCIMB 8053	~$1.0x10^6$ CFU/mL	Distilled water	200-600 MPa 40-60ºC 30 min	CUT = 180 s DT = 120 s	Less than 1 log reduction after 600 MPa-60ºC for 30 min. About 67.7% of spore population was germinated at this condition.	Mills et al. (1998)
C. sporogenes PA 3679 (ATCC 7955)	$1.0x10^6$ to $3.0x10^6$ CFU/mL	Meat broth	400-1,200 MPa 90-110ºC 0-60 min	CUT = N/A DT = N/A	1.3 log reduction was obtained after 1,200 MPa-90°C for 5 min, while 2.8 log reduction was observed after 500 MPa-100ºC for 20 min.	Rovere et al. (1998)
C. sporogenes ATCC 7955 (PA 3679)	~$1x10^6$ CFU/mL	McIlvaine citrate phosphate buffer (pH 4.0-7.0)	404MPa 25, 45, 70, 90ºC 15, 30 min	CUT = N/A DT = N/A	Spore suspension at pH 4.0 provided 6 log reduction after 404 MPa-70ºC for 15 min, while less than 0.5 log reduction was obtained with those at pH 6.0 and 7.0.	Stewart et al. (2000)
C. sporogenes PA 3679	~$1.0x10^6$ CFU/mL	Scrambled egg patties	688 MPa 105-121ºC 0-5 min	CUT = N/A DT = N/A	6 log reduction was obtained after 688 MPa-121ºC for 3 min.	Koutchma et al. (2005)
C. sporogenes ATCC 7955	$1.2x10^7$ CFU/mL	Deionized water	0.1-700 MPa 105, 121ºC 0-5 min	CUT = 35 s DT < 1 s	About 3.3 and 6.3 log reduction was obtained after CUT of 700MPa at 105 and 121°C, respectively.	Ahn et al. (2007)
C. sporogenes ATCC 7955	$1.0x10^4$, $1.0x10^6$, $1.0x10^8$ CFU/mL	Deionized water	700 MPa 105ºC 0-5 min	CUT = 35 s DT < 1 s	At 700 MPa-105ºC for 1 min, 3.8 to 6.3 log reduction was reported.	Ahn and Balasubrama-niam (2007a)

Continued

Table 2.2. continued

C. sporogenes ATCC 3584, ATCC 7955	~$5x10^6$ CFU/mL	0.1M citric (pH 4.75, 6.5) and PBS (pH 7.4)	550-650 MPa 55-75ºC 15, 30 min	CUT = 90 s DT < 20 s	Acidic condition (pH 4.75) effectively enhanced spore inactivation when combined with 650 MPa-75ºC for 30 min resulting in 5.8 log reduction.	Paredes-Sabja et al. (2007)
C. perfringens type A (4 P-*cpe* isolates and 5 C-*cpe* isolates)	~$5x10^6$ CFU/mL	0.1M citric (pH 4.75, 6.5) and PBS (pH 7.4)	550-650 MPa 55-75ºC 15, 30 min	CUT = 90 s DT < 20 s	About 5.1 and 2.8 log reduction was obtained from P-*cpe* and C-*cpe* isolates (pH 4.75), respectively, after treated at 650 MPa-75ºC for 30 min.	Paredes-Sabja et al. (2007)
Geobacillus stearothermophilus IFO 12550	~$1x10^6$ CFU/mL	1 mg/mL of Bovine serum albumin, ovalbumin, and β-lactoglobulin	0.1-1,000 MPa 20, 60, and 70ºC 20-60 min	CUT= N/A DT < 1 s	More than 3.8 log reduction after 800 MPa-60ºC for 60 min.	Hayakawa et al. (1994)
G. stearothermophilus ATCC 7953	~$1x10^7$ CFU/g	Mashed broccoli and cocoa mass	0.1-600 MPa 60-120ºC 0-160 min	CUT = 100 s DT = N/A	More than 6 log reduction of G. *stearothermophilus* suspended in mashed broccoli after 600 MPa-120ºC for 20 min. Almost 6 log reduction of *G. stearothermophilus* suspended in cocoa mass after 600 MPa-90ºC for 60 min. Protective effect of fat from cocoa mass yielded limited spore inactivation.	Ananta et al. (2001)
G. stearothermophilus ATCC 7953	~$1x10^6$ CFU/g	Egg patties	400-700 MPa, 105ºC 0-5 min	CUT = 144 s DT = N/A	About 4 log reduction was observed after 700 MPa-105ºC for 5 min, whereas 1.2 log reduction was obtained at 0.1 MPa-121ºC after 15 min. PATP treatment did not have any protective effect.	Rajan et al. (2006b)

Continued

Table 2.2. continued

*Thermoanaerobacterium thermosaccharolyticu*m TMW 2.299	$2.0x10^6$ to $4.5x10^8$ CFU/g	Mashed carrots (pH 5.15)	800 MPa 80 ºC 0-16 min	CUT = 400 s DT = 400 s	*T. thermosaccharolyticu*m spores were more heat resistant but less pressure resistant than spores of *C. botulinum* and *B. amyloliquefaciens*.	Margosch et al. (2004a)
*T. thermosaccharolyticu*m ATCC 27384	$1.2x10^7$ CFU/mL	Deionized water	0.1-700 MPa 105, 121ºC 0-5 min	CUT = 35 s DT < 1 s	5 log reduction of *T. thermosaccharolyticu*m was observed at 700 MPa-105°C for 5 min, while no survivors of *C. sporogenes* were detected under the same condition.	Ahn et al. (2007)

Chapter 3: Influence of pressurization rate and pressure pulsing on the inactivation of *Bacillus amyloliquefaciens* spores during pressure-assisted thermal processing

Abstract

Pressure-assisted thermal processing (PATP) is an emerging sterilization technology where a combination of pressure (500 to 700 MPa) and temperature (90 to 121°C) are used to inactivate bacterial spores. The objective of this study was to examine the role of pressurization rate and pressure pulsing in enhancing spore inactivation during PATP. *Bacillus amyloliquefaciens* TMW 2.479 Fad 82 spore suspensions were prepared in deionized water at three inoculum levels (~$1.1x10^9$, $1.4x10^8$, and $1.3x10^6$ CFU/mL), treated at two pressurization rates (18.06 and 3.75 MPa/s), and held at 600 MPa and 105°C for 0, 0.5, 1, 2, 3, and 5 min. Experiments were carried out using custom-fabricated high-pressure microbial kinetic testing equipment. Single and double pulses with equivalent pressure holding times (1 to 3 min) were investigated using the spore suspension containing ~$1.4x10^8$ CFU/mL. Spore survivors were enumerated by pour plating using Trypticase soy agar after incubation at 32°C for 48 h. During short pressure holding times (≤ 2 min), PATP treatment with the slow pressurization rate provided enhanced spore reduction than that of the fast pressurization rate. However,

these differences diminished with extended pressure holding times. After 5-min pressure holding time, *B. amyloliquefaciens* spores decreased about 6 log CFU/mL regardless of pressurization rate and inoculum level. Double-pulse treatment enhanced PATP lethality by approximately 2.4 to 4 log CFU/mL in comparison to single-pulse for a given pressure holding time. In conclusion, pressure pulsing considerably increases the efficacy of PATP treatment. Contribution of pressurization rate to PATP lethality varies with duration of pressure holding.

3.1 Introduction

Pressure-assisted thermal processing (PATP) is one of the emerging technologies for processing shelf-stable low-acid foods. During PATP, the food material is subjected to a combination of elevated pressure (500 to 700 MPa) and temperature (90 to 121°C) for a specified holding time. The unique advantages of this technology include a rapid increase in the temperature of treated food samples and the expansion cooling of products upon depressurization (Rajan et al., 2006). Consequently, PATP better preserves product attributes such as color, flavor, texture, and nutritional values when compared to conventional thermal processing.

A number of previous studies demonstrated the role of process pressure, temperature, and holding time on spore inactivation (Margosch et al., 2004a, 2004b, 2006; Ahn et al., 2007; Ahn and Balasubramaniam, 2007; Black et al., 2007), but very limited information is available on the role of pressurization rate on spore inactivation. Similar to thermal processing, the total treatment time during PATP includes both

pressure come-up time and pressure holding time. The time required to increase the pressure of the sample from atmospheric pressure to the target process pressure is often described as pressure come-up time (Farkas and Hoover, 2000; Balasubramaniam et al., 2004). The process come-up time is primarily a function of the target pressure and the pump horsepower. In general, the pressurization rate of the high-pressure equipment is fixed and normally governed by equipment design parameters. Typical come-up time in commercial scale high-pressure equipment falls in the range of 130 to 240 s for reaching 600 to 700 MPa pressure. Excessively longer come-up times (> 180 s) can increase the total process time and reduce the process throughput. Variation in come-up time may also affect the inactivation kinetics of microorganisms. Therefore, consistency and awareness of pressurization and depressurization rates are important in PATP process development.

The role of the pressurization and depressurization rate on vegetative bacteria inactivation during high-pressure pasteurization has been studied previously (Hayakawa et al., 1998; Noma et al., 2002; Rademacher et al., 2002; Chapleau et al., 2006). Possibly due to variation in experimental setup and process conditions used by various researchers, often contradictory and inconclusive trends were reported. Smelt (1998) hypothesized that a slow pressurization rate might induce a stress response that would render pressurization less effective, while a faster pressurization rate might contribute to higher inactivation. Author further observed that the presence or absence of gas vacuoles can alter the results. Hayakawa et al. (1998) reported that rapid decompression (1.30 to 1.65 ms) enhanced microbial lethality of *Bacillus stearothermophilus* spores. Rapid depressurization (1 ms) was more effective than slow decompression (> 30 s) in

inactivating vegetative bacteria and in lowering treatment pressure requirements (Noma et al., 2002). Inactivation of *Listeria innocua* by pressure was similar when the bacterium was subjected to either a fast pressurization (500 MPa/min) followed by a slow depressurization (100 MPa/min) or a slow pressurization (100 MPa/min) followed by a faster depressurization (500 MPa/min) (Rademacher et al., 2002).

Pressure pulsing, also referred to as oscillatory pressurization, pressure cycle process, or non-continuous pressurization, is another approach to enhance microbial lethality (Hayakawa et al., 1994; Farkas and Hoover, 2000; Furukawa et al., 2000; Meyer et al., 2000; Lopez-Caballero et al., 2000; Furukawa et al., 2003; Shao et al., 2007). Lopez-Caballero et al. (2000) reported that a two pulse (400 MPa at 7°C in two 5 min pulse) process produced no apparent advantage over a single pulse in reducing oyster's microbiota. Pressure pulsing was effective in pectin methyl esterase inactivation in orange juice (Basak and Ramaswamy, 2001). Chapleau et al. (2006) stated that pressure holding time is a more important parameter than pressure pulsing for inactivation of *Salmonella* Typhimurium and *Listeria monocytogenes.* Repeated pressure cycles enhanced spore inactivation, presumably by a pressure-induced germination and subsequent inactivation of germinated spore (Smelt, 1998). Mild pressure treatments (50 to 500 MPa, at 25 to 60°C) induced spore germination through the activation of spore's nutrient receptors (Smelt, 1998; Wuytack et al., 2000; Black et al., 2007). Hayakawa et al. (1994) reported six pulses at 400 MPa and 70°C for 5 min reduced *B. stearothermophilus* spores by 4 log CFU/mL. With the exception of a few studies, in many earlier pulsing investigations, temperature during pulsing was not reported.

Systematic studies, therefore, are needed to document the influence of pressurization rate and pressure pulsing on spore inactivation under controlled pressure-thermal conditions. The objectives of this study were to examine the effect of pressurization rate on enhancing PATP spore inactivation. Additionally, the combined efficacy of pressurization rate and pressure pulsing on spore lethality was also investigated.

3.2 Materials and methods

3.2.1 Spore preparation

Bacillus amyloliquefaciens TMW 2.479 Fad 82 spores (M. Gänzle, Lehrstuhl für Technische Mikrobiologie, Technische Universität München, Freising, Germany) were used in the study due to their high pressure-thermal resistance (Margosch et al., 2004b, 2006; Rajan et al., 2006). Cultures were grown in trypticase soy broth supplemented with 0.1% yeast extract (TSBYE; BD Diagnostic Systems, Sparks, MD) with aerobic incubation at 32°C for 24 h. After the second transfer in TSBYE, the cultures were used for spore production. Portions (100 µl) of *B. amyloliquefaciens* culture were spread-plated on Trypticase soy agar (TSA; BD Diagnostic Systems) supplemented with 10 ppm $MnSO_4 \cdot H_2O$ (Fisher Scientific, Pittsburgh, PA). The inoculated plates were then aerobically incubated at 32°C for 10-14 days. The spores were harvested after 95% of the population was sporulated. This was verified by using a phase-contrast microscopy. The surface of inoculated plates was flooded with 10 mL of sterile distilled water and the spores were scraped with a sterile glass spreader. The spore suspension was washed five times by differential centrifugation that ranged from 2,000 to 8,000 x *g* for 20 min each at

4°C. Spore pellets were resuspended in sterile deionized water to obtain approximately 10^9 spores/mL. The suspension was sonicated for 10 min using a sonicator (SM275HT, Crest, ETL Testing Laboratory, Cortland, NY) and heated at 80°C for 10 min to destroy any remaining vegetative cells. The spore suspension was stored in a refrigerator at 4°C until used.

3.2.2 High-pressure microbial kinetic tester

Spores were treated in a high-pressure microbial kinetic tester (pressure test unit PT-1, Avure Technology Inc., Kent, WA) as described previously (Rajan et al., 2006). An intensifier (M-340 A, Flow International, Kent, WA) was used to generate the desired pressure. A 54-mL stainless steel pressure chamber was immersed in a temperature-controlled bath to maintain the desired process temperature condition (kept at 105°C for this study). Propylene glycol (57-55-6, Avatar Corporation, University Park, IL) was used as the heating medium in the temperature-controlled bath. Fast (18.06 MPa/s) and slow (3.75 MPa/s) pressurization rates were achieved by suitably adjusting the amount of air supply to the hydraulic pump (model PO45/45-OGPM-120, Interface Devices, Milford, CT) using a needle valve. The depressurization rate during the study was fixed (~ 2 s). The sample temperature, bath temperature, and chamber pressure were recorded every 1 s with a K-type thermocouple sensor (model KMQSS-04OU-7, Omega Engineering, Stamford, CT) and a pressure transducer (model 3399 093 006, Tecsis, Frankfurt, Germany). A data acquisition computer equipped with relevant hardware (Daq-Board/2000 16-bit, 200 kHz PCI card, DBK 81 seven-channel thermocouple

expansion card, and DBK 203 expansion card; IOTech, Cleveland, OH) and software (DasyLab 7.00.04; National Instruments Corp., Austin, TX) was used to record the data.

3.2.3 Sample preparation for pressure-assisted thermal processing

Spore suspensions of *B. amyloliquefaciens* (0.2 mL) and sterile deionized water (1.8 mL) were aseptically transferred into a pouch (5 x 2.5 cm, polyethylene bags, 01-002-57, Fisher Scientific). Final concentrations in suspensions were $1.1x10^9$, $1.4x10^8$, and $1.3x10^6$ spores per mL. The sample pouches were then heat-sealed using impulse heat sealer (American International Electric, Whittier, CA) and the sample content was manually mixed thoroughly. The packaged pouches were kept (< 1 h) in an ice-water bath (4°C) until PATP processed. Prior to PATP treatment, the spore-containing pouch was placed inside a 10-mL polypropylene syringe (model 309604, Becton, Dickinson and Company, Franklin Lakes, NJ), which served as sample holder. After loading the pouch inside the sample holder, ~8 mL of water was used to fill the remainder of the holder to ensure that the immediate vicinity of the sample pouch had similar heat of compression characteristics as that of spore suspension. In addition, the sample holder was wrapped with two layers of insulating material (Sports Tape, CVS® Pharmacy Inc., Woonsocket, RI) to minimize heat exchange with the environment during PATP treatment (Nguyen et al., 2007). The syringe containing the sample pouch was then preheated to the pre-processing temperature (T_1 = 58°C; Figure 3.1.A) in a water bath (Isotemp 928, Fisher Scientific). After pre-heating for 2 min, the syringe was then immediately loaded into the microbial kinetic tester pressure chamber and the vessel was closed. Sample temperature history was continuously monitored (Figure 3.1). The pressurization started when the

sample temperature reached the predetermined value T_2 (Figure 3.1.A). This temperature was estimated based on a trial-and error experimental approach using the following relationship (Rajan et al., 2006; Nguyen et al., 2007):

$$T_2 = T_3' - \left(CH \cdot \Delta P + \Delta T_H\right) \tag{3.1}$$

where T_3' is the target temperature, CH is the heat of compression value of the sample (defined as temperature increase per 100 MPa during sample pressurization), and ΔP is the process pressure. The spore suspensions are assumed to have the same heat of compression as that of water (Rasanayagam et al., 2003). ΔT_H is the temperature gain by the test sample during loading within the pressure chamber as well as pressurization. A trained equipment operator effectively controlled and minimized the ΔT_H values through a set of preliminary experiments (Nguyen et al., 2007).

3.2.4 Experimental approach

Experiments were conducted to study the influence of the pressurization rate (fast, 18.06 MPa/s; slow, 3.75 MPa/s) on the inactivation of *B. amyloliquefaciens* spores processed at 105°C and 600 MPa for various pressure-holding times (0, 0.5, 1, 2, 3, and 5 min) and spore inoculum levels (1.1×10^9, 1.4×10^8, and 1.3×10^6 CFU/mL). For experiments comparing the microbial efficacy of single- and double-pulse, inoculum level was kept at 1.4×10^8 CFU/mL. Single- and double-pulse experiments were conducted at 600 MPa and 105°C for selected equivalent pressure-holding times (1 and 3 min). In all cases, pressure-holding times did not include the pressure come-up time or the depressurization time. During pressure pulsing experiments, the time interval between the first and second pulse (t_{pause}) was chosen as 60 s (Figure 3.1.B). This time interval

allowed the researchers to ensure that the sample was depressurized during first pulse and to start the pressurization cycle for the second pulse at a consistent temperature (Table 3.1.B). After depressurization, the sample was immediately cooled in an ice-water bath. The untreated control sample was used for the determination of initial inoculum. All spore suspensions (treated and untreated) were analyzed for the total viable spore count within 2 h after the treatment. Each experiment was repeated at least three times.

3.2.5 Enumeration of survivors

The surface of each pouch was sanitized with 70% ethanol before being aseptically opened. After mixing the pouch content thoroughly, 1 mL of the sample content was serially diluted in 0.1% peptone water. The 1 mL aliquots of the appropriate dilutions were pour-plated in duplicate using TSA plates, which were then incubated at 32°C for 48 h for enumeration of spore survivors. Colonies were counted on a dark-field Quebec colony counter (Leica Microsystems, Richmond Hill, Ontario, Canada). The detection limit of the spore enumeration technique was 10 CFU/mL.

3.2.6 Integration of pressure- and temperature-time

To evaluate relative significance of pressure-temperature profiles during PATP treatment, areas under pressure-time ($\int_{t_1}^{t_2} P\,dt$) and temperature-time ($\int_{t_1}^{t_2} T\,dt$) plots were calculated. These areas represent the magnitude of pressure and thermal dosages received by the test samples during the treatment. According to an earlier study (Rajan et al., 2006), lethality of *B. amyloliquefaciens* spores was likely to occur when pressures and temperatures were kept above these threshold levels: ($P(t) \geq 500$ MPa; $T(t) \geq 95$°C). Below this threshold, limited or insignificant spore inactivation occurred. Accordingly, lethal areas

($\int_{t_1}^{t_2}[P(t)-500]\,dt$ and $\int_{t_1}^{t_2}[T(t)-95]\,dt$) were calculated over various pressure-holding times. Areas were calculated by the trapezoidal rule (with linear interpolation) using MATLAB version 7.1 (The MathWorks, Inc., Natick, MA). The lower and upper limits of integration (t_1 and t_2) corresponds to the time interval when the temperature or pressure values reach specific target process values (P = 500 MPa; T = 95°C).

3.2.7 Statistical analysis

All data were analyzed with the Statistical Analysis System software (SAS 9.1, SAS Institute Inc., Cary, NC). The independent variables included inoculum level (I_i; $1.1\text{x}10^9$, $1.4\text{x}10^8$, and $1.3\text{x}10^6$ CFU/mL), pressurization rate (PR_j; fast and slow), pressure-holding time (t_k; 0, 0.5, 1, 2, 3, and 5 min), or type of pressure treatment (P_i; single- and double-pulse). The decrease in spore population (Δ log CFU/mL) in response to the process treatment was the dependent variable (Y_{ijk}). The following statistical models were analyzed using the general linear model (GLM) procedure.

$$Y_{ijk} = \mu + I_i + PR_j + t_k + \varepsilon_{ijk} \tag{3.2}$$

$$Y_{ijk} = \mu + P_i + PR_j + t_k + \varepsilon_{ijk} \tag{3.3}$$

where ε_{ijk} is the error term. Mean comparison for treatment effects were evaluated with the Tukey test at a 5% significant level (P = 0.05).

3.3 Results and discussion

3.3.1 Pressure and temperature histories during fast and slow pressurization rate

A typical temperature history during pre-processing and pressure processing is shown in Figure 3.1 and Table 3.1 In both cases, the spore suspensions were preheated to the same initial pre-process temperature (T_I=57.4°C, Table 3.1). After loading spore pouches into the pressure chamber, the slow pressurization rate samples were adjusted to 65.4°C whereas the fast pressurization samples were heated to 70.6°C. This discrepancy was necessary to achieve equal processing temperatures for both treatments during pressure holding. However, once the target pressure was reached, the temperature of the samples increased slightly for another 20 s before the target temperature was maintained. This observation was in agreement with that of Nguyen et al. (2007). Since process temperature is an integral part of the inactivation of bacterial spores during PATP (Ting et al., 2002; Reddy et al., 2003; Margosch et al., 2004b; Rajan et al., 2006), the target temperature should be well-controlled during the holding times.

Compression or decompression of the food material changes its internal energy and alters temperature distribution within the pressure chamber (Pehl and Delgado, 1999). The extent of temperature gradient within the pressure chamber is influenced by parameters such as chamber material and geometry, type of food material, compression or decompression rate, and initial temperature of the product (Rademacher et al., 2002; Ting et al., 2002). From a thermodynamic perspective, two extreme processes describe the thermal effects at high pressure (Rademacher et al., 2002). First, the isothermal process involves an infinitely slow pressurization step ($dp/dt \rightarrow 0$). The second process,

the adiabatic process, involves rapid pressurization rate ($dp/dt \rightarrow \infty$). However, conducting experiments under both of those conditions can be challenging and may require complex equipment and process control. The real process will usually fall somewhere between these two limits.

During double-pulse experiments, pressure holding time for each pulse was kept as half of the pressure holding time during single-pulse experiments. Pressure-temperature histories during pulsing experiments were similar to single-pulse experiments as described previously. The time interval between double pulses (t_{pause}) was kept as 60 s at atmospheric pressure. However, it was experimentally difficult to control temperature during the second pulse. Since the external glycol bath and pressure vessel was maintained at 105°C (to minimize heat loss during extended pressure holding times), the sample continued to gain heat energy during the time interval between two pulses as well as during the second pulse come-up time. Thus, temperature of the sample just before pressurization of the second pulse was approximately 77-79°C, resulting in the process temperature (T_3 to T_4) during the second pulse of 111-113°C (Table 3.1.B and Figure 3.1.B). This increase in temperature of the second pulse was not expected when planning the experiments of pressure pulsing. Therefore, to address the influence of this additional increase in temperature, the single-pulse experiment at 112°C was conducted to compare its contribution to the treatments (i.e., single pulse at 105°C and double pulses at 105 and 112°C). It was important to note that this additional lethal temperature exposure during the pressure pause and second pulse should be considered when evaluating any potential benefits of double pulse on spore inactivation.

3.3.2 Effect of pressurization rate on spore inactivation

Figure 3.2 highlights the effect of the pressurization rate on viability of *B. amyloliquefaciens* suspended in deionized water. In general, viable spore population decreased with increased pressure holding time. Inoculum level, pressurization rate, and pressure holding time significantly influenced the inactivation of *B. amyloliquefaciens* spores ($P < 0.05$) (Table 3.2).

The estimated $D_{105°C}$ value of *B. amyloliquefaciens* spores was 24.2±0.3 min. Similarly, a previous study showed negligible inactivation of these spores at 105°C for 5-min holding time (Rajan et al., 2006). Current study showed considerable spore inactivation when the thermal treatment at 105°C was combined with pressure. However, degree of lethality enhancement was affected by pressurization rate (Table 3.2 and Figure 3.2). The slow pressurization rate resulted in higher spore inactivation than that of the fast pressurization rate when inactivation was measured immediately after pressure come-up time ($P < 0.05$). This trend was observed in all three inoculum levels tested (Figure 3.2). During slower pressurization rates, spores have been exposed to a lethal temperature region (T > 95°C) for a longer time than under faster pressurization rates (Table 3.3). This prolonged thermal exposure under pressure undoubtedly caused the enhanced lethality. Samples processed using the slow pressurization rate continued to show enhanced lethality up to 2 min of pressure holding time. Subsequently, the difference between microbial efficacies of the pressurization rates diminished for all inoculum levels, specifically after 5 min holding time. At 1.1 x 10^9 CFU/mL inoculum and after 5 min of pressure holding, the fast pressurization rate reduced *B. amyloliquefaciens* spores

by 5.4 log CFU/mL, whereas approximately 6.1 log CFU/mL was inactivated with the slow pressurization rate. The interaction effect between inoculums level and pressurization rate was not significant ($P = 0.35$). Regardless of pressurization rate, treating the spores with 600 MPa for 5 min pressure holding time inactivated approximately 6.5 log CFU/mL in the spore suspension containing 1.4 x 10^8 CFU/mL, whereas no survivors were detected in the suspension containing 1.3 x 10^6 CFU/mL.

Spores of *B. amyloliquefaciens* TMW 2.479 have an ability to retain dipicolinic acid (DPA) content during pressure treatments, thus making this bacterium highly pressure-resistant (Margosch et al., 2006). It is likely that the heat-pressure combinations tested in this study helped the release of DPA, thus caused substantial lethality to these spores. Once spores release more than 90% of their DPA content, further spore inactivation by pressure diminishes (Margosch et al., 2004b; Subramanian et al., 2007), which might explain the decrease in lethality with a longer pressure-holding time (Figure 3.2). Subramanian et al. (2007) observed significant changes of several absorbance signals from *B. amyloliquefaciens* TMW 2.479 during the pressure come-up time of 700 MPa and 121ºC. Those peaks were attributed to calicum dipicolinate (1281, 1378, and 1440 cm^{-1}) and DPA (1568 cm^{-1}). Moreover, there was a complete loss of signals from the DPA-related bands after the come-up time, indicating the release of most DPA-related compounds from the spore cores (Subramanian et al., 2007).

Chapleau et al. (2006) correlated the efficiency of high-pressure treatment with microbial reduction by using barometric power (BP), which was estimated from the area under the pressure-time curve. The authors observed the highest microbial inactivation of

S. Typhimurium *and L. monocytogenes* when the slowest pressurization (1 MPa/s) and depressurization (5 MPa/s) were applied; these correspond to the highest BP. In the present study, the areas under the curve of temperature-time and pressure-time were calculated and termed as thermal and pressure dosages. The lethal region (T > 95°C and P > 500 MPa) only of pressure and temperature histories was selected for the estimation of thermal and pressure dosages since there was no or limited spore inactivation below this threshold (Rajan et al., 2006). These thermal and pressure dosages seem to correlate well with spore inactivation from various PATP treatments. Increasing the pressure-holding time or decreasing the pressurization rate produced large thermal and pressure dosages, and spore inactivation correspondingly increased (Table 3.3). Linear relationship of both thermal and pressure dosage was obtained with log reduction of the initial spore population containing 1.1 x 10^9 CFU/mL, whereas those from 1.4 x 10^8 and 1.3 x 10^6 CFU/mL displayed the linear relationship up until 2 min pressure-holding time followed by a decrease in spore inactivation (Table 3.3).

3.3.3 Role of pressure pulsing in enhancing spore inactivation

It may be hypothesized that pulsed pressure during PATP causes spore germination during the first pulse followed by inactivation of germinated spores upon subsequent pulsing. Moderate pressure treatment (50 to 300 MPa) at 25 to 50°C is known to cause spore germination (Smelt, 1998; Wuytack et al., 1998; Wuytack et al., 2000; Black et al., 2007) but no information is available indicating this transformation could occur during PATP application. An experiment was conducted to determine if germination of *B. amyloliquefaciens* spores occurs during PATP (Figure 3.3). Spores

that have been treated with heat (105°C) with or without pressure (700 MPa) were tested for germination. Subsequently, treatment survivors were enumerating before and after a heat shock at 80°C for 10 min to kill germinated spores (Wuytack et al., 1998; Wuytack et al., 2000). No spore germination was evident during PATP or the thermal treatment alone (Figure 3.3).

The influence of single- and double-pulse treatment at 105°C and 600 MPa on spore inactivation is presented in Figure 3.4. Double-pulse pressurization was more effective ($P < 0.05$) in inactivating bacterial spores than single-pulse, with an added substantial reduction with a slow pressurization rate. A fast pressurization rate with 0-min pressure holding time reduced 0.5 and 1.0 log CFU/mL for single and double pulses, respectively (Figure 3.4.C). Log survivors from the slow pressurization rate with or without pulsing were statistically different, where 1.3 and 5.7 log CFU/mL were inactivated from single and double pulses, respectively, after the pressure come-up time ($P < 0.05$). Pressure holding time played a major role in spore inactivation for both single- and double-pulse treatments that employed the fast pressurization rate. However, the importance of pressure holding time diminished for the double-pulse treatment that employed the slow pressurization rate (Figure 3.4.C). Treatment of spore suspensions with the first pulse for 1.5 min, which was followed by the second pulse for another 1.5 min, inactivated spores to undetectable level irrespective of pressurization rates. In general, double pulses enhanced PATP lethality by approximately 2.4 to 4 log CFU/mL when compared to a single pulse at an equivalent pressure holding time. The increased lethality of double pulses could be explained by the large values of thermal and pressure

dosages (Figure 3.4.A and 3.4.B). Enhanced lethality was also noticed when using the slow pressurization rate.

The double pulse along with slow pressurization rate provided a synergistic effect on spore inactivation. To further verify this observation, an additional single-pulse experiment at 600 MPa and 112°C was performed. The effect of treatments (single pulse at 105°C, single pulse at 112°C, and double pulses at 105 and 112°C) were statistically different (Table 3.2.B). As expected, further increasing the process temperature from 105 to 112°C enhanced spore inactivation. About 1.9×10^1 and 1.2×10^1 CFU/mL of spores survived after treatment at 600 MPa and 112°C for 3 min using a fast and slow pressurization rate, respectively (Figure 3.5). However, spore survivors were not detectable by direct plating from a double-pulse experiment conducted at 600 MPa, 105 and 112°C for 3 min holding time, regardless of pressurization rates. This suggests that increasing the temperature alone from 105 to 112°C, without pressure pulsing, is not sufficient to enhance PATP spore inactivation (Figure 3.5).

Currently, the mechanism of spore inactivation is actively investigated. Lethal treatment can alter physiological state of bacterial spores. This may include structural changes, e.g., alternations in spore cortex and membranes, and functional modifications, e.g., ability of the spores to recover by culturing on microbiological media. Mathys et al. (2007) reported that combined pressure (up to 600 MPa) and heat (up to 77^{o}C) modified *B. licheniformis* spore physiology through germination and spore cortex hydrolysis, and physically compromised spore's inner membrane. PATP conditions employed in the current study did not yield significant germination in *B. amyloliquefaciens* spores (Figure

3.3). However, it appears that the first pulse sensitized the spores to the remainder of the lethal treatment. It is also likely that the high temperature during the pause-time interval between pressure pulses (~ 78°C) and the second pulse come-up time contributed to this sensitization. Although it is not apparent in this study, spore injury may be proposed as the cause of this sensitization. Detailed mechanistic studies are needed to explain the enhanced microbial lethality during pulsed pressurization, compared to the single-pulse treatment.

3.4 Conclusions

Within a range of experimental conditions in this study (600 MPa, 105°C, for up to 2-min pressure holding time), the slow (compared to fast) pressurization rate was effective in enhancing the inactivation of *B. amyloliquefaciens* spore. Extending the pressure holding times diminished the influence of pressurization rate on spore inactivation. Regardless of the pressurization rate studied, the double-pulse treatment enhanced PATP lethality by additional 2.4 to 4 log reduction, when compared to the equivalent treatment time from the single pulse. The most effective sporicidal treatments in the study were associated with slow pressurization rate and double pulsing. The enhanced spore inactivation should be weighed against the increased cost associated with potential equipment wear due to the severe process conditions encountered during double-pulse treatment.

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Pressurization rate (MPa/s)	Temperature at different stages during processing (°C) [a]					Time required at different stages of pre-processing (s)		
	Pre-process (T_1)	Immediately before pressurization (T_2)	Immediately after pressurization (T_3)	Pressure holding (T_3~T_4)	Depressurization (T_5) [b]	Pre-process (t_1) [c]	Pressure come-up time (t_2)	Pause-time between pulses (t_{pause})
(A) Single pulse								
Fast (18.06)	57.7 ± 0.5	70.6 ± 0.4	103.8 ± 0.5	105.4 ± 0.5	79.2 ± 0.8	236 ± 6.0	33 ± 1.0	-
Slow (3.75)	57.1 ± 0.9	65.4 ± 0.8	105.2 ± 0.6	105.5 ± 0.4	79.0 ± 0.9	188 ± 7.0	160 ± 2.0	-
(B) Double pulse								
Fast (18.06)								
1st pulse	58.0 ± 0.6	70.2 ± 0.7	103.0 ± 0.5	105.2 ± 0.3	79.6 ± 0.9	236 ± 6.0	33 ± 1.0	62 ± 1.0
2nd pulse	-	78.9 ± 0.4	108.6 ± 0.7	110.8 ± 0.8	82.4 ± 0.5	-	33 ± 1.0	-
Slow (3.75)								
1st pulse	57.6 ± 0.2	64.7 ± 0.3	105.1 ± 0.5	105.5 ± 0.4	79.5 ± 0.5	188 ± 7.0	160 ± 2.0	69 ± 2.0
2nd pulse	-	77.0 ± 0.5	111.9 ± 0.4	112.4 ± 1.0	81.6 ± 0.8	-	160 ± 2.0	-

[a] Glycol bath temperature was maintained at 105.5°C.
[b] Depressurization time (<2 s) is not included.
[c] t_1 is the sum of preheating time for the sample in the water bath, and time spent in the pressure chamber just before the commencement of pressurization.

Table 3.1. Temperature histories at different stages of pre-processing and pressure-assisted thermal processing at 600 MPa and 105°C.

Parameter [a]	Probability
(A) Inactivation of different spore concentrations by selected processing variables	
Inoculum level (I_i)	<0.0001
Pressurization rate (PR_j)	<0.0001
Holding time (t_k)	<0.0001
Inoculum level x Pressurization rate ($I*PR$)	0.3513
Inoculum level x Holding time ($I*t$)	<0.0001
Pressurization rate x Holding time ($PR*t$)	<0.0001
Inoculum level x Pressurization rate x Holding time ($I*PR*t$)	0.0029
(B) Inactivation of spores (1.4 x 10^8 CFU/mL) by pulsed pressure and other processing variables	
Pressure pulsing (P_i)	<0.0001
Pressurization rate (PR_j)	<0.0001
Holding time (t_k)	<0.0001
Pressure pulsing x Pressurization *rate ($P*PR$)*	0.2317
Pressure pulsing x Holding time ($P*t$)	0.0001
Pressurization rate x Holding time ($PR*t$)	<0.0001
Pressure pulsing x Pressurization rate x Holding time ($P*PR*t$)	0.0028

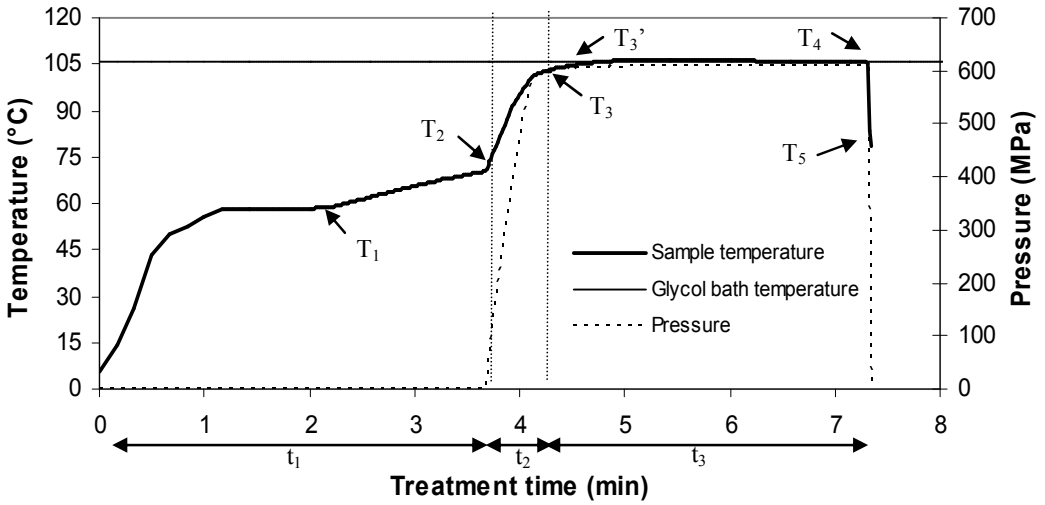
[a] influence of individual processing parameter and their interaction terms.

Table 3.2. Summary of statistical analysis results regarding the contribution of various processing parameters to the spore lethality (Δ log CFU/mL).

Pressurization rate (MPa/s)	Pressure holding time (min)	Thermal dosage (°C·s)	Pressure dosage (MPa·s)	Inoculum level (1.1. x 10^9 CFU/mL)	Inoculum level (1.4 x 10^8 CFU/mL)	Inoculum level (1.3 x 10^6 CFU/mL)
				Corresponding log reduction (log CFU/mL)		
Fast (18.06)	0	117 ± 12	1041 ± 119	0.5 ± 0.2	0.6 ± 0.1	0.6 ± 0.2
	0.5	370 ± 16	4013 ± 266	0.9± 0.3	1.5 ± 0.1	1.2 ± 0.1
	1	669 ± 38	6954 ± 20	1.4 ± 0.5	2.4 ± 0.2	2.0 ± 0.4
	2	1420 ± 77	13621 ± 189	2.8 ± 0.2	3.9 ± 0.4	4.1 ± 0.3
	3	2034 ± 71	19265 ± 249	3.7 ± 0.5	5.2 ± 0.5	4.9 ± 0.2
	5	3189 ± 75	32973 ± 1359	5.4 ± 0.6	6.8 ± 0.1	5.1 ± 0.1
Slow (3.75)	0	667 ± 79	5202 ± 587	1.1 ± 0.2	2.6 ± 0.3	2.0 ± 0.1
	0.5	898 ± 71	8080 ± 83	1.4 ± 0.1	2.9 ± 0.5	2.2 ± 0.2
	1	1158 ± 157	10744 ± 338	2.5 ± 0.6	3.4 ± 0.3	3.2 ± 0.3
	2	1949 ± 56	17006 ± 614	3.4 ± 0.1	4.6 ± 0.1	4.5 ± 0.2
	3	2596 ± 134	23479 ± 300	4.6 ± 0.3	5.8 ± 0.3	5.1 ± 0.1
	5	3803 ± 161	36361 ± 274	6.1 ± 0.4	6.6 ± 0.1	5.1 ± 0.1

Table 3.3. Thermal and pressure dosage of the lethal region (T > 95°C and P > 500 MPa) at various pressure holding times and their corresponding log reduction from the single-pulse experiment. Data presented are mean ± standard deviation of three independent replications.

(A)



(B)

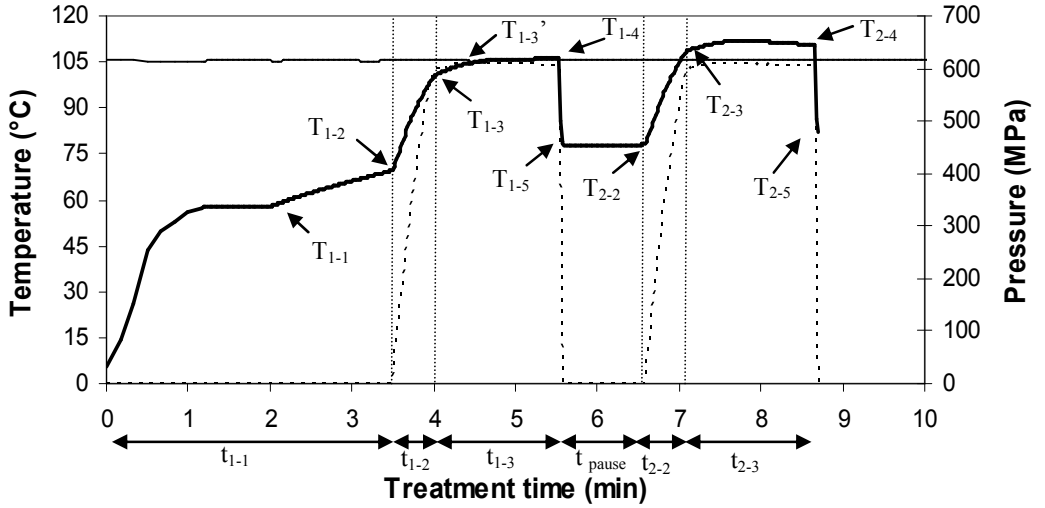


Figure 3.1. Comparison of pressure and temperature histories of spore samples subjected to fast pressurization rate during single- and double-pulse treatment at PATP conditions (600 MPa at 105°C for 3 min). Single-pulse treatment (A) and double-pulse treatment (B).

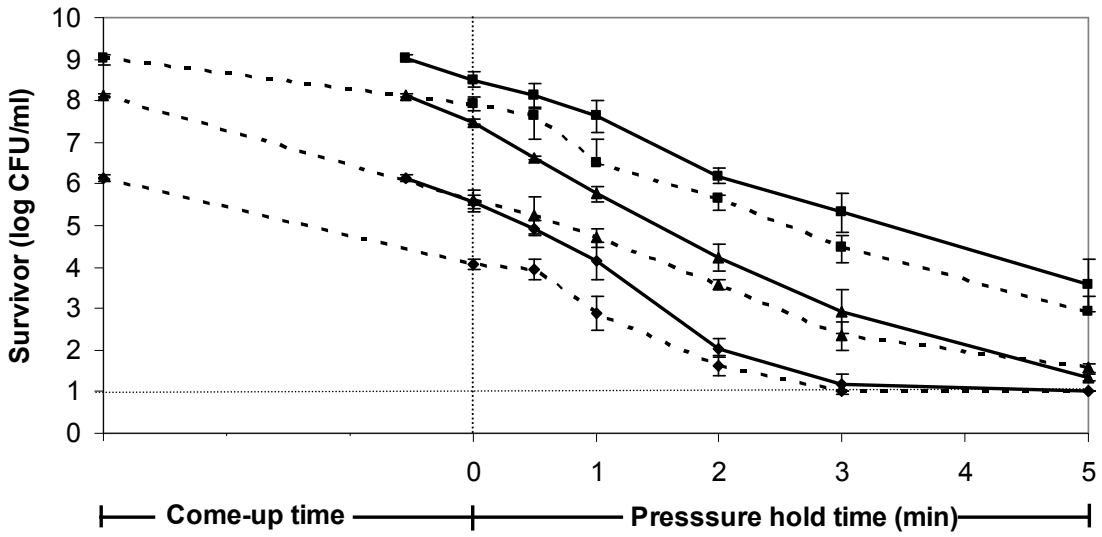


Figure 3.2. *Bacillus amyloliquefaciens* TMW 2.479 spore survivors in deionized water subjected to 600 MPa and 105°C with two pressurization rates and three inoculation levels. Pressurization rates: (———) fast; (---------) slow. Inocula: (■) 1.1 x 10^9 CFU/mL; (▲) 1.4 x 10^8 CFU/mL; (◆) 1.3 x 10^6 CFU/mL. The horizontal dotted line indicates the detection limit (< 10 CFU/mL).

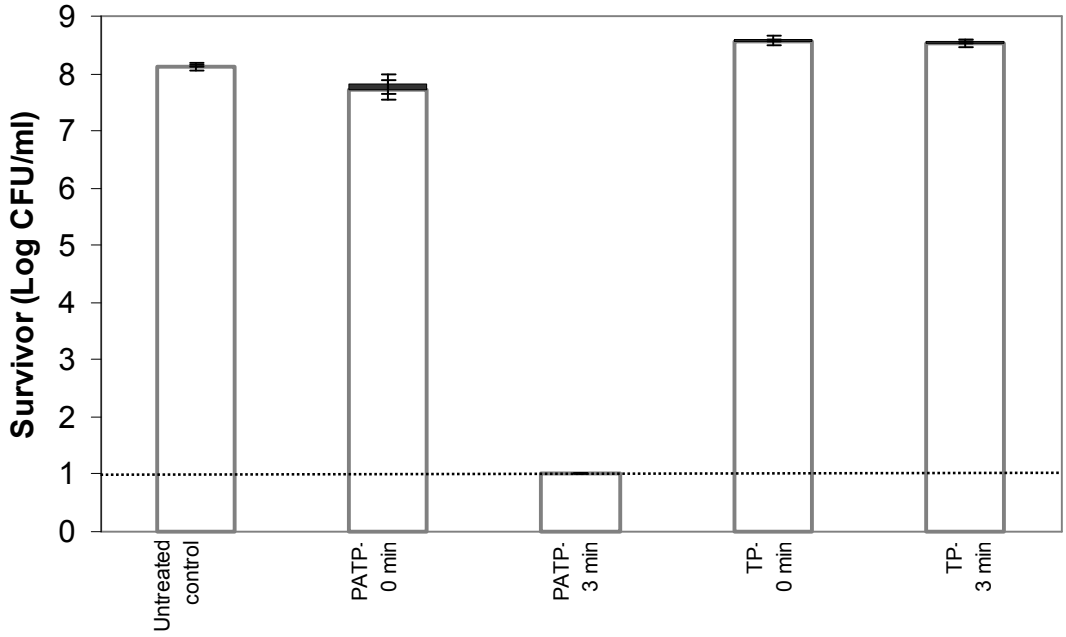
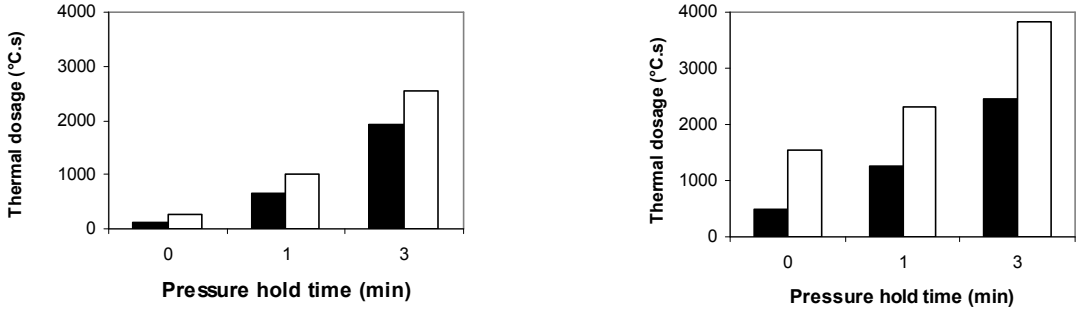


Figure 3.3. Spore count when suspensions were subjected to PATP (700 MPa-105°C) or TP (105°C-0.1 MPa), for 0 and 3 min, and subsequently tested for determining germinated sub-populations. Spore populations: (☐) dormant spores; (■) germinated spores. The horizontal dotted line indicates the detection limit (< 10 CFU/mL).

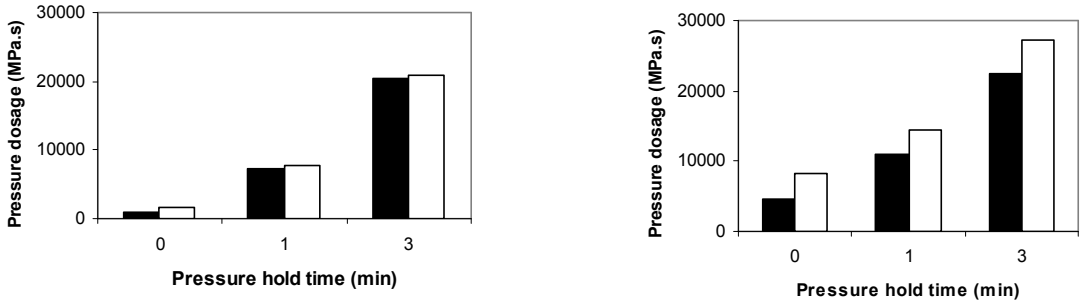
Figure 3.4. Thermal dosage (A), pressure dosage (B) of the lethal region (T > 95°C and P > 500 MPa) at various pressure holding times during (■) single- and (□) double-pulse experiments. Spore survival curve (C) of *B. amyloliquefaciens* TMW 2.479 after treatment with 600 MPa and 105°C with (—◆—) single and (-----◇-----) double pulses. Pressurization rates: (18.06 MPa/s) fast; (3.75 MPa/s) slow. $^{a-c}$ Mean (log CFU/mL) ± standard deviation: different superscript within treatment (single or double pulses) represents significant difference among means ($P < 0.05$). $^{A-C}$ Mean (log CFU/mL) ± standard deviation: different superscript within time represents significant difference among means ($P < 0.05$). The initial population was 1.4 x 10^8 CFU/mL. The horizontal dotted line indicates the detection limit (< 10 CFU/mL). The asterisk (*) represents spore counts under detectable level.

Fast rate (18.06 MPa/s) Slow rate (3.75 MPa/s)

(A)



(B)



(C)

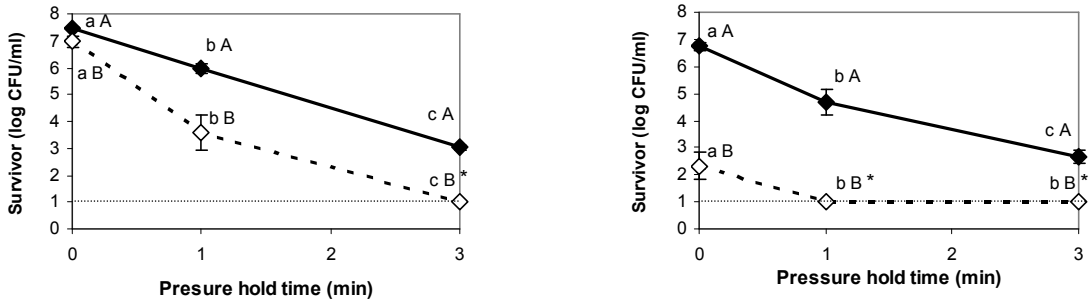


Figure 3.4.

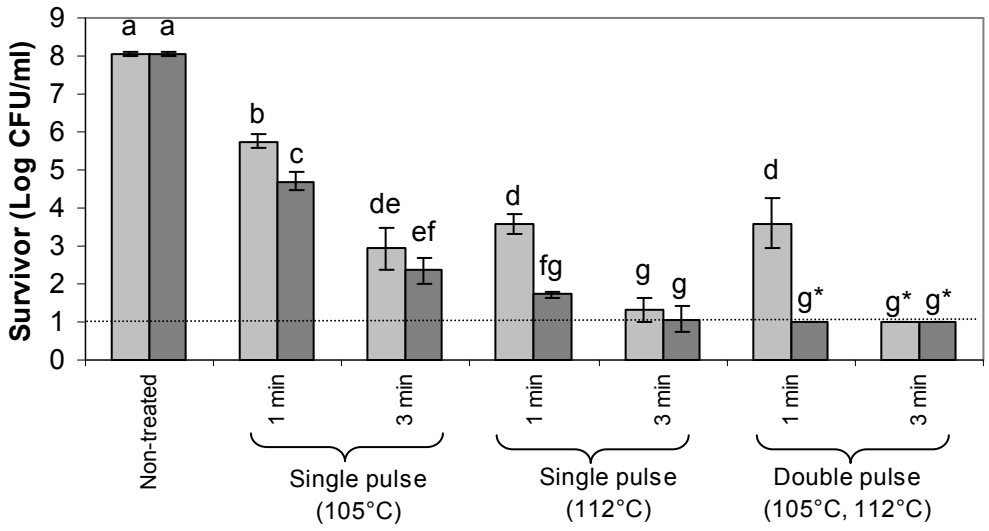


Figure 3.5. *Bacillus amyloliquefaciens* TMW 2.479 spore survivors in deionized water processed at different treatment conditions (single pulse, 105ºC; single pulse, 112ºC; double pulse, 105 and 112ºC) and two PATP holding times (1 and 3 min). Pressurization rates: (☐) fast; (■) slow. $^{a\text{-}g}$ Mean (log CFU/mL) ± standard deviation: different superscript within treatment represents significant difference among means ($P < 0.05$). Control demonstrates the initial population at 1.4 x 10^8 CFU/mL. The horizontal dotted line indicates the detection limit (< 10 CFU/mL). The asterisk (*) represents spore counts under detectable level.

Chapter 4: Efficacy of pressure-assisted thermal processing, in combination with organic acids, against *Bacillus amyloliquefaciens* spores suspended in deionized water and carrot puree

Abstract

Effect of organic acids (acetic, citric, and lactic; 100 mM, pH 5.0) on spore inactivation by pressure-assisted thermal processing (PATP; 700 MPa and 105°C), high pressure processing (HPP; 700 MPa and 35°C) and thermal processing (TP; 105°C and 0.1 MPa) was investigated. *Bacillus amyloliquefaciens* spores were inoculated into sterile organic acid solutions to obtain a final concentration of 1.3 x10^8 CFU/mL. *B. amyloliquefaciens* spores were inactivated to undetectable levels with or without organic acids after 3-min PATP holding time. At a shorter PATP treatment time (~ 2 min), inactivation was greater when spores were suspended in citric and acetic acids than in lactic acid or deionized water. Presence of organic acids during PATP resulted in 33% to 80% germination in the population of spores that survived the treatment. In contrast to PATP, neither HPP nor TP, for up to 5-min holding time with or without addition of organic acids, was sporicidal. In a separate set of experiments, carrot puree was tested, as a low-acid food matrix, to study spore recovery during extended storage following PATP. Results showed that organic acids were effective in inhibiting spore recovery in treated

carrot puree during extended storage (up to 28 days) at 32°C. In conclusion, addition of some organic acids provided significant lethality enhancement ($P < 0.05$) during PATP treatments and suppressed spore recovery in the treated carrot puree.

4.1 Introduction

Pressure-assisted thermal processing (PATP) provides an opportunity for food processors to produce commercially sterile shelf-stable low-acid foods at modest treatment conditions. The process involves simultaneous application of pressures (500 to 700 MPa) and temperatures (90 to 121°C) to a pre-heated food product over a short holding time. One of the unique advantages of the technology is the ability to increase product temperature quasi-instantaneously with the heat of compression. This reduces the severity of the thermal effect such as that encountered during conventional thermal processing. The technology has potential applications in processing value-added heat-sensitive low-acid products such as soups, egg products, coffee, tea, and mashed potatoes (Balasubramaniam et al., 2008).

Several authors have investigated PATP efficacy against bacterial spores (Ananta et al., 2001; Cléry-Barraud et al., 2004; Margosch et al., 2006; Rajan et al., 2006; Ahn et al., 2007; Mathys et al., 2007; Paredes-Sabja et al., 2007; Zhu et al., 2008; Bull et al., 2009). It is desirable to reduce the severity of the pressure-thermal treatment to minimize process impact on product quality. Reducing process severity will also make the technology commercially attractive to the food industry for processing various shelf-stable, low-acid foods.

Traditional thermal processing techniques such as canning use acidification as a means of reducing process severity and extending product shelf-life. Acetic, citric, and lactic acids are weak organic acids that can be used as food additives in most foods without any specific limitations (Casadei et al., 2000). Influence of these acidulants on microbial inactivation mostly depends on the acid's dissociation constant (K_a) and pH of the medium. Moreover, the inhibitory effects of various types of organic acids could be substantially dissimilar due to the variation in the numbers of carboxyl and hydroxyl groups, and double bonds in their molecular structures (Hsiao and Siebert, 1999). Leguerinel and Mafart (2001) reported the effectiveness of different organic acids in lowering the heat resistance of *B. cereus* spores. Spores of *Paenibacillus polymyxa* were found to be susceptible to the undissociated form of lactic acid during heat treatment (Casadei et al., 2000). To select appropriate organic acids, pH of the food should be comparable or lower than organic acid's pK_a values. The objectives of this study were to investigate the feasibility of enhancing the efficacy of PATP in combination with selected organic acids against bacterial spores and to explore the potential of extending product shelf-life by the combined treatment.

4.2 Materials and methods

4.2.1 Bacterial strain and preparation of spores

Bacillus amyloliquefaciens TMW 2.479 Fad 82 culture was obtained from M. Gänzle (Department of Agricultural, Food and Nutritional Science, University of Alberta, Edmonton, Alberta, Canada). The culture was activated in trypticase soy broth

supplemented with 0.6% yeast extract (TSBYE; Becton, Dickinson and Company, Sparks, MD) and aerobically incubated at 32°C for 24 h. After two transfers, 100 µl of vegetative cells was spread-plated on trypticase soy agar supplemented with 0.6% yeast extract (TSAYE; Becton, Dickinson and Company) and 10 ppm $MnSO_4 \cdot H_2O$ (Fisher Scientific, Pittsburgh, PA). The inoculated plates were incubated at 32°C for 14 days. Spores were collected by flooding the plates with cold sterile deionized water and washed five times by differential centrifugation (2,000 to 8,000 x *g*, 20 min, 4°C). The spore pellets were then resuspended in sterile deionized water to obtain ~10^9 spores/mL. The suspension was sonicated for 10 min (SM275HT, Crest, ETL Testing Laboratory, Cortland, NY). Heat treatment (80°C for 15 min) was applied to destroy any remaining vegetative cells. The spore suspension in deionized water was stored at 4°C until used.

4.2.2 Organic acids

Food-grade acetic acid (glacial, FCC; Spectrum®, Fisher Scientific), citric acid (anhydrous powder, FCC; Tate & Lyle, Decatur, IL), and lactic acid (FCC 88; Purac®, Lincolnshire, IL) were prepared to a final concentration of 100 mM. Organic acids (100 mM) were also prepared from chemical-grade material: acetic acid (glacial, ACS; Fisher Scientific), citric acid (Acros Organics, Morris Plains, NJ), and lactic acid (Acros Organics). The pH values of the solutions were measured using a pH meter (Accumet® model 15, Fisher Scientific). A final pH value of 5.0 was achieved by an addition of 1N to 5N sodium hydroxide (Fisher Scientific) to various organic acid solutions. All solutions were filter-sterilized with a 0.22 µm-pore-size membrane filter (Fisher Scientific).

4.2.3 Preparing spores for processing

Prior to the treatments, the spore suspension was sonicated for 10 min to prevent clumping. Aliquots (0.2 mL) of *B. amyloliquefaciens* spore suspension and 1.8 mL of sterile organic acid solution (acetic, citric, and lactic; 100 mM, pH 5.0, and deionized water; pH 6.8) were aseptically transferred into a sterile pouch (5 x 2.5 cm, polyethylene bags, 01-002-57, Fisher Scientific) to obtain ~ 1.3 x10^8 CFU/mL. The pouches were then heat sealed using an impulse heat sealer (American International Electric, Whittier, CA), while minimizing occluded air as much as practically possible. All inoculated pouches were stored up to 2 h in an ice-water bath (4°C) before treatment.

4.2.4 Pressure-assisted thermal processing and high pressure processing

High pressure microbial kinetic tester (PT-1, Avure Technologies Inc., Kent, WA) was used in this study. The unit is equipped with an intensifier (M-340 A, Flow International, Kent, WA) that can generate pressures up to 700 MPa. A 54-mL stainless steel pressure chamber was immersed in a temperature-controlled bath. Propylene glycol (Houghton Safe-620TY, Houghton International Inc., Valley Forge, PA) was used as the heat transfer medium in the temperature-controlled bath. The glycol was also used as the pressure-transmitting medium within high pressure processor. For PATP applications, the temperature of the external glycol bath was set at 105°C (target process temperature) to minimize any loss during the test. The high pressure processor had a pressurization rate of 14.3 MPa/s, while depressurization occurred within 2 s for all treatments. The sample temperature, bath temperature, and chamber pressure were recorded every second with a K-type thermocouple sensor (model KMQSS-04OU-7, Omega Engineering, Stamford,

CT) and pressure transducer (model 3399 093 006, Tecsis, Frankfurt, Germany) using a data acquisition computer.

For various combined pressure-heat treatment experiments, the pouch was placed inside a sample holder which was made of a 10-mL polypropylene syringe (model 309604, Becton, Dickinson and Company) wrapped with two layers of insulating material (Sports Tape, CVS® Pharmacy Inc., Woonsocket, RI). The void volume in the syringe was filled with ~8 mL of water to ensure that the spore suspension and the syringe water in the vicinity of the sample experience similar thermal response during processing. Insulating syringe also helped to minimize the heat exchange between the syringe containing the spore sample and the surrounding glycol bath, which has higher heat of compression than that of water. The syringe containing the pouch was pre-heated at an empirically determined pre-processing temperature (T_1 ~ 54.6 ºC; Table 4.1.A) in a water bath (Isotemp 928, Fisher Scientific) for 2 min. The syringe was then immediately loaded into the pressure chamber. The pressure-temperature histories in the vicinity of the sample within the syringe were recorded. After reaching the predetermined temperature, T_2 (Table 4.1.A), the pressurization process started. The T_2 was estimated using the following relationship:

$$T_2 = T_3 - \left(CH \cdot \Delta P + \Delta T_H\right) \tag{4.1}$$

where T_3 is the desired target temperature (°C), CH is the heat of compression value of water (temperature increase in °C per 100 MPa pressurization), and ΔP is the process pressure (MPa). ΔT_H is the temperature gained by the test sample during loading within the pressure chamber as well as pressurization (Nguyen et al., 2007).

Test samples were treated at various PATP (700 MPa-105°C for 0, 1, 2, 3, and 5 min) and HPP (700 MPa-35ºC for 0, 2, and 5 min) conditions. The pressure holding time did not include the pressure come-up time or the depressurization time. After depressurization, the spore suspension was immediately removed from the pressure chamber and cooled in an ice-water bath (4°C) to prevent further inactivation. Surviving *B. amyloliquefaciens* populations were enumerated as described in a later section within 3 h after the treatments. All experiments were independently repeated at least three times.

4.2.5 Thermal processing

Thermal processing (TP) experiments were carried out at 105°C and 0.1 MPa using a 35-L circulating oil bath (NESLAB EX-35 Digital One, Thermo Fisher Scientific Inc., Waltham, MA). Thermal process experiments utilized custom-fabricated thermal-death-time disks (TDT disk; 18 mm diameter, 4.5 mm height) as sample holders (Jin et al., 2008). The sample temperature was monitored and recorded by inserting a K-type thermocouple (Omega Engineering) attached to a data logger (IOtech Inc., Cleveland, OH) into a TDT disk containing sterile deionized water without spores. Two oil baths were used to manipulate thermal pre-processing time to that of PATP (Nguyen et al., 2007). Temperature of the first oil bath was maintained at 113 ± 1ºC. Once the sample temperature reached ~ 100°C, all TDT disks were shifted to the second oil bath which was maintained at 105°C. At specific holding times, the disk was removed from the second oil bath and immediately immersed into an ice-water bath (4°C) to avoid further inactivation. Temperature histories were automatically recorded by the data logger. Come-up time of the samples to reach 105°C was approximately 1.2 min and it took

about 3 min to bring the thermal-processing temperature from 105°C to 4°C (Table 4.1.B). Surviving *B. amyloliquefaciens* populations were enumerated within 3 h after the treatments. All experiments were independently repeated at least three times.

4.2.6 Enumeration of survivors and spore germination

After treatments, 1 mL of the treated spore suspension was serially diluted in sterilized 0.1% peptone water. The 1-mL aliquots of the appropriate dilutions were then pour-plated on duplicate TSAYE. Portion of the treated samples were also heat shocked at 80°C for 15 min to kill germinated and sensitized spores occurring during the PATP treatments. Colonies of survivors were counted after incubation at 32°C for 48 h.

Heat sensitivity was used as a criterion for spore germination (Wuytack et al., 2000; Van Opstal, et al., 2004; Black et al., 2008). The percentage of spore germination was estimated using equation 4.2 from the knowledge of the total surviving population after PATP (N_{PATP}) and the number of dormant spores survived PATP treatment followed by a heat shock ($N_{PATP+HT}$) at 80°C for 15 min.

$$\%\ Spore\ Germination = \frac{(N_{PATP} - N_{PATP+HT})}{N_{PATP}} \times 100 \qquad (4.2)$$

4.2.7 Most-proable number (MPN) technique

When the treatment with PATP inactivated bacterial spores to undetectable level by the plating method (≤ 10 CFU/mL), survivor population was estimated using the MPN technique (FDA, 2001). Briefly, sample pouches were aseptically cut open and its contents were serially diluted in sterilized 0.1% peptone water and inoculated into 9 TSBYE tubes at 3 x 0.1, 3 x 0.01, and 3 x 0.001 mL inocula. Samples processed in deionized water were used as the control. The inoculated MPN tubes were incubated at

32°C up to 15 days to allow the pressure-heat injured spores to recover, germinate, and grow. All inoculated tubes were monitored for turbidity. The turbid tubes (indicating growth) were then streaked onto TSA plates to confirm the purity of culture and observe typical *B. amyloliquefaciens* colonies after incubation at 32°C for 48 h.

4.2.8 Microbial stability of PATP processed carrot puree

The combined effect of PATP and organic acid was tested using inoculated carrot puree (Gerber®, Fremont, MI) at pH 5.4. Through a separate set of preliminary experiments, it was found that untreated carrot puree supported the growth of the *B. amyloliquefaciens* spores. Spores inoculated into carrot puree at low levels (~10^2 and 10^3 CFU/mL) were able to grow to > 10^7 CFU/mL after 2 days of storage at 32°C.

Aliquots of organic acids (0.18 mL, 1 M) were added to the sterile carrot puree (1.62 mL) and thoroughly mixed to obtain a final concentration of 100 mM. When adding acid solutions to the carrot puree, its pH dropped to 3.3 to 2.8. The pH of the carrot puree-organic acid mixture was then adjusted to pH 5.0 by using 1N to 5N sodium hydroxide. Carrot puree-organic acid solution (1.8 mL) and *B. amyloliquefaciens* spores (0.2 mL) were mixed and packaged as described earlier.

Various carrot puree samples inoculated with *B. amyloliquefaciens* spores were processed at 700 MPa and 105°C for 5 and 15 min. Treated pouches were stored at 32°C up to 28 days. Sample pouches were withdrawn at 0, 2, 7, 14, 21, and 28 days of storage. During each withdrawal, samples were enumerated using both plate count and the MPN technique as follows. PATP-treated samples (2 mL) were mixed with 18 mL of sterilized 0.1% peptone water. The samples were then homogenized in a stomacher (Seward Lab

Stomacher, Norfolk, UK) at 230 rpm for 2.5 min. Portions of processed samples were also heat shocked at 80ºC for 15 min to inactive any germinated or sensitized spores. After serial dilution, 0.1 mL aliquots of appropriate dilutions were spread-plated on duplicate TSAYE plates. Enumeration was carried out after incubation at 32°C for up to 72 h. The MPN analysis (similar to that described earlier) was also carried out for estimating surviving spores that were not recovered by the plate count technique.

4.2.9 Statistical analysis

Data analysis was performed using the Statistical Analysis System software (SAS 9.1, SAS Institute Inc, Cary, NC). The independent variables were suspension medium (i.e., deionized water, acetic, citric, and lactic acid) and pressure holding times (i.e., untreated, 0, 1, 2, 3, and 5 min). The spore survivors (log CFU/mL) in response to the combined pressure-heat process treatment served as the dependent variable (Y_{ijk}) and the experimental replications were also included as a blocking factor (β_i) in the analysis. The following statistical model was used to analyze the data by the general linear model (GLM) procedure of SAS.

$$Y_{ijk} = \mu + \beta_i + S_j + T_k + ST_{jk} + \varepsilon_{ijk} \tag{4.3}$$

where Y_{ijk} is the dependent variable, β_i is the blocking factor, S_j is the suspension medium, T_k is the holding time, ST_{jk} is the interaction term between suspension medium and holding time, and ε_{ijk} is the error term. Mean comparison were evaluated with the Student-Newman, Keuls (SNK) test at a 5% significant level ($P = 0.05$).

4.3 Results and discussion

Chemical- and food-grade organic acids, including citric (pK_a = 4.75), lactic (pK_a = 3.88), and acetic (pK_a = 4.76), at 100 mM level and pH 5.0, were tested in combination with PATP. Preliminary experiments verified that addition of organic acids to the spore suspension (without TP or PATP treatment) did not cause any statistically significant inactivation or germination. Further, for a given process condition, microbial efficacy of chemical- and food- grade organic acids were not statistically different (data not shown). Therefore, subsequent studies were carried out using food-grade organic acids only.

4.3.1 Influence of organic acids on enhancing spore inactivation during pressure-assisted thermal processing

Figure 4.1 compares the spore survivors after PATP treatments in combination with organic acids for various treatment times. During the pressure come-up time (~ 48 s), up to 0.4 log reduction was observed (Figure 4.1). Under the conditions of the experiments, the spores suspended in deionized water did not show any significant germination ($P > 0.05$) over a 5-min pressure holding time; spores enumerated immediately after PATP treatments were similar in counts to those further subjected to the heat shock at 80°C for 15 min. Increasing pressure holding time increased *B. amyloliquefaciens* inactivation. For example, pressure holding time of 1 and 2 min resulted in 4.0 and 5.8 log reduction, respectively. *Bacillus amyloliquefaciens* spores were ultimately inactivated to below the detection level (≤ 10 CFU/mL) after 3-min pressure holding time.

Among the organic acids tested, acetic and citric acids (100 mM) were found to be effective in enhancing spore inactivation even at the shorter holding time (2 min) (Figure 4.1). Within the range of the experimental conditions tested, lactic acid (100 mM) did not enhance PATP lethality ($P > 0.05$). It provided similar inactivation as the control (i.e., spores suspended in deionized water). Increasing concentrations of the citric, lactic, and acetic acids up to 200 mM during PATP provided similar lethality as that of 100 mM (data not shown).

Combining the organic acids (100 mM) with pressure (700 MPa and 35°C) or thermal (105°C and 0.1 MPa) treatments did not enhance *B. amyloliquefaciens* spore inactivation. A 5-min pressure treatment at 700 MPa-35°C caused spore activation up to 0.3 log CFU/mL. Similarly, about 0.1-0.4 log CFU/mL spore activation was observed after heat treatment at 105°C-0.1 MPa up to 5 min.

The sporicidal efficacy of organic acids during thermal processing is well documented (Casadei et al., 2000; Leguerinel and Mafart, 2001; Moussa-Boudjemaa et al., 2006), but their efficacy during PATP is not known. The efficacy of organic acids during thermal processing was attributed to acidulant type and concentration, pH and composition of the medium, inoculum level, and bacterial strains (Leguerinel and Mafart, 2001; Moussa-Boudjemaa et al., 2006). Palop et al. (1997) found that citric acid was more effective against *B. coagulans* while heating at 100°C for 10 s, whereas lactic acid was more sporicidal with the longer heating times (≥ 2 min). Within the range of the experimental conditions, combination of PATP with acetic or citric acids appears to yield

more sporicidal effect than does the PATP treatment of spores in deionized water (Figure 4.1).

4.3.2 Germination of surviving population of *B. amyloliquefaciens* spores after pressure-assisted thermal processing in the presence of organic acids

In general, the organic acids tested induced germination among the surviving population of *B. amyloliquefaciens* spores after PATP treatment (Figure 4.1). The magnitude of germination varied with the type of organic acid and pressure holding time. Acetic acid induced germination of 38% (0 min) to 80% (2 min) of surviving spore population, whereas citric acid caused 44% (after 0 min) and 75% (after 2 min) of surviving spore population to germinate (Table 4.2). For lactic acid, spore germination was detected at 0 and 1 min (33 to 64%), but the level of spore germination was not detectable at 2-min pressure holding time.

A number of earlier researchers reported that moderate pressure (100 to 400 MPa) induced germination in bacterial spores, such as those of *B. subtilis* and *B. cereus*, at moderate process temperature (25 to 60°C) (Wuytack et al., 2000; Van Opstal et al., 2004). At 100-300 MPa, germination was induced through the activation of spore's nutrient receptors even without the presence of nutrients (Paidhungat et al., 2002). At higher pressure levels (400 to 800 MPa), the release of dipicolinic acid (DPA) was initiated, which lead to the later steps in spore germination (Wuytack et al., 1998; Paidhungat et al., 2002). Black et al. (2008) observed about 4 log CFU/mL of germination from *B. subtilis* spores suspended in milk after treated at 500 MPa-40°C for 5 min and about 85% of DPA was released. In our earlier studies on *B. amyloliquefaciens*

spores (as well as the current study), spores suspended in deionized water did not produce any detectable spore germination after treatments at 600-700 MPa at 105°C for about 5-min pressure holding time (Ratphitagsanti et al., 2009). It appears that addition of organic acids facilitated the germination in PATP-treated *B. amyloliquefaciens* spores.

When pH values before and after PATP treatments were compared, all organic acid investigated in this study had similar pH values (~ pH 5.0). Pressure-induced transient pH shift has been reported in the literature. For example, Min (2008) used a real-time in-situ pH measurement technique and observed that citric (pH 4.55, 570 mM) and phosphoric acids (pH 6.90, 58 mM) when treated under pressure (400 MPa at 25^{o}C) transiently shifted their pH towards acidic side by 0.57 and 1.24 pH units, respectively. Upon depressurization, pH values returned close to initial values. Similarly, Paredes-Sabja et al. (2007) theoretically predicted a 0.7 pH units shift towards acidic conditions for citric acid (initial pH 4.75) when subjected to the 650 MPa-treatment. The pressure-induced pH-shift directly impacts the dissociation constant (pK_a) of an acid which could be explained by the rearrangement of water molecules. When increasing pressure, water molecules are aligned into a compact structure and condensed layers are formed around the charged ions. This leads to the dissociation of uncharged acids (HA) into additional H^+ and A^- ions (Neuman et al., 1973; El'yanov and Hamann, 1975; Paredes-Sabja et al., 2007). Since ionization occurs under pressure processing, the beneficial influence of the undissociated acid forms as an antimicrobial entity become less effective (Earnshaw et al., 1995). On the contrary, it was also reported that the undissociated forms of organic acids might be more active under pressure (Smelt, 1998).

Temporary exposure to lower pH values under pressure possibly sensitized spores to PATP. Native spores might transform into demineralized forms (also referred to as H-spores) when high pressure-induced ionization occurs (Marquis and Bender, 1985; Paredes-Sabja et al., 2007). The transient pH shift might impact spore inner membrane by increasing their permeability barriers, leading to the decrease in resistance of spores to thermal and pressure treatments (Paredes-Sabja et al., 2007). Very limited data are currently published on pressure-temperature induced pH shift; techniques for estimating the transient pH shift under pressure are still under development. Foods may contain number of organic and amino acids that can act as buffering agents and each with different pKa values. Thus, the net pH shift effect of various organic acid constituents present in the food during pressurization is not known. More research is needed to understand the transient pH shift under pressure and its impact on the processed food.

4.3.3 Microbial stability during storage

Table 4.3 presents the MPN results of *B. amyloliquefaciens* spores suspended in deionized water and various organic acid solutions (100 mM) when these suspensions are treated with 700 MPa-105^{o}C for 3 and 5 min holding times and stored up to 15 days at 32°C. During this storage period, greater numbers of viable spores were generally recovered from spores inoculated in deionized water than those from organic acid solutions. For example, after PATP for 3 min and 15 days of storage, viable spores were 2.4, 1.2, 0.63, and 0.50 log MPN/mL in deionized water, acetic, citric, and lactic acids, respectively. PATP-treated spores in acid solutions, after 5-min pressure holding time as well as those in deionized water yielded < 10^1 MPN/mL viable spore populations.

In the absence of organic acids, treatment of carrot puree at 700 MPa-105°C for 5 or 15 min inactivated *B. amyloliquefaciens* spores to the undetectable level of the plate count method ($<10^2$ CFU/mL). Subsequent storage of the suspension subjected to the 5-min PATP at 32°C for 7 days and beyond resulted in significant growth of *B. amyloliquefaciens* (> 7 log CFU/mL) (Figure 4.2). This indicated possible repairing and recovery of injured population in the presence of nutrient rich food matrix over storage time when organic acids were not present. However, there was no recovery of surviving spores when pressure holding increased from 5 min to 15 min; spore population level remained below the detection limit over 28 days of storage at 32°C. When spores of *B. coagulans* were processed at 100°C for less than 2 min, spores underwent repair from the associated thermal injury (Palop et al., 1997). Valero et al. (2003) also observed the growth of *B. cereus* spores in vegetable-based products (e.g. carrot broth, zucchini broth, and carrot puree) over storage times at normal (≤ 8°C) or elevated (12-16°C) refrigerated temperatures. In the current study, adding organic acids to inoculated carrot puree inhibited spore recovery during 28 days storage at 32°C (Table 4.4.A and 4.4.B). Based on these results, organic acids appear quite effective in inhibiting the recovery of the spore population that survived PATP treatment of carrot puree. Further research is needed to establish the effectiveness of PATP-organic acid combinations on variety of low-acid food matrices.

4.4 Conclusions

Organic acids, particularly citric and acetic acids reduced the resistance of *B. amyloliquefaciens* spores to the combined pressure-thermal (700 MPa-105°C for 2 min) treatment. Organic acids also facilitated the germination of the remaining surviving population after PATP treatment. HPP (700 MPa-35°C) and TP (105°C-0.1 MPa), with or without organic acids, did not cause detectable spore inactivation or germination. Organic acids tested in this study were effective in preventing the recovery of the remaining population of PATP-treated *B. amyloliquefaciens* spores that were inoculated in a model low-acid food (carrot puree) and stored up to 28 days at 32°C. In conclusion, the use of organic acids in combination of with pressure-thermal treatment may be a useful approach for enhancing spore inactivation during treatment and extending microbial shelf-life of the processed products. More research is needed to understand the interaction between various organic acids and the natural food constituents during post-PATP treatment.

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(A)

Holding time (min)	Come-up time (min)	Pre-process (T_1, ºC)	Immediately before pressurization (T_2, ºC)	Immediately after pressurization (T_3, ºC)	Pressure holding (T_3~T_4, ºC)[a]	Depressurization (T_5, ºC)[b]
0	0.8 ± 0.02[c]	54.3 ± 1.0	66.3 ± 0.2	105.3 ± 0.2	-	84.1 ± 0.5
1	0.8 ± 0.02	54.8 ± 0.9	61.8 ± 0.5	101.4 ± 0.8	104.6 ± 0.7	76.5 ± 0.3
2	0.8 ± 0.02	54.9 ± 0.2	62.1 ± 0.9	101.8 ± 1.0	105.5 ± 1.1	78.8 ± 0.3
3	0.8 ± 0.05	54.7 ± 0.6	61.5 ± 0.1	100.9 ± 0.6	105.2 ± 0.4	76.6 ± 0.2
5	0.8 ± 0.04	55.4 ± 1.4	62.1 ± 0.6	101.9 ± 0.3	105.7 ± 0.5	76.0 ± 0.5

[a] External glycol bath surrounding the pressure chamber was maintained at 105ºC.
[b] Depressurization time occurred within 2 s.
[c] Data presented are means ± standard deviation of three independent trials.

(B)

Holding time (min)	Come-up time (min)	Pre-process (T_1, ºC)	Immediately before thermal treatment (T_2, ºC)	Thermal holding (T_3~T_4, ºC)[d]	Cool-down temperature (T_5, ºC)	Cool-down time (min)
0	1.2 ± 0.1[b]	57.2 ± 1.1	72.2 ± 0.7	105.3 ± 0.1	5.9 ± 1.4	2.9 ± 0.3
2	1.2 ± 0.2	58.2 ± 0.5	67.0 ± 1.1	105.4 ± 0.1	4.8 ± 0.5	3.0 ± 0.4
5	1.2 ± 0.2	56.1 ± 0.1	66.6 ± 0.3	105.3 ± 0.2	7.1 ± 1.3	2.8 ± 0.4

[b] Data presented are means ± standard deviation of three independent trials.
[d] Temperature of oil bath was maintained at 105°C.

Table 4.1. Temperature histories at different stages of pre-processing and pressure-assisted thermal processing for samples processed at 700 MPa and 105ºC (A) and during thermal processing at 105ºC and 0.1 MPa (B).

Holding time (min)	Spore germination (%)		
	Acetic acid	Citric acid	Lactic acid
0	38 ± 2.0 [a]	44 ± 7.3	33 ± 2.6
1	60 ± 29	74 ± 8.3	64 ± 27
2	80 ± 8.4	75 ± 8.3	Nd [b]

[a] Data presented are means ± standard deviation from at least two independent trials.
[b] Nd = not detectable.

Table 4.2. Percent germination in the surviving *Bacillus amyloliquefaciens* spore populations in the presence of organic acids after 700 MPa-105°C treatment for various pressure holding times.

Treatment	Log MPN/mL*			
	2 days	5 days	10 days	15 days
PATP for 3 min:				
- Deionized water	2.1 ± 0.27 Aa	2.4 ± 0.29 Aa	2.4 ± 0.20 Aa	2.4 ± 0.20 Aa
- Acetic acid	0.90 ± 0.50 Ba	1.0 ± 0.36 Ba	1.0 ± 0.36 Ba	1.2 ± 0.29 Ba
- Citric acid	0.63 ± 0.20 Ba	0.63 ± 0.20 Ba	0.63 ± 0.20 Ba	0.63 ± 0.20 Ba
- Lactic acid	< 0.48 ** Ba	< 0.48 ** Ba	0.50 ± 0.05 Ba	0.50 ± 0.05 Ba
PATP for 5 min:				
- Deionized water	0.79 ± 0.47 Ba	0.92 ± 0.42 Ba	0.92 ± 0.42 Ba	0.92 ± 0.42 Ba
- Acetic acid	0.96 ± 0.45 Ba	0.96 ± 0.45 Ba	0.96 ± 0.45 Ba	0.96 ± 0.45 Ba
- Citric acid	0.69 ± 0.31 Ba	0.69 ± 0.31 Ba	0.69 ± 0.31 Ba	0.69 ± 0.31 Ba
- Lactic acid	< 0.48 ** Ba	< 0.48 ** Ba	< 0.48 ** Ba	< 0.48 ** Ba

* MPN tubes were incubated at 32°C. Recovery of injured spores was monitored throughout 15 days.

** Numbers are below method's detection limit (≤ 0.48 log MPN/mL).

A-B Means ± SD: different superscript within a column represents significant difference among suspension media ($P < 0.05$).

a Means ± SD: different superscript within a row represents significant difference among incubation times ($P < 0.05$).

Table 4.3. Survivors of *Bacillus amyloliquefaciens* spores that were suspended in food-grade organic acids, PATP-processed for 3 and 5 min at 700 MPa and 105°C, and enumerated following a heat-shock treatment (80°C for 15 min).

(A) 700 MPa-105°C-5min

Storage (days)	After PATP treatment (Germinated + Dormant spores)		After PATP treatment following heat shock (Dormant spores only)	
	Citric acid	Lactic acid	Citric acid	Lactic acid
0	1.2 ± 0.12^{AB}	0.90 ± 0.44^{AB}	1.3 ± 0.33^{AB}	0.80 ± 0.49^{AB}
2	1.6 ± 0.40^{AB}	1.5 ± 0.66^{AB}	1.5 ± 0.14^{AB}	1.8 ± 0.41^{AB}
7	1.9 ± 0.30^{AB}	1.7 ± 0.62^{AB}	2.2 ± 0.50^{A}	1.8 ± 0.37^{AB}
14	0.50 ± 0.04^{B}	1.1 ± 0.72^{AB}	1.4 ± 0.52^{AB}	2.0 ± 0.54^{A}
21	$0.48^{**\ B}$	1.1 ± 0.77^{AB}	2.1 ± 0.78^{AB}	2.2 ± 0.36^{A}
28	1.1 ± 0.54^{AB}	0.75 ± 0.34^{AB}	1.7 ± 0.79^{AB}	0.94 ± 0.81^{AB}

(B) 700 MPa-105°C-15min

Storage (days)	After PATP treatment (Germinated + Dormant spores)		After PATP treatment following heat shock (Dormant spores only)	
	Citric	Lactic	Citric	Lactic
0	0.93 ± 0.06^{C}	0.99 ± 0.05^{BC}	1.2 ± 0.60^{BC}	1.2 ± 0.58^{ABC}
2	1.7 ± 0.64^{ABC}	1.5 ± 0.73^{ABC}	2.0 ± 0.38^{ABC}	2.5 ± 0.14^{AB}
7	1.5 ± 0.78^{ABC}	1.8 ± 0.41^{ABC}	2.7 ± 0.33^{A}	2.0 ± 0.87^{ABC}
14	0.64 ± 0.28^{C}	0.66 ± 0.26^{C}	1.7 ± 0.65^{ABC}	1.5 ± 0.66^{ABC}
21	0.96 ± 0.20^{BC}	1.0 ± 0.84^{BC}	0.84 ± 0.35^{C}	1.1 ± 0.77^{BC}
28	1.1 ± 0.26^{BC}	0.64 ± 0.28^{C}	1.3 ± 0.82^{ABC}	0.98 ± 0.19^{BC}

[*] Treated samples were stored at 32°C, MPN tubes were incubated for 5 days at 32°C.
[**] Numbers are below method's detection limit (≤ 0.48 log MPN/mL).
[A-C] Means ± SD: different superscript represents significant difference among means ($P < 0.05$).

Table 4.4. Recovery (log MPN/mL) of *Bacillus amyloliquefaciens* spores, inoculated in carrot puree, during 28-day storage after PATP treatment in combination with organic acids.

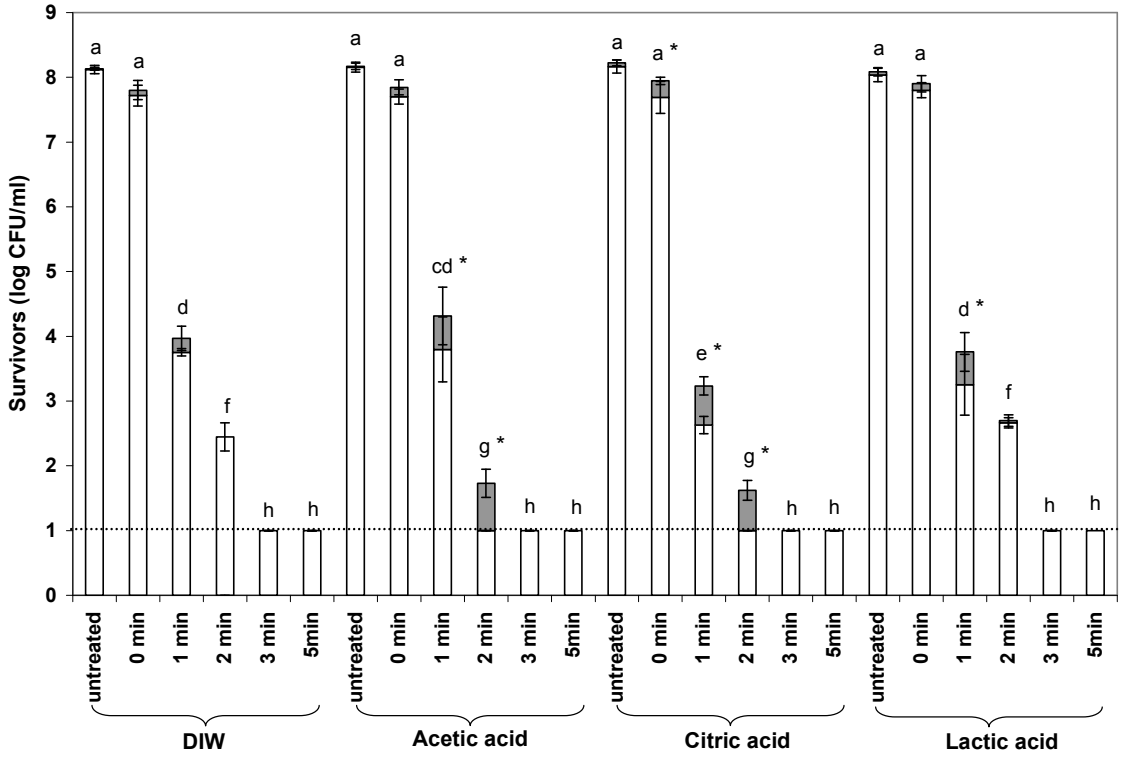

Figure 4.1. *Bacillus amyloliquefaciens* spore survivors (log CFU/mL) inoculated in food-grade organic acids (acetic, citric, and lactic) and treated at 700 MPa and 105°C. Spores inoculated in deionized water served as control. (☐) Dormant spores and (■) germinated spores. Initial inocula were 8.1 ± 0.05 log CFU/mL. The horizontal dotted line indicates the detection limit (10 CFU/mL). Different letters represents significant difference among means at $P < 0.05$. An asterisk (*) indicates statistically significant spore germination.

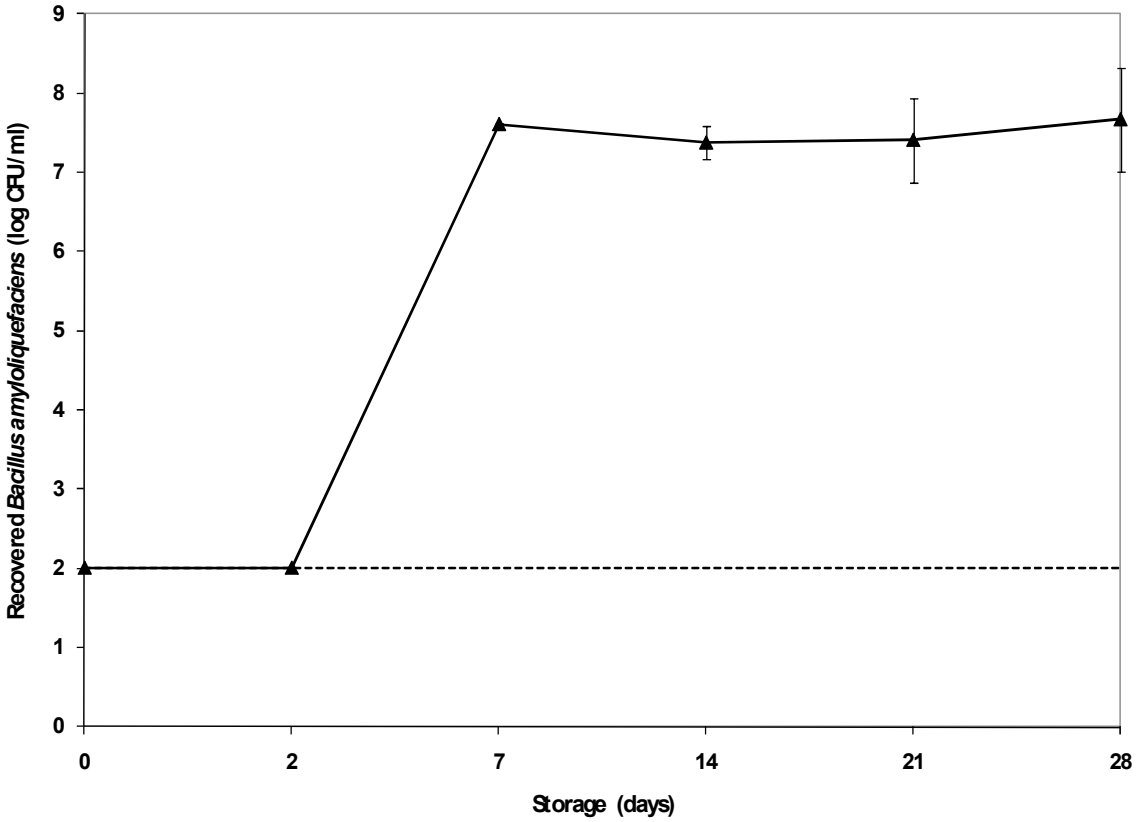

Figure 4.2. Recovery of *Bacillus amyloliquefaciens* on Trypticase soy agar supplemented with 0.6% yeast extract during various storage times at 32°C. The inoculated spore-carrot puree samples were subjected to PATP treatment (700 MPa-105°C at either 5 or 15 min) and then stored at 32°C before enumeration. —▲— (PATP 5 min); ------- (method's detection limit = 100 CFU/mL) PATP 15 min-treated samples remained under detection limit throughout storage time.

Chapter 5: Fourier-transform infrared microspectroscopy and multivariate analysis of *Bacillus amyloliquefaciens* spore inactivation during pressure-assisted thermal processing

Abstract

Changes in spore composition of *Bacillus amyloliquefaciens* grown in different sporulation media (TSAYE and NAYE) as influenced by pressure-assisted thermal processing (PATP), high pressure processing (HPP), and thermal processing (TP) were investigated using Fourier-transform infrared spectroscopy (FT-IR). Additional experiments were carried out to compare and contrast the biochemical changes during single- and double-pulse PATP treatments with equivalent holding times. FT-IR spectra discriminated spore samples grown in two sporulation media as well as treated by different processing techniques (PATP, HPP, and TP). PATP and TP treatments caused major changes in calcium dipicolinate (CaDPA) structures as determined by FT-IR bands at 1381, 1415, and 1442 cm^{-1}. These bands corresponds to the contribution of COO^- vibration of CaDPA chelate, the interaction of Ca^{2+} with COO^- group, and pyridine ring vibration of DPA (dipicolinic acid), respectively. Ratio of peak heights at 1381 cm^{-1} and 1442 cm^{-1} indicated the higher amount of CaDPA release by the double-pulse treatment. In addition, impact on amide bands (1540-1650 cm^{-1}) of protein were detected in TP and PATP treated samples. While FT-IR spectra were able to predict microbiological lethality

for PATP (single-pulse) and TP treatments, it did not predict lethality changes during double-pulse PATP treatment. This may be possibly due to differences in mechanism of inactivation during single- and double-pulse treatment.

5.1 Introduction

Consumers demand minimally processed shelf-stable low-acid foods, with better color, texture, appearance, nutritional values, with minimal or no preservatives. During traditional thermal processing, the product is heated by conduction or convection process and the severity of thermal treatment adversely degrade product quality and destroy heat sensitive ingredients. The food industry is investigating alternative sterilization technologies that can meet consumer demand for minimally processed safe low-acid food products. Among the alternative sterilization technologies investigated, pressure-assisted thermal processing (PATP) gained industrial interest in the recent years. It has a potential for manufacturing shelf-stable low-acid foods such as soups, mashed potatoes, coffee, and tea (Balasubramaniam et al., 2008). In February 2009, its application for sterilization of low-acid shelf-stable products was approved by U.S. Food and Drug Administration (FDA) (Anonymous, 2009). The process preserves low-acid foods by using a combination of pressure (500 to 700 MPa) and temperature (90 to 121ºC) over a short holding time (<10 min). One of the unique advantages of this technology is its ability to provide rapid and uniform temperature increase in the treated food as a result from heat of compression. Expansion cooling also occurs upon depressurization. This limits severity of thermal effect and provides superior product quality.

Application of pressure treatment at ambient temperature effectively inactivates variety of vegetative pathogenic and spoilage microorganisms. On the other hand, pressure in combination with heat is needed for bacterial spore inactivation. Number of earlier studies documented the effectiveness of PATP on inactivation of various bacterial spores including *Bacillus subtilis*, *B. amyloliquefaciens*, *Clostridium botulinum*, *C. sporogenes*, and *Geobacillus stearothermophilus* (Margosch et al., 2006; Reddy et al., 2006; Ahn et al., 2007; Zhu et al., 2008; Akhtar et al., 2009; Bull et al., 2009). Pressure pulsing or cycling reported to further enhance spore inactivation (Hayakawa et al., 1994; Meyer et al., 2000). Ratphitagsanti et al. (2009) reported that spore inactivation of *B. amyloliquefaciens* TMW 2.479 Fad 82 was enhanced by 2 to 4 log CFU/mL when double-pulse treatment was employed over single-pulse treatment at 600 MPa-105ºC for equivalent holding times. Very limited studies have been conducted to evaluate the mechanistic factors contributing to enhanced lethality during double-pulse treatment.

Advances in FT-IR spectroscopic instrumentation and multivariate analysis provide capability for rapid detection, identification, and characterization of spoilage and pathogenic microorganisms. FT-IR spectroscopy allows the simultaneous data collection from all frequencies, thus improving its sensitivity. Other advantages of FT-IR spectroscopy include simplicity, rapidity, and high throughput (Naumann 2000). Specific spectral patterns based on chemical and biological composition of samples are obtained and these unique biochemical fingerprints are the key for discrimination and identification among different biological specimen. The FT-IR microspectroscopy (FT-IR coupled with microscope) is effective to detect, identify, and classify various

microorganisms at strain levels (Perkins et al., 2005; Schiza et al., 2005; Ngo Thi and Naumann, 2007; Brooke et al., 2008). Subramanian et al. (2006) employed attenuated total reflectance (ATR)-IR spectroscopy as an analytical tool to predict the viable spore counts after thermal and PATP treatments with correlation coefficient (r) of > 0.99 and standard errors of cross-validation ($10^{0.2}$ – $10^{0.5}$ CFU/mL). The authors also found correlation between PATP spore resistance and calcium dipicolinate (CaDPA) content of the spores when five different bacterial strains were investigated (Subramanian et al., 2007).

The objective of this study was to employ ATR-FTIR microspectroscopy to differentiate biochemical changes of bacterial spores during the inactivation by pressure-assisted thermal processing (especially single- and double-pulse treatments), high pressure processing, and thermal processing.

5.2 Materials and methods

5.2.1 Spore production

Culture of *B. amyloliquefaciens* TMW 2.479 Fad 82 was provided by M. Gänzle (Department of Agricultural, Food and Nutritional Science, University of Alberta, Edmonton, Alberta, Canada). Activation and isolation of the bacterial culture was done by the 3-phase-streak plate method on Trypticase soy agar supplemented with 0.6% yeast extract (TSAYE; Becton, Dickinson and Company, Sparks, MD). The plate was then incubated in an aerobic condition at 32°C for 24 h. An isolated single colony was selected and transferred to a test tube containing Trypticase soy broth supplemented with

0.6% yeast extract (TSBYE; Becton, Dickinson and Company). The spore crops were grown in two different sporulation media (Table 5.1). The first batch of the spore crop was prepared by spread-plating the 100 µL portions of *B. amyloliquefaciens* culture on TSAYE supplemented with 10 ppm $MnSO_4 \cdot H_2O$ (Fisher Scientific, Pittsburgh, PA) (Ahn et al., 2007; Ratphitagsanti et al., 2009). The second spore crop was grown on nutrient agar supplemented with 0.6% yeast extract (NAYE; Becton, Dickinson and Company) and 10 ppm $MnSO_4 \cdot H_2O$ (adapted from Mazas et al., 1995 and Cazemier et al., 2001). The inoculated plates on TSAYE were aerobically incubated at 32°C for 10-14 days, whereas those on NAYE were incubated for 3-5 days to obtain 95% sporulated population. The sporulation was verified by using a phase-contrast microscopy. The surface of inoculated plates was flooded with 10 mL of cold sterile deionized water and the spores were scraped with disposable plastic spreaders. The spore suspension was washed five times by differential centrifugation that ranged from 2,000 to 8,000 x *g* for 20 min each at 4°C. Spore pellets were resuspended in sterile deionized water to obtain ~10^9 spores/mL inocula. The suspension was sonicated for 10 min (SM275HT, Crest, ETL Testing Laboratory, Cortland, NY) following by heat treatment at 80°C for 15 min to destroy any remaining vegetative cells. Both spore suspensions were stored in a 4°C refrigerator.

5.2.2 Sample preparation for PATP, HPP, or TP treatment

For PATP and HPP experiments, spore suspensions (0.2 mL) were inoculated in deionized water (1.8 mL) and packaged in sterile polyethylene pouches (5cm x 2.5cm, 01-002-57, Fisher Scientific) at inoculum levels of ~1.6×10^8 CFU/mL for TSAYE crop

and ~2.5x10^8 CFU/mL for NAYE crop. The pouches were then heat-sealed by an impulse heat sealer (American International Electric, Whittier, CA). After manually mixed the sample content, all pouches were submersed into an ice-water bath (4°C). Samples were treated within 2 h after preparation.

Sample preparation for TP experiments were done by aseptically transferred 0.2 mL spore suspension of *B. amyloliquefaciens* and 1.8 mL sterile deionized water into an aluminum-TDT-tube to obtain the final spore concentration of ~2.3x10^8 and ~3.4x10^8 CFU/mL for TSAYE and NAYE crops, respectively. All aluminum-TDT-tubes containing the spore suspension were kept in an ice-water bath (4°C) up to 2 h before thermal treatments.

5.2.3 Combined pressure-thermal treatment

Combined pressure-thermal experiments were conducted using a microbial kinetic tester (PT-1 test unit, Avure Technology Inc., Kent, WA). The spore samples were treated to various combined pressure-thermal treatment conditions by adapting published methods reported earlier (Ahn et al., 2007; Ratphitagsanti et al., 2009). The come-up time of the PT-1 unit was 0.7 ± 0.1 min. The sample pouch was placed inside a 10-mL polypropylene syringe (model 309604, Becton, Dickinson and Company) wrapped with insulating material (Sports Tape, CVS® Pharmacy Inc., Woonsocket, RI). The rest of the syringe was filled with ~ 8 mL of water to ensure uniform temperature within the syringe during treatment.

For PATP treatments, the syringe containing spore sample was heated to a pre-processing temperature of 58ºC using water bath (Isotemp 928, Fisher Scientific) for 2

min. Preheated syringe was immediately loaded into the PT-1 unit. The pressure chamber was suspended in propylene glycol (Houghto-Safe-620-TY, Houghton International Inc., Troy, MI) bath (maintained at 105.5°C). The propylene glycol was also used as the pressure-transmitting fluid within the PT-1 unit. PATP experiments were conducted at 600 MPa-105°C up to 8 min pressure holding times. Additional experiments were conducted to compare the microbial efficacy of single- and double-pulse treatments at an equivalent pressure holding time of 3 min. During double-pulse treatment, samples were taken for microbial and FT-IR analyses after various stages (C_1, D_1, B_2, C_2, and D_2; Figure 5.1.B). Spore sample pouches subjected to pressure-thermal treatment were immediately immersed into an ice-water bath to cool the samples. Microbial analysis was conducted on the same day of experiments, while FT-IR samples were kept in a refrigerator up to 24 h before the analysis.

High pressure processing experiments (600 MPa-35°C up to 70 min holding time) were also performed by using a similar approach to that of PATP samples. The pre-processing temperature for HPP spore samples was 4.9 ± 0.8°C. An untreated spore suspension of each spore crop was used as controls.

5.2.4 Thermal treatment

Thermal inactivation of spores was conducted at 105°C-0.1 MPa using a 35L heating bath circulator (NESLAB EX-35 Digital One, Thermo Fisher Scientific Inc., Waltham, MA). Bath oil (Temperature range: -7 to 176°C, O220, Fisher Scientific) was used as the heating fluid. The aluminum-TDT-tubes containing spore samples were pre-heated for 2 min at 58°C water bath before further subjected to 105°C oil bath. Sample

temperature was monitored by inserting a K-type thermocouple attached to a data logger into a control aluminum cell containing sterile deionized water without spores. The thermal come-up time to reach 105°C was 2.3 min. The tubes were removed from the oil bath after the come-up time and different holding times (up to 240 min) and then immediately immersed into an ice-water bath. Standard plate count method was used to enumerate spores surviving the thermal treatment as well as untreated spore suspensions on the same day of processing. Samples for FT-IR analysis were kept in a refrigerator and the FT-IR analysis was carried out within 24 h after thermal treatments.

5.2.5 Enumeration of spore survivors

Spore samples grown on different sporulation media (TSAYE and NAYE supplemented with 10 ppm $MnSO_4$) and processed by various treatments were enumerated as follows. Sample contents (1 mL) were serially diluted in 0.1% peptone water and then spread-plated in duplicate on TSAYE. After incubation at 32°C up to 72 h, the viable count of spore survivors were enumerated.

5.2.6 Fourier-transform infrared microspectroscopy

FT-IR analysis of treated spore samples was modified from Subramanian et al. (2006) and Männig et al. (2008). Aliquots (500 μL) were centrifuged at 13,000 rpm and 4°C for 4.5 min. After removing the supernatant, the spore pellet was washed with 100 μL sterile deionized water and re-centrifuged at the same condition. The pellet was then re-suspended with 5 μL sterile deionized water, applied onto hydrophobic Neo-grid membrane filters (HGMFs; Neogen Corporation, Lansing, MI). The membrane filters with deposited spores were then vacuum-dried to form a thin film. Infrared spectroscopic

studies were carried out by using an Excalibur 3500GX FT-IR microscopy spectrometer (Varian, Palo Alto, CA) in Attenuated Total Reflectance (ATR) mode. The spectra were collected from the wavenumbers of 4000 to 700 cm^{-1} (mid-infrared) region with a total of 128 scans at a resolution of 8 cm^{-1}. The FT-IR spectrometer was equipped with a PERMAGLOW mid-IR source, an extended-range KBr beam splitter, and a deuterated triglycine sulfate detector. Aliquots from each treated sample were applied onto three individual spots on the membrane and two measurements were taken at different locations on each spot. At least two to five independent replications of PATP, HPP, and TP treatments were carried out resulting in 12-30 spectra per sample per treatment time (six spectra per replication).

5.2.7 Multivariate analyses

Pirouette® (version 3.11, Infometrix Inc., Woodville, WA) comprehensive chemometrics modeling software was employed to transform the spectra to their 2^{nd} derivatives using a Savitzky-Golay polynomial filter (five-point window), mean-centered, and vector-length normalized. Classification analysis of samples processed at various conditions was further analyzed using principal component analysis algorithm (soft independent modeling by class analogy; SIMCA). Principal component analysis extracts information from the data set onto few dimensions, which are accounted for maximum possible variance (Mark, 2001). The spectral wavenumbers and their associated functional groups responsible for the classification of the spores could be identified using the discriminating power plot based on the measure of variable importance by minimizing the difference between samples within clusters and maximizing those from

different clusters (Dunn and Wold, 1995). Correlation between specific spectral information (900 – 1800 cm^{-1} region) and spore survivors (obtaining from the standard plate count) were determined using partial least squares regression (PLSR), which utilized large number of dependent variables to predict the viable spores surviving the treatments. A nonlinear iterative partial least-squares (NIPALS) algorithm was employed. PLSR with cross-validation (iterative recalculation of the model omitting a different sample point each time) was used to test for the model sensitivity.

5.2.8 Statistical analysis

Statistical Analysis System software (SAS 9.1, SAS Institute Inc., Cary, NC) was used for data analysis. Independent variables were treatment (PATP single- and double-pulse, TP, and HPP), holding time, and sporulation media (TSAYE and NAYE), whereas log reduction (Δ log CFU/mL) in response to the treatments served as the dependent variable. The data was analyzed by the SAS program with the general linear model (GLM) procedure. The mean comparison were evaluated with the Tukey's test at a 5% significant level (P = 0.05).

5.3 Results and discussion

5.3.1 Pressure and temperature history during PATP pulsing treatments and spore inactivation

Figure 5.1 provides sample pressure and temperature history of single- and double-pulse treatment at 600 MPa-105°C for 3 min holding time. Process temperature of the single-pulse treatment was well maintained at 105°C as the external glycol bath was

heated to the same temperature. Even though both single- and double-pulse treatments had an equivalent time of 3 min under pressure, double-pulse treatment had higher total treatment time (~ 5.4 min) which included the pause time between two pulses (1 min) as well as an additional pressurization time for the 2nd pulse (0.7 min). This longer treatment time during double-pulse may also lead to higher process temperature values during the 2nd pulse. For example, during the 1st pulse holding time (1.5 min), the process temperature (105.5 ± 0.4°C) was maintained. Upon depressurization of the 1st pulse, the temperature dropped to ~77°C. Subsequently during the pause time between pulses (D_1-B_2), spore sample temperature increased to ~78°C due to heat transfer from the surrounding glycol bath which was kept at 105.5°C. This resulted in higher process temperature of 112 ± 0.9°C during the 2nd pulse holding time (C_2-D_2). It is further worth to note that the temperature history obtained during the double-pulse treatment likely further influenced by pressure equipment design parameters (such as chamber volume, pressurization rate, chamber insulation characteristics, etc.). The current study utilized a pressure chamber volume of ~20 mL and had relatively faster pressurization rate (~14 MPa/s). Accordingly, care must be taken in extrapolating the results of this study to larger pilot scale equipments.

At the equivalent 3 min-holding time, enhanced spore inactivation were observed in the double-pulse treatment (Table 5.2). Spores grown from TSAYE and NAYE media provided additional 2.6 log reduction from the double-pulse treatment than that from the single-pulse PATP treatment (600 MPa at 105°C for 3 min). It is worth to note that the majority of the spore inactivation during double-pulse treatment (Table 5.2) took place

during the 2nd pulse holding time where the spores were subjected to 600 MPa-112°C treatment for 1.5 min.

5.3.2 Combined pressure-thermal resistance influenced by sporulation media

Among the two sporulation media, differences in spore resistant property were observed. Spore crops grown on NAYE produced higher PATP and TP resistant spores. $D_{105°C\text{-}0.1\ MPa}$ values of the spore crop grown on TSAYE and NAYE were 28.1 ± 1.2 min and 36.8 ± 1.5 min, respectively. Similarly, D values of PATP treated spores grown on TSAYE and NAYE media were 1.0 ± 0.1 min and 1.4 ± 0.2 min at 600 MPa-105°C.

It has been well-documented that sporulation media impact spore heat resistance (Cazemier et al., 2001; Mah et al., 2008). Figure 5.2.A shows that FT-IR could differentiate the two spore crops based on their resistant properties as influenced by different sporulation media. The corresponding peaks differentiating the properties among these two spore crops were found at 1388 and 1577 cm^{-1} from the discriminating power plot (data not shown). These bands are associated to the stretching bands of COO^- group of CaDPA chelate and the C-N vibrations of the pyridine ring, respectively. The discriminating power is a measure of variable importance (i.e., IR frequency), which contributes to the development of the SIMCA pattern recognition and classification (Dunn and Wold, 1995). Different levels of CaDPA chelate might be inherited in the spore cores during sporulation. Moreover, FT-IR could also be used to ensure consistency and reliability of pressure-thermal resistance of each new spore crop. Differences in resistant property among TSAYE grown spores from various spore crop preparations

were observed (Figure 5.2.B). This may facilitate the evaluation of process resistance of the untreated spore crops before being treated.

5.3.3 Classification of bacterial spores treated by thermal and pressure-assisted thermal processing

Hydrophobic grid membrane filters (HGMFs) overlaid on a selective medium was previously used to isolate a single colony of *Salmonella* serovars (Männig et al., 2008). In this current study, a protocol was developed for the classification of bacterial spores treated by various processing methods by combining hydrophobic grid membrane filtration with infrared spectroscopy (Subramanian et al., 2006). Use of the HGMFs enabled direct spectroscopic observation of the biochemical changes in treated spores. The membrane confined spores within the barriers of the grid, while vacuum drying helps to limit the interference from water absorption bands. This sample preparation method was simple and improved signal intensity.

Figure 5.3.A and 5.3.B provides a sample SIMCA model illustrating consecutive changes in PATP and TP treated bacterial spores. FT-IR spectroscopy was clearly able to discriminate changes in untreated spore samples against that of PATP or TP treated samples. In TP treated samples, the biochemical changes gradually occurred over 240 min holding time (Figure 5.3.B). Based on interclass distance (≤ 3), the treated samples with similar changes in biochemical properties were grouped together resulting various distinct clusters (Figure 5.3.A and 5.3.B). Interclass distances are Euclidian distances between centers of clusters, which could be used as an indicator in SIMCA classification model (Kvalheim and Karstang, 1992). In general, large interclass distances (above 3)

demonstrate well separation among the classes. PATP resulted in rapid spore inactivation over a short time. Similarity in biochemical composition of the treated spores was observed after 2 min-PATP treatment as indicated by the same cluster containing the treated samples from 2-8 min holding times (Figure 5.3.A). As expected, pressure alone (600 MPa-35^{o}C, up to 70 min) did not clearly distinguish the untreated and treated spore samples into groups since *B. amyloliquefaciens* spores were not inactivated at this condition (data not shown).

5.3.4 Biochemical changes associated with PATP treated spores

The spectral wavenumbers and the associated functional groups that were responsible for the classification of the spores in SIMCA class projections were identified using the discriminating power plot (Figure 5.4). The higher the value of the discriminating power, the greater is the influence of that wavenumber in classifying the samples that are in the model (Lavine, 2000). In comparison to untreated control, PATP caused predominant changes in the region of 1384, 1415, 1446 cm^{-1} in both TSAYE and NAYE grown spores. The identified bands represent changes in dipicolinic acid (pyridine-2,6-dicarboxylic acid; DPA) structure, especially the contribution of COO^{-} vibration of CaDPA chelate (1384 cm^{-1}), the COO^{-} stretching vibration in the presence of Ca^{2+} (1415 cm^{-1}), and the DPA pyridine ring vibration (1442-1446 cm^{-1}) (Byler and Farrell, 1989; Cheung et al., 1999; Goodacre et al., 2000; Perkins et al., 2005). DPA is always chelated with divalent cations, especially calcium ions in a 1:1 ratio as CaDPA. This unique component is only present in bacterial spores and it represents about 5-10% of the dry weight of *Bacillus* spores (Setlow et al., 2006).

Highly resistant PATP-treated NAYE grown spores showed additional discriminating power peaks at 1350 cm^{-1} (absorption due to lipids) and 1577 cm^{-1} (C-N vibrations of the DPA pyridine ring or COO^- group of acidic amino acid residues of spore proteins) (Cheung et al., 1999; Wolfangel et al., 1999; Sahu et al., 2006) (Figure 5.4). NAYE grown spores also caused changes in the protein region, specifically at 1635 cm^{-1} (amide I of β-pleated sheet of secondary proteins), 1543 cm^{-1} (amide II involved stretching vibration of C-N groups), and 1273 cm^{-1} (amide III band of proteins or CaDPA band) (Helm and Naumann, 1995; Cheung et al., 1999; Schiza et al., 2005).

Table 5.3 presents discriminating power values at selected CaDPA bands (~1377-1384, ~1411-1415, and ~1438-1446 cm^{-1}) and the corresponding log reduction of bacterial spores grown in different sporulation media during PATP, TP, and HPP. In general, discriminating power values increased with increase in holding times, within the same treatment condition and sporulation media. Although this observation appeared to hold good in most cases, the variation in the band intensity during holding times was also observed. This might be due to the heterogeneity of spore populations as well as variability in pressure-thermal histories during PATP and TP replications.

5.3.5 Contrast between single- and double-pulse PATP treatments

Figure 5.5 compares the discriminating power in classification of bacterial spores grown in TSAYE and NAYE media and subjected to single- and double-pulse PATP treatments. In general, regardless of the growth media, both single- and double-pulse treatments showed similar changes on the bands associated to CaDPA chelate, in particular at 1377-1381, 1411, and 1435-1442 cm^{-1} (Figure 5.5). However, single-pulse

treatment produced significantly higher discriminating power values than double-pulse treatment. According to Cheung et al. (1999), the ratio of peak heights at wavenumbers 1379 and 1443 cm^{-1} could be used as an indicator of the CaDPA levels in bacterial spores. The higher the ratio, the more the CaDPA exists in the spores. Presence of high levels of CaDPA chelate, the relatively low content of core water, and the saturation of spore DNA with a group of small acid-soluble proteins (SASPs) play major roles in spore resistance properties (Setlow and Setlow, 1995). Specifically, the DNA-α/β-type SASP complex was the primary contributor to spore thermal stabilities, while divalent cations (Ca^{2+}, Mn^{2+}, Mg^{2+}) and DPA provide synergistic effect on stability protecting the spore DNA against heat (Setlow et al., 2006). Both sporulation media demonstrated higher ratio of peak height associated to COO^- vibration of CaDPA (~1377-1381 cm^{-1}) and pyridine ring vibration (~1435-1442 cm^{-1}) in the single-pulse treatment. The peak ratios of single- and double-pulse treatments were 3.0 and 2.5 for TSAYE grown spores, as well as 1.5 and 1.2 for NAYE grown spores, respectively. The ratio suggested less CaDPA release from the single-pulse treatment than that of the double-pulse treatment, indicating less lethality at the equivalent holding time.

5.3.6 Biochemical changes associated with TP treated spores

At 105°C-0.1 MPa, it took more than 70 min to obtain any measurable spore inactivation (1.1-2.5 log reduction) in both spore crops. After 180 min-holding time at 105°C, TSAYE grown spores were inactivated to undetectable level and NAYE grown spores had about 4.7 log reduction. FT-IR analysis of the TP spores indicated that discrimination was largely influenced by the similar bands observed from the PATP

treatment, specifically at 1384, 1411, and 1442 cm^{-1} (Figure 5.6). Biochemical changes among particular samples could be gradually monitored (data not shown). Three distinct groups of PATP treated samples were classified based on their interclass distance. Samples were grouped together when the interclass distance was lower than 3. Differences observed between the untreated control and the 1st group of treated samples (0, 2, 5 min) was mainly associated with the interaction of the Ca^{2+} with COO^- groups and the stretching of COO^- group of CaDPA chelate (1419 and 1381 cm^{-1}). When comparing changes between the 1st group (0, 2, 5 min) and the 2nd group (30, 70, 120 min), it was evident that bands associated with clustering of samples were related to the DPA pyridine ring vibration (~ 1446 cm^{-1}), which became very prominent for the discrimination of the 2nd group (30, 70, 120 min) from the 3rd group (180, 240 min) (data not shown).

Figure 5.7 demonstrates the influence of specific PATP and TP treatments that yield comparable 3 log reduction to *B. amyloliquefaciens* TMW 2.479 spores (NAYE grown). It required at least 120 min at 105°C-0.1 MPa to achieve ~ 3 log reduction, while 5 min-holding time PATP treatment (600 MPa-105°C) provided similar result. Both PATP and TP specifically acted upon CaDPA chelate (1276, 1373, 1411, and 1612 cm^{-1}) and its pyridine ring (1438 and 1573 cm^{-1}) (Figure 5.7). FT-IR results supported that there were similar changes happening in the structural level of DPA and CaDPA among the two treatments at the same lethality. However, the bands obtained from PATP were clearly much higher in discriminating power values (~ 45,000 arbitrary units) than that

from TP treatment (~ 14,000 arbitrary units). This indicated that the biochemical changes of bacterial spores taking place during PATP was at the greater intensity than TP.

5.3.7 Quantification of spore survivors from infrared spectra and validation of PLSR models

Surviving spore populations of *B. amyloliquefaciens* TMW 2.479 after PATP and TP treatments could be estimated by cross-validated PLSR models using spectral region (900 to 1800 cm^{-1}) (Figure 5.8 - 5.9). The leave-one-out cross validation generally removes one sample from the training set, performed PLSR on the remaining samples. Then, it predicts the log spore survivors from the left-out sample and sums up the error until the total samples in the training set is analyzed. Good correlation on spore survivors obtained from the standard plate count and the mid-infrared spectral regions was found for both PATP and TP treatments (Figure 5.8 - 5.9). High coefficients of correlation ($r > 0.96$) and low standard errors of cross-validation (SECV ~ $10^{0.16}$ – $10^{0.26}$ CFU/mL) were obtained from all PLSR models.

To verify whether or not single- and double-pulse treatments followed similar mechanism of inactivation, the developed PLSR model based on single-pulse PATP treatment was used to predict the spore survivors during various stages of the double-pulse treatment. Figure 5.10 shows the predicted spore survivors by FT-IR spectra and the experimental values obtained from the standard plate count during each stage of double-pulse treatment of *B. amyloliquefaciens* TMW 2.479 spores grown on different sporulation media. FT-IR spectra microbial lethality model based on single-pulse could not predict double-pulse lethality changes during 2^{nd} pulse pressure holding time (D_2),

possibly due to differences in respective spore inactivation mechanisms. It is further possible that some of the biochemical changes were not detected during double-pulse treatment by FT-IR microspectroscopy. The single-pulse based PATP lethality model was only successful in predicting (< 0.6 log CFU/mL) up to C_2 (the 2nd pulse come-up time; Figure 5.1.B and Figure 5.10). More studies are needed using advanced high resolution spectroscopic techniques such as Raman to further understand the nature of biochemical changes under these conditions.

5.4 Conclusions

A study was conducted to investigate PATP, HPP, and TP treatment effects on biochemical changes of *B. amyloliquiefaciens* TMW 2.479 spores. Both PATP and TP caused rise of CaDPa bands at approximately 1384, 1415, and 1442 cm^{-1}. The intensity of these bands in general increased with increasing treatment times. For an equivalent log reduction, PATP showed higher intensities than that of TP. Changes in lipids and polypeptides were also evident in PATP-treated highly resistant NAYE grown spores. Release of CaDPA from the spore core served as a key component indicating the inactivation of *B. amyloliquefaciens* TMW 2.479 spores. FT-IR spectra of the bacterial spores not only influenced by the processing conditions, but also influenced by the sporulation media.

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Media component	Amount (g/L)
Difco™ Tryptic soy agar (TSAYE)	
Pancreatic digest of casein	15
Papaic digest of soybean	5
Sodium chloride	5
Agar	15
Yeast extract	6
Difco™ Nutrient agar (NAYE)	
Beef extract	3
Peptone	5
Agar	15
Yeast extract	6

Both media were supplemented with 10 ppm $MnSO_4$.
Source: Becton, Dickinson and Company (Sparks, MD)

Table 5.1. Approximate formula per liter of media used for sporulation.

Total treatment time*	Log N/No	
	TSAYE grown†	NAYE grown†
Single-pulse		
0.7 min (B-C)	0.2±0.3 ^{A}	0.3±0.1 ^{A}
3.7 min (B-D)	-3.2±0.1 ^{E}	-2.5±0.3 ^{D}
Double-pulse		
0.7 min (B_1-C_1; after 1st pulse come-up time)	0.2±0.3 ^{A}	0.3±0.1 ^{A}
2.2 min (B_1-D_1; after 1st pulse)	-1.5±0.2 ^{BC}	-1.1±0.2 ^{B}
3.2 min (B_1-B_2; after pausing time)	-1.6±0.2 ^{C}	-1.2±0.1 ^{BC}
3.9 min (B_1-C_2; after 2nd pulse come-up time)	-2.2±0.3 ^{D}	-1.6±0.3 ^{C}
5.4 min (B_1-D_2; after 2nd pulse)	-5.8±0.3 ^{G}	-5.1±0.3 ^{F}

* See Figure 5.1.A and 5.1.B for nomenclature. Total processing time includes pressure come-up time, pressure holding time, and depressurization time.

† Initial population of untreated controls: TSAYE - 1.6 x 10^8 CFU/mL; NAYE - 2.5 x 10^8 CFU/mL.

Log N/No was calculated where N representing spore count measured after exposure to the PATP treatment for a specific holding time and No representing initial spore count without the PATP treatment.

Means with the same letter are not significantly different.

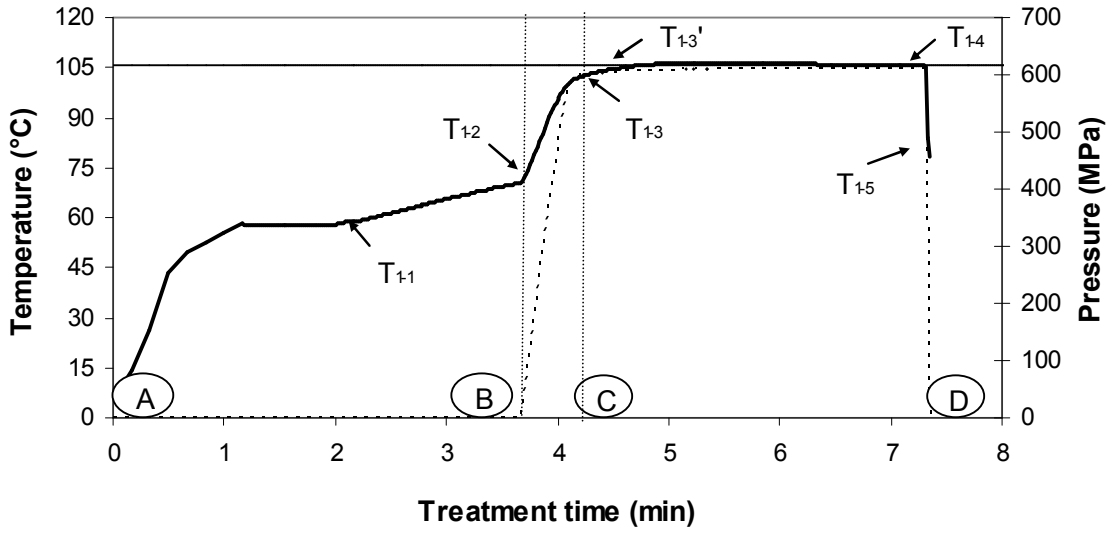
Table 5.2. Log reduction of *Bacillus amyloliquefaciens* TMW 2.479 spores after single- and double-pulse treatments at 3 min equivalent pressure holding time.

Treatment condition and holding time	TSAYE grown spores					NAYE grown spores				
	Wavenumber (cm^{-1})			Ratio of peak height $\frac{1384}{1442}$ cm^{-1}	Log N/No	Wavenumber (cm^{-1})			Ratio of peak height $\frac{1384}{1442}$ cm^{-1}	Log N/No
	~1377-1384	~1411-1415	~1438-1446			~1377-1384	~1411-1415	~1438-1446		
PATP (600 MPa-105°C)										
0 min	67	179	48	1.4	-0.1±0.2 ^{AB}	212	621	8	26	0.1±0.2 ^{AB}
0.5 min	348	1399	645	0.5	-0.7±0.2 ^{BCD}	938	6192	59	16	-0.1±0.2 ^{AB}
1 min	1325	2433	2851	0.5	-1.5±0.2 ^{E}	2216	10829	168	13	-0.9±0.0 ^{CDE}
2 min	1304	3226	2301	0.6	-2.5±0.3 ^{F}	4195	11146	620	7	-1.7±0.0 ^{E}
5 min	2369	4152	3016	0.8	-5.1±0.3 ^{I}	4919	31230	570	9	-3.4±0.2 ^{G}
8 min	1417	2779	2943	0.5	-5.8±0.3 ^{J}	4363	42730	657	7	-5.2±0.1 ^{IJ}
TP (105°C-0.1 MPa)										
0 min	51	260	103	0.5	0.2±0.2 ^{A}	8	20	22	0.3	0.3±0.2 ^{A}
2 min	140	229	184	0.8	0.2±0.0 ^{A}	63	271	241	0.3	0.3±0.2 ^{A}
5 min	1956	1524	969	2.0	0.2±0.2 ^{A}	190	680	309	0.6	0.3±0.1 ^{A}
30 min	608	1335	1411	0.4	-1.1±0.0 ^{DE}	583	2164	1554	0.4	-0.1±0.1 ^{ABC}
70 min	750	2501	2359	0.3	-2.5±0.1 ^{F}	743	2446	2516	0.3	-1.1±0.4 ^{DE}
120 min	1001	2884	1949	0.5	-3.9±0.1 ^{GH}	2641	9643	6334	0.4	-2.6±0.5 ^{F}
180 min	798	6725	4179	0.2	< D.L.	6430	28580	30803	0.2	-4.7±0.4 ^{HI}
240 min	1040	4888	3136	0.3	< D.L.	4571	46611	21886	0.2	-5.1±0.1 ^{IJ}
HPP (600 MPa-35°C)										
0 min	n/a	n/a	n/a	-	-0.1±0.1 ^{AB}	n/a	n/a	n/a	-	0.02±0.0 ^{AB}
5 min	n/a	n/a	n/a	-	0.2±0.0 ^{A}	n/a	n/a	n/a	-	0.1±0.1 ^{AB}
30 min	n/a	n/a	n/a	-	0.2±0.1 ^{A}	n/a	n/a	n/a	-	0.2±0.1 ^{A}
70 min	n/a	n/a	n/a	-	0.03±0.1 ^{AB}	n/a	n/a	n/a	-	0.3±0.1 ^{A}

< D.L. represents spore survivors under method detection limit (< 10^2 CFU/mL).
n/a: data not available. SIMCA could not differentiate the treated samples into distinctive groups.
Means with the same letter are not significantly different.

Table 5.3. Discriminating power values at bands associated to CaDPA structures and log N/No corresponding to treatments.

(A)



(B)

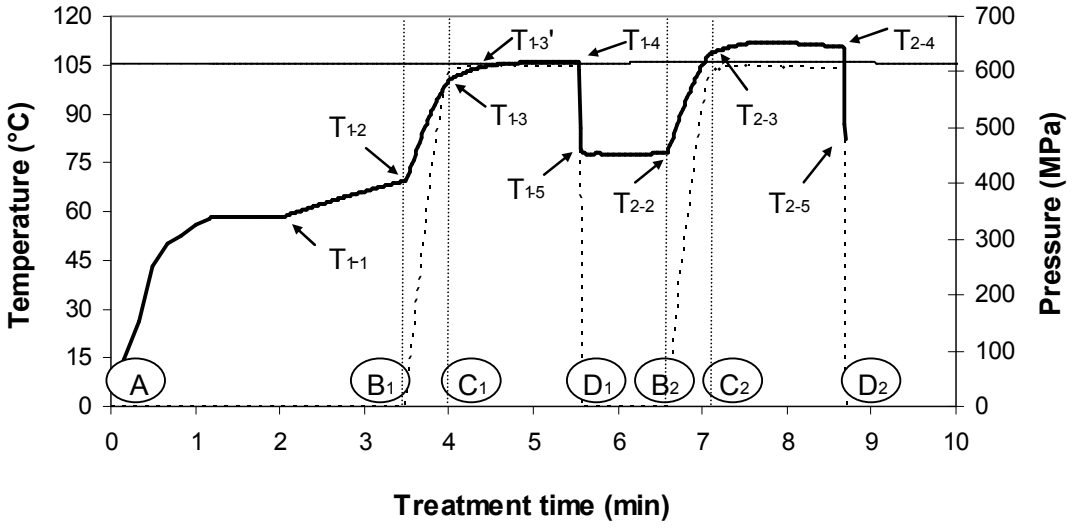
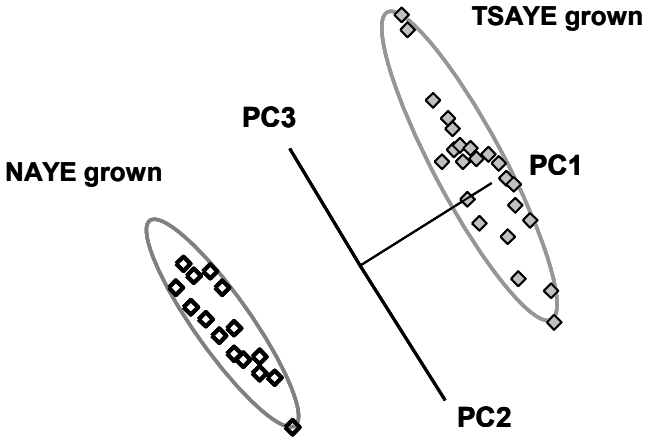


Figure 5.1. Sample pressure (----------) and temperature (———) histories during single- (A) and double-pulse (B) treatment. Processing times include pre-process time in a conditioning bath and pressure chamber (A-B_1), pressure come-up time (B_1-C_1 and B_2-C_2), pressure holding time (C_1-D_1 and C_2-D_2), and depressurization time (< 2 s). Glycol bath temperature was maintained at 105.5°C. Time pausing between two pulses (D_1-B_2) was kept at 1 min.

(A)



(B)

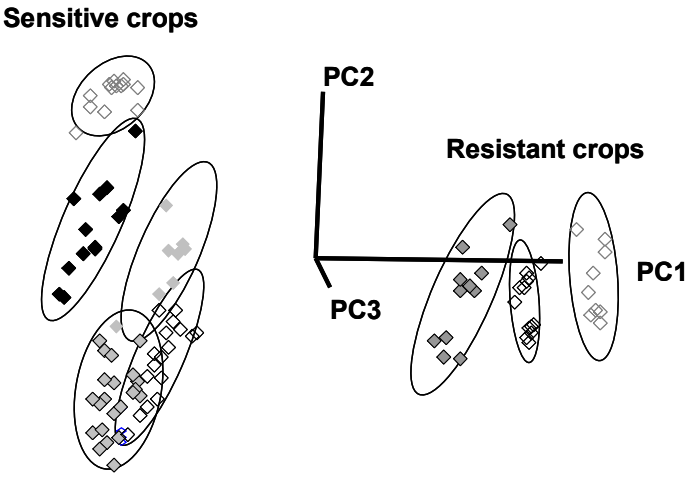
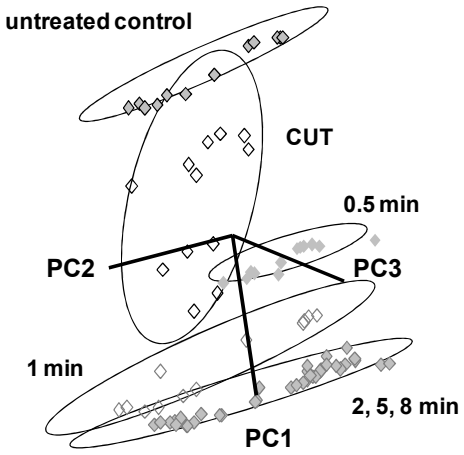


Figure 5.2. Soft independent modeling by class analogy on resistant property of untreated *Bacillus amyloliquefaciens* TMW 2.479 spores as influenced by different sporulation media (A) and different spore crop preparations using TSAYE as a sporulation medium (B).

(A)



(B)

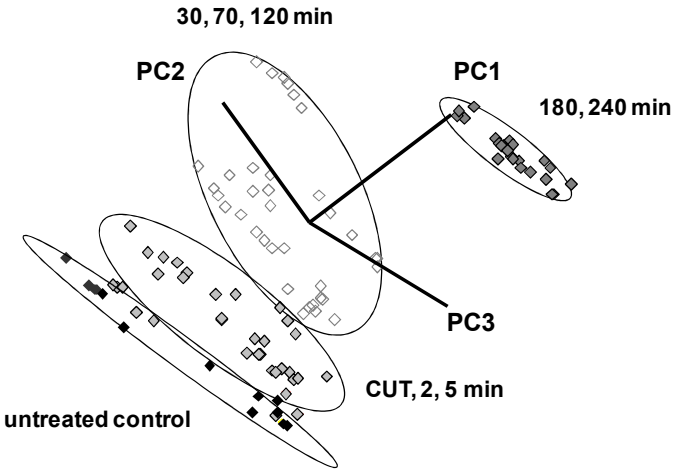


Figure 5.3. Soft independent modeling by class analogy of different class projections of *Bacillus amyloliquefaciens* TMW 2.479 spores (NAYE grown) after pressure-assisted thermal processing at 600 MPa-105°C (A) and thermal processing at 105°C-0.1 MPa (B).

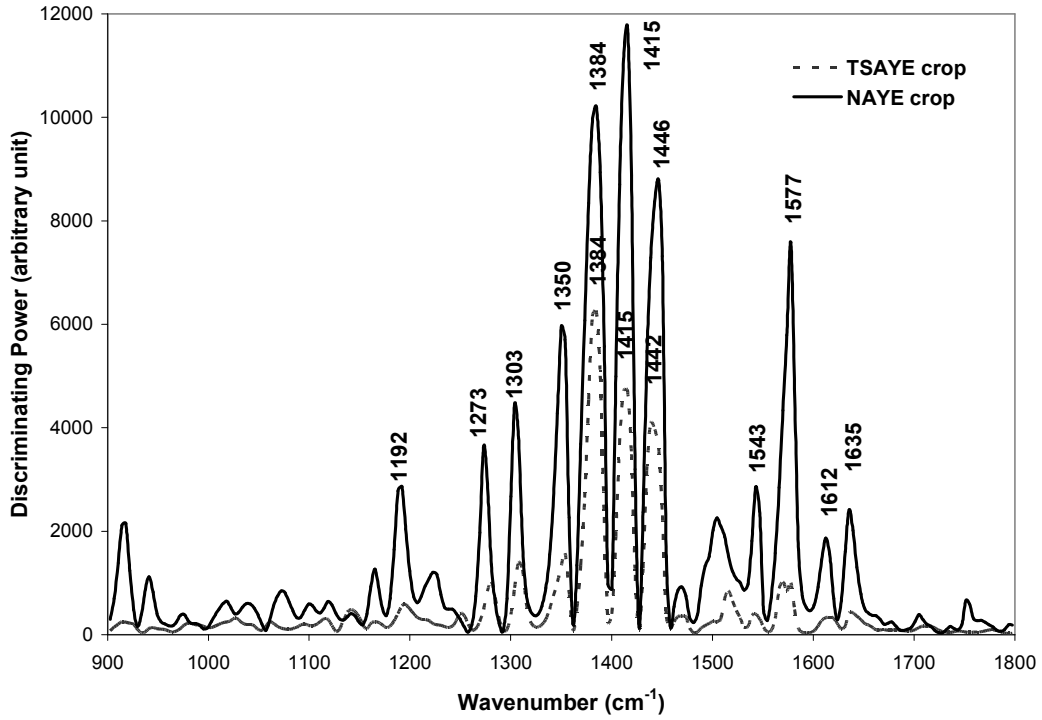


Figure 5.4. Discrimination power plot in classification of pressure-assisted thermal processing treated *Bacillus amyloliquefaciens* TMW 2.479 spores grown on two different media.

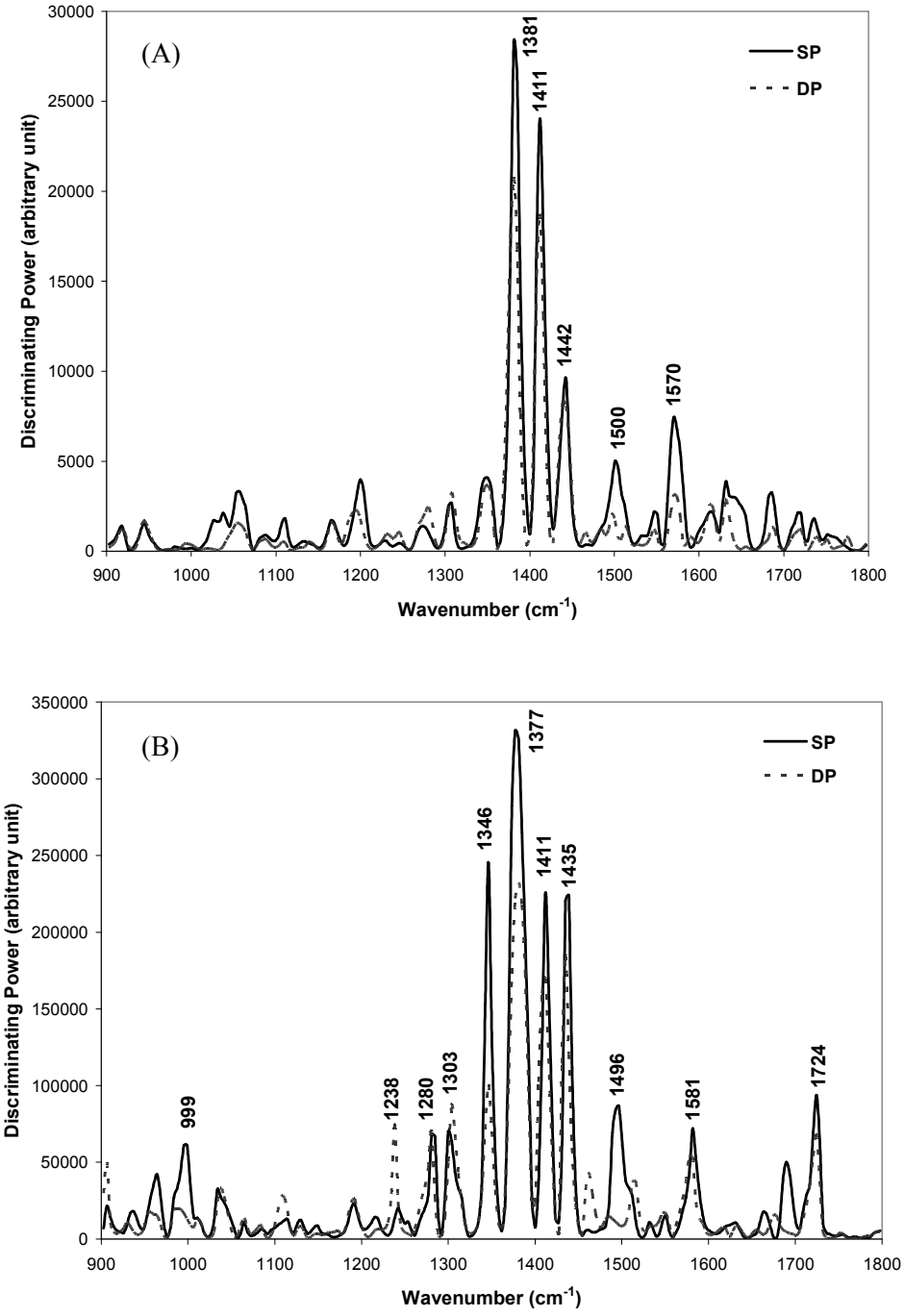


Figure 5.5. Comparison of single- (SP) and double-pulse (DP) treatments on *Bacillus amyloliquefaciens* TMW 2.479 spores processed at an equivalent holding time of 3 min; TSAYE grown spores (A) and NAYE grown spores (B).

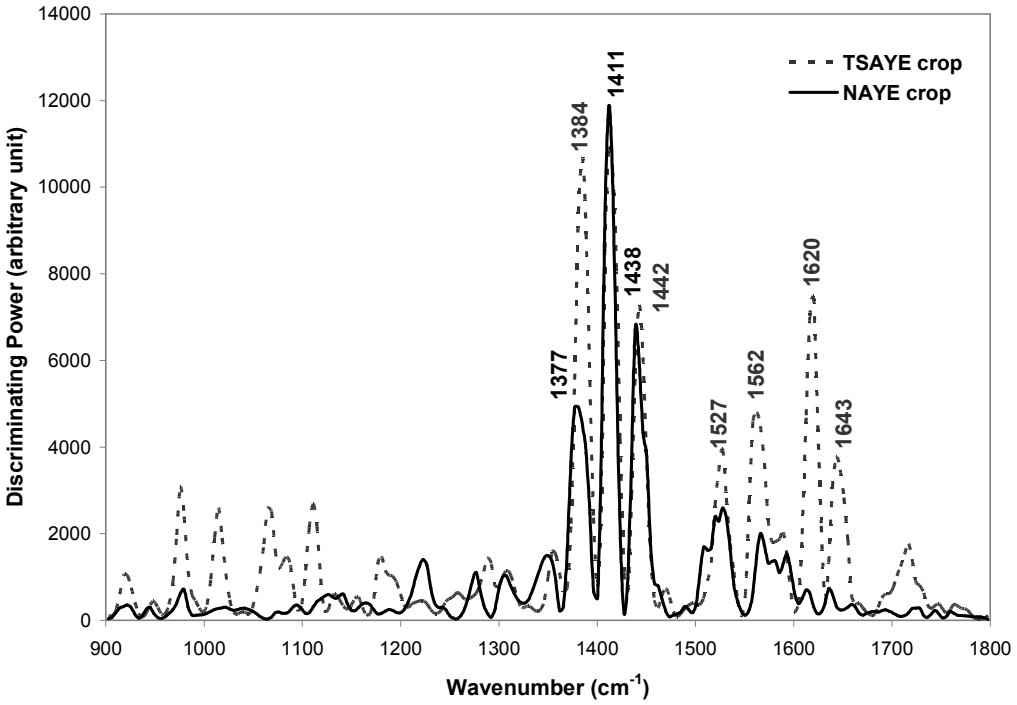


Figure 5.6. Discrimination power plot in classification of *Bacillus amyloliquefaciens* TMW 2.479 spores grown on two different media after thermal processing at 105°C-0.1 MPa.

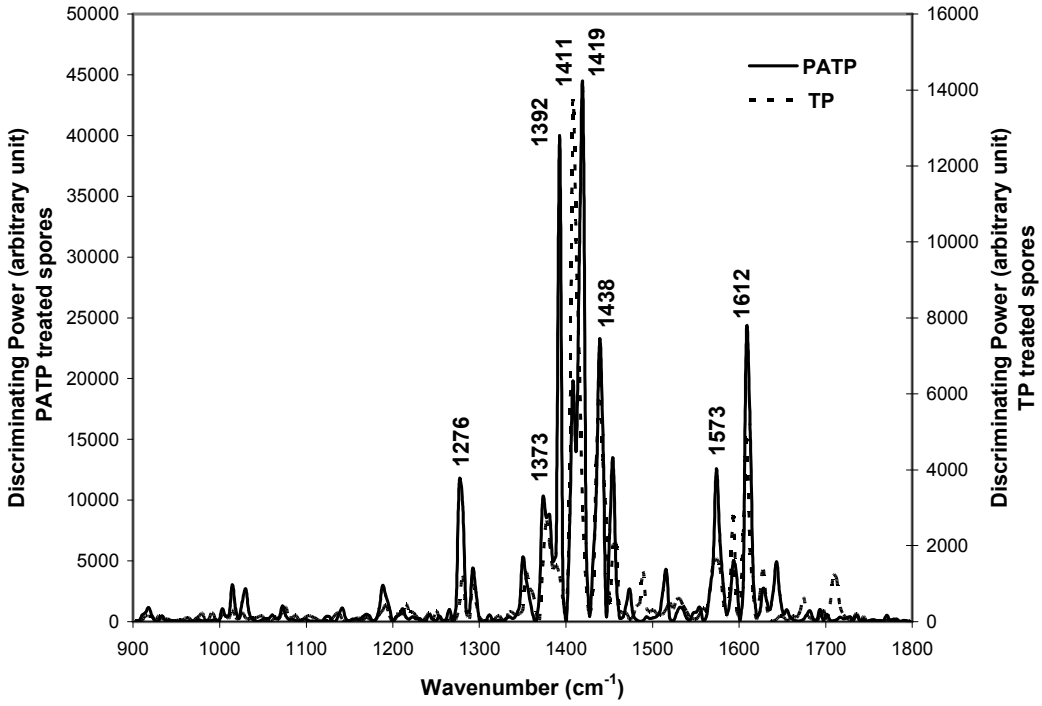
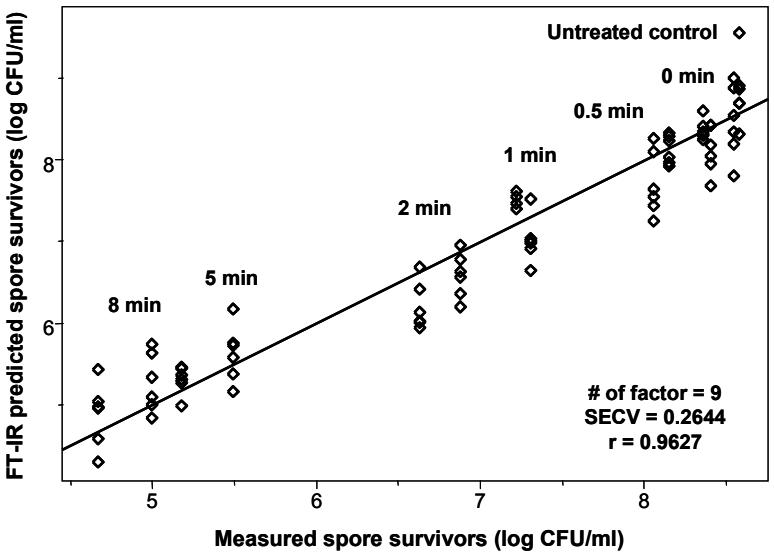


Figure 5.7. Comparison of discriminating power plot of *Bacillus amyloliquefaciens* TMW 2.479 spores grown on NAYE at 3 log reduction after TP (105°C at 0.1 MPa for 120 min) and PATP (600 MPa at 105°C for 5 min) treatments.

(A)



(B)

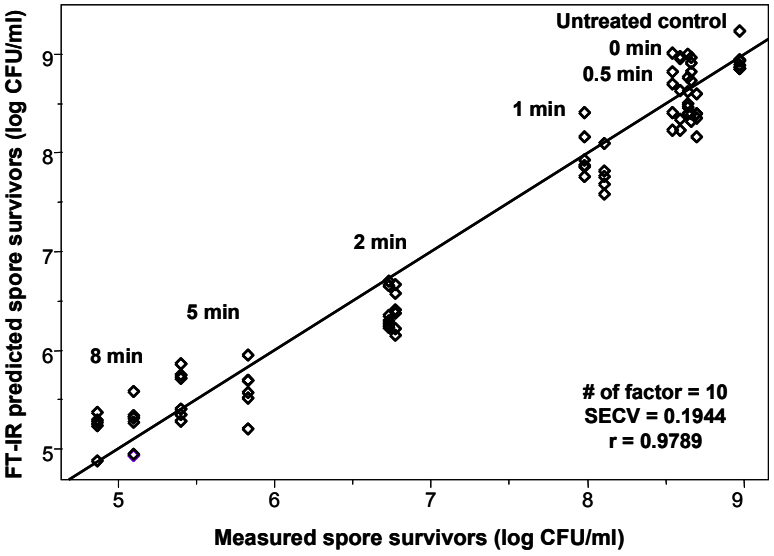
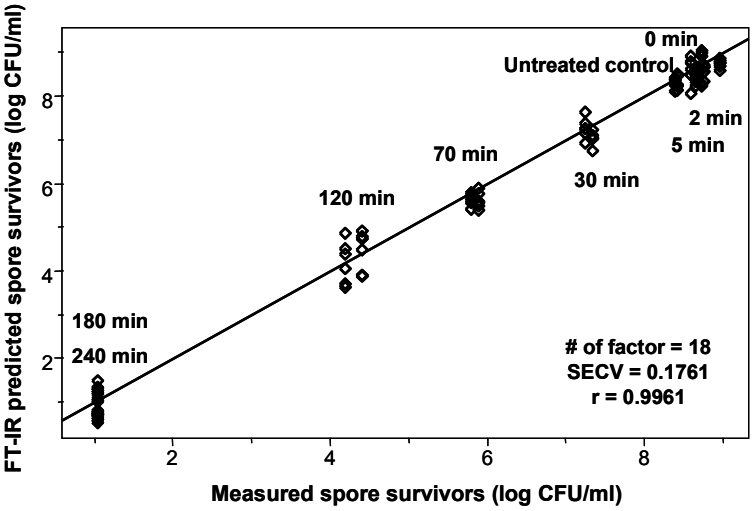


Figure 5.8. Cross-validated (leave-one-out) partial least squares regression plots for spore inactivation by pressure-assisted thermal processing; TSAYE grown spores (A) and NAYE grown spores (B).

(A)



(B)

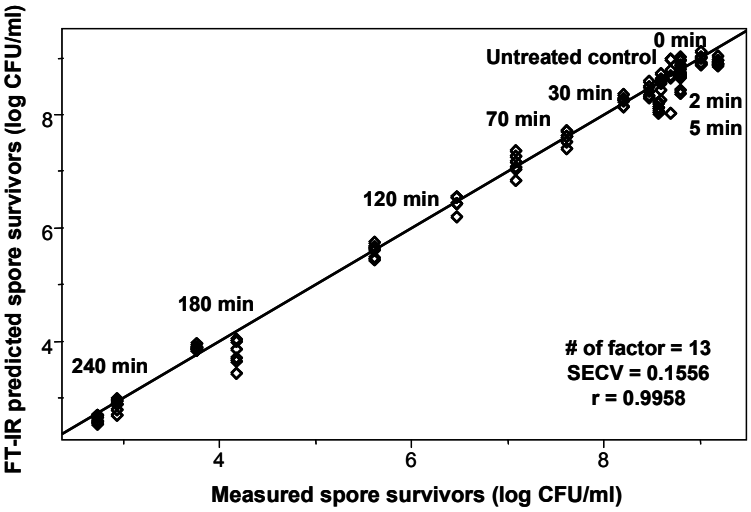
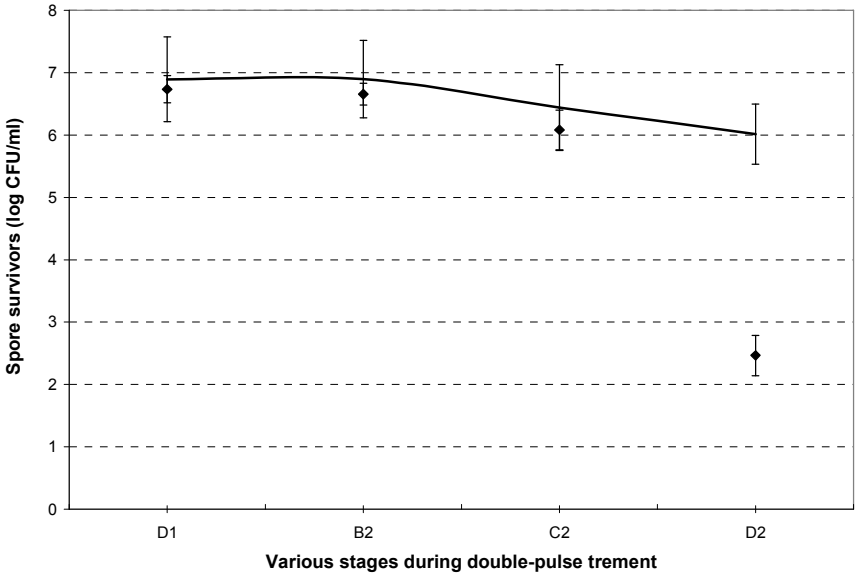


Figure 5.9. Cross-validated (leave-one-out) partial least squares regression plots for spore inactivation by thermal processing; TSAYE grown spores (A) and NAYE grown spores (B).

(A)



(B)

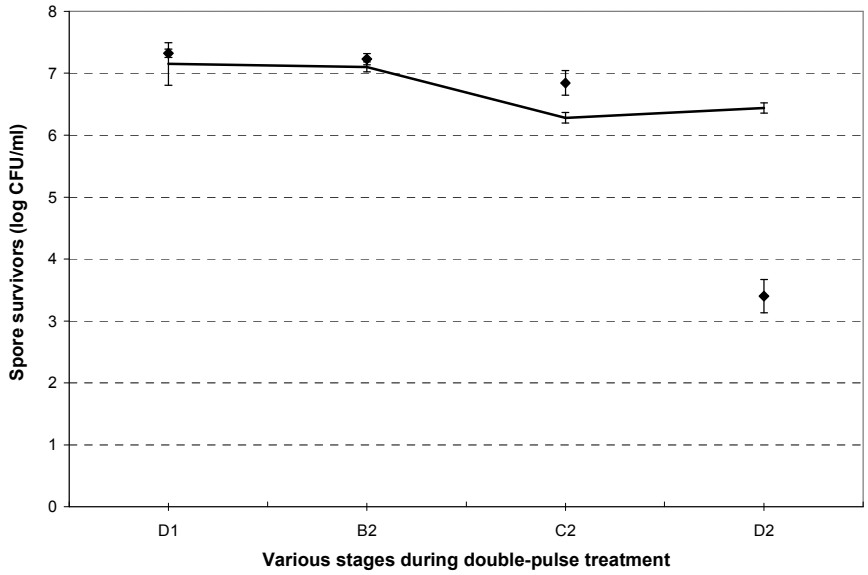


Figure 5.10. Predicting microbial efficacy of double-pulse PATP treatment for *Bacillus amyloliquefaciens* TMW 2.479 spore survivors based on single-pulse FT-IR spectra model. Predicted spore survivors by FT-IR spectra (_____) and measured spore survivors by standard plate count (◆); TSAYE grown spores (A) and NAYE grown spores (B). Refer to Figure 5.1.B for nomenclature during the double-pulse treatment.

Chapter 6: Conclusions

Role of pressurization rate and pressure pulsing on spore inactivation

- Regardless of pressurization rate, the 600 MPa-105°C treatment for 5 min reduced *B. amyloliquefaciens* spores by approximately 6.5 log CFU/mL in the spore suspension containing ~ 1.4 x 10^8 and ~ 1.1x10^9 CFU/mL. Similar process conditions resulted in inactivation to undetectable level when a lower spore suspension level (~ 1.3 x 10^6 CFU/mL) was used.
- Slow pressurization rate (3.75 MPa/s), showed enhanced inactivation within short pressure holding time (≤ 2 min). During slower pressurization rates, spores have been exposed to a lethal temperature region (T > 95°C) for a longer time than that of faster pressurization rates (18.06 MPa/s). This prolonged thermal exposure under pressure might have contributed to enhanced inactivation.
- Double-pulse treatment (600 MPa-105°C, with two 1.5 min-pressure holding times) significantly enhanced PATP spore inactivation than single-pulse treatment (600 MPa-105°C, with 3 min-pressure holding time). Double-pulse treatment generally provided additional 2.4 to 4 log CFU/mL more than that from single-pulse treatment. Regardless of pressurization rates, double-pulse treatment inactivated *B. amyloliquefaciens* spores to below the method's detection limit (≤ 10 CFU/mL) after a total of 3 min-holding time. Process temperature during 2nd

pulse increased from 105°C to 112°C due to heat gain from the surroundings as well as heat of compression. However, increasing process temperature from 105°C to 112°C during single-pulse treatment was not adequate to cause similar log reduction as that of double-pulse treatment. Different inactivation mechanisms may have been responsible for the spore inactivation during single- and double-pulse treatment.

Role of organic acid in enhancing PATP spore inactivation

- Addition of organic acids (100 - 200 mM at pH 5.0) to the spore suspension (without any treatment) did not cause any statistically significant inactivation or germination.
- Chemical- and food-grade organic acids produced similar microbial efficacy during PATP treatment.
- Organic acid facilitated spore germination and the magnitude of spore germination varied with type of organic acid and pressure holding time.
- Among the organic acids tested, acetic and citric acids was found to be the most effective in enhancing spore inactivation after 2 min-pressure holding time at 700 MPa-105°C. Lactic acid exhibited similar spore reduction levels as that of spores suspended in deionized water.
- In a low-acid food model system (carrot puree), recovery of the remaining population was observed during extended storage (up to 28 days at 32°C) but the presence of organic acids suppressed spore recovery.

Biochemical changes of PATP treated spores through FT-IR microspectroscopy

- A protocol was developed for the classification of bacterial spores treated by various processing methods (PATP, HPP, and TP) by combining hydrophobic grid membrane filtration with infrared microspectroscopy. This enabled direct spectroscopic observation of the biochemical changes in treated spores.
- FT-IR spectra of the bacterial spores were not only influenced by the PATP, TP, and HPP processing conditions, but also by the sporulation media.
- The main functional groups associated to spore inactivation by PATP and TP treatments were identified as those related to calcium dipicolinate (CaDPA). Associated FT-IR bands were identified as 1381, 1415, and 1442 cm^{-1}, implying the contribution of COO^- vibration of CaDPA chelate, the interaction of Ca^{2+} with COO^- group, and pyridine ring vibration of dipicolinic acid (DPA), respectively.
- Surviving spores from standard plate count were well-correlated with the FT-IR spectra. High coefficients of correlation ($r > 0.96$) and low standard errors of cross-validation ($\sim 10^{0.16} - 10^{0.26}$ CFU/mL) were obtained from the developed partial least square regression models.
- Single-pulse based PATP FT-IR PLSR model could not predict double-pulse lethality changes during the 2^{nd} pulse pressure holding time, possibly due to differences in respective spore inactivation mechanisms.
- More studies are needed using advanced high resolution spectroscopic techniques such as Raman to further understand the nature of biochemical changes during double-pulse treatment.

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Appendix A: SAS commands

Appendix A1:

Inactivation of different spore concentrations by selected processing variables (see Table 3.2)

Independent variables = inoculum levels (10^9, 10^8, and 10^6 CFU/mL); pressurization rate (fast and slow); PATP holding times (0, 0.5, 1, 2, 3, and 5 min)
Dependent variable (Y) = log reduction (CFU/mL)

```
data single_pulse;;
input inoculum rate time @;
do i=1 to 3;
input Y @;
output;
end;
datalines;
1       1       1       -0.40 -0.32 -0.80
1       1       2       -0.67 -0.71 -1.3
1       1       3       -1.3  -1.0  -1.9
1       1       4       -2.6  -2.9  -3.0
1       1       5       -3.3  -3.6  -4.3
1       1       6       -4.7  -5.6  -6.0
1       2       1       -0.94 -1.0  -1.3
1       2       2       -1.3  -1.5  -1.4
1       2       3       -1.9  -2.6  -3.0
1       2       4       -3.4  -3.2  -3.4
1       2       5       -4.3  -4.5  -4.9
1       2       6       -5.7  -6.2  -6.4
2       1       1       -0.60 -0.58 -0.78
2       1       2       -1.5  -1.5  -1.5
2       1       3       -2.6  -2.2  -2.4
2       1       4       -3.8  -3.6  -4.3
2       1       5       -5.5  -5.5  -4.7
2       1       6       -6.8  -6.7  -6.9
2       2       1       -2.8  -2.3  -2.6
2       2       2       -2.4  -3.0  -3.3
2       2       3       -3.2  -3.3  -3.7
2       2       4       -4.4  -4.7  -4.6
2       2       5       -6.1  -5.8  -5.5
2       2       6       -6.5  -6.5  -6.7
3       1       1       -0.79 -0.37 -0.49
3       1       2       -1.1  -1.2  -1.3
3       1       3       -1.8  -1.8  -2.4
3       1       4       -4.2  -4.3  -3.8
3       1       5       -4.8  -5.1  -5.0
3       1       6       -5.2  -5.1  -5.1
3       2       1       -2.0  -2.2  -2.0
3       2       2       -2.2  -2.5  -2.0
3       2       3       -2.9  -3.5  -3.3
3       2       4       -4.5  -4.8  -4.3
3       2       5       -5.2  -5.1  -5.1
3       2       6       -5.2  -5.1  -5.1
```

```
;
proc print; run;
proc glm;;
class inoculum rate time;
model Y = inoculum rate time inoculum*rate inoculum*time rate*time inoculum*rate*time;
means inoculum rate time/tukey;
means inoculum*rate inoculum*time rate*time inoculum*rate*time/tukey;
run;
```

Inactivation of spores (~1.4 x 10^8 CFU/mL) by pulsed pressure and other processing variables (see Table 3.2)

Independent variables = treatments: single-105°C, single-112°C, double-105°C-112°C; pressurization rate: fast and slow; PATP holding times: 1 and 3 min
Dependent variable (Y) = log reduction (CFU/mL)

```
data pulsing_logreduction;;
input treatment rate time @;
do i=1 to 3;
input y @;
output;
end;
datalines;
1       1       1       -2.6    -2.2    -2.2
1       1       2       -5.5    -5.5    -4.5
1       2       1       -3.2    -3.3    -3.6
1       2       2       -6.1    -5.7    -5.4
2       1       1       -4.2    -4.5    -4.7
2       1       2       -7.0    -6.9    -6.3
2       2       1       -6.5    -6.3    -6.3
2       2       2       -7.2    -6.6    -7.2
3       1       1       -4.9    -4.8    -3.7
3       1       2       -7.1    -7.0    -7.0
3       2       1       -7.1    -7.0    -7.0
3       2       2       -7.1    -7.0    -7.0
;
proc print; run;
proc glm;;
class treatment rate time;
model Y = treatment rate time treatment*rate treatment*time rate*time treatment*rate*time;
means treatment rate time/tukey;
means treatment*rate treatment*time rate*time treatment*rate*time/tukey;
run;
```

Mean comparison of spore survivors processed at different treatment conditions (see Figure 3.5)

Independent variables = treatment condition; replication
Dependent variable (Y) = log survivor (CFU/mL)

```
data pulsing_logN;;
```

```
input treatment rep survivor;
datalines;
1       1       8.1
1       2       8.0
1       3       8.0
2       1       8.1
2       2       8.0
2       3       8.0
3       1       5.5
3       2       5.9
3       3       5.8
4       1       4.9
4       2       4.8
4       3       4.4
5       1       2.7
5       2       2.6
5       3       3.5
6       1       2.0
6       2       2.4
6       3       2.7
7       1       3.9
7       2       3.5
7       3       3.3
8       1       1.6
8       2       1.8
8       3       1.8
9       1       1.1
9       2       1.2
9       3       1.7
10      1       0.90
10      2       1.4
10      3       0.85
11      1       3.2
11      2       3.3
11      3       4.3
12      1       1.0
12      2       1.0
12      3       1.0
13      1       1.0
13      2       1.0
13      3       1.0
14      1       1.0
14      2       1.0
14      3       1.0
;
proc print; run;
proc glm;;
class treatment rep;
model survivor = treatment;
means treatment/tukey;
run;
```

Appendix A2:

Spore survivors in various suspension media after PATP treatment (see Figure 4.1)

Independent variables = suspension media (DIW, acetic, citric, lactic); replications (1, 2, 3); pressure holding times (untreated, 0, 1, 2, 3, 5 min)
Dependent variable (Y) = log survivor (CFU/mL)

```
option ls=120;
data FCCBH;
input sol rep time cfu;
cards;
1       1       -1      8.0
1       2       -1      8.1
1       3       -1      8.1
1       1       0       7.8
1       2       0       7.8
1       3       0       7.8
1       1       1       3.6
1       2       1       4.0
1       3       1       4.1
1       1       2       2.1
1       2       2       2.7
1       3       2       2.3
1       1       3       1.0
1       2       3       1.0
1       3       3       1.0
1       1       5       1.0
1       2       5       1.0
1       3       5       1.0
2       1       -1      8.2
2       2       -1      8.1
2       3       -1      8.2
2       1       0       7.8
2       2       0       7.8
2       3       0       8.0
2       1       1       4.3
2       2       1       4.4
2       3       1       4.2
2       1       2       2.0
2       2       2       1.6
2       3       2       1.6
2       1       3       1.0
2       2       3       1.0
2       3       3       1.0
2       1       5       1.0
2       2       5       1.0
2       3       5       1.0
7       1       -1      8.3
3       2       -1      8.3
3       3       -1      8.2
3       1       0       8.1
```

```
3       2       0       8.1
3       3       0       7.6
3       1       1       3.1
3       2       1       3.5
3       3       1       3.1
3       1       2       1.8
3       2       2       1.6
3       3       2       1.5
3       1       3       1.0
3       2       3       1.0
3       3       3       1.0
3       1       5       1.0
3       2       5       1.0
3       3       5       1.0
4       1       -1      8.1
4       2       -1      8.1
4       3       -1      8.1
4       1       0       7.8
4       2       0       7.8
4       3       0       8.1
4       1       1       3.5
4       2       1       3.9
4       3       1       3.9
4       1       2       2.7
4       2       2       2.9
4       3       2       2.3
4       1       3       1.0
4       2       3       1.0
4       3       3       1.0
4       1       5       1.0
4       2       5       1.0
4       3       5       1.0
;
proc glm;
class sol rep time;
model cfu = sol rep time sol*time;
means sol rep time sol*time/SNK;
run;
```

Mean comparison after PATP treatment: (see Figure 4.1)

```
option ls=120;
data FCCBH;
input trt rep cfu;
cards;
1       1       8.0
1       2       8.1
1       3       8.1
2       1       7.8
2       2       7.8
2       3       7.8
3       1       3.6
3       2       4.0
```

3	3	4.1
4	1	2.1
4	2	2.7
4	3	2.3
5	1	1.0
5	2	1.0
5	3	1.0
6	1	1.0
6	2	1.0
6	3	1.0
7	1	8.2
7	2	8.1
7	3	8.2
8	1	7.8
8	2	7.8
8	3	8.0
9	1	4.3
9	2	4.4
9	3	4.2
10	1	2.0
10	2	1.6
10	3	1.6
11	1	1.0
11	2	1.0
11	3	1.0
12	1	1.0
12	2	1.0
12	3	1.0
13	1	8.3
13	2	8.3
13	3	8.2
14	1	8.1
14	2	8.1
14	3	7.6
15	1	3.1
15	2	3.5
15	3	3.1
16	1	1.8
16	2	1.6
16	3	1.5
17	1	1.0
17	2	1.0
17	3	1.0
18	1	1.0
18	2	1.0
18	3	1.0
19	1	8.1
19	2	8.1
19	3	8.1
20	1	7.8
20	2	7.8
20	3	8.1
21	1	3.5
21	2	3.9

```
21      3       3.9
22      1       2.7
22      2       2.9
22      3       2.3
23      1       1.0
23      2       1.0
23      3       1.0
24      1       1.0
24      2       1.0
24      3       1.0
;
proc glm;
class trt rep;
model cfu = trt rep;
means trt rep/SNK;
run;
```

Spore survivors as influenced by suspension media (seeTable 4.3)

Independent variables = treatment; replication
Dependent variable (Y) = log survivor (MPN/mL)

```
option ls=120;
data FCC_HT_bysol;
input trt rep mpn;
cards;
1       1       1.8
1       2       2.2
1       3       2.3
2       1       2.1
2       2       2.7
2       3       2.3
3       1       2.3
3       2       2.7
3       3       2.3
4       1       2.3
4       2       2.7
4       3       2.3
5       1       1.4
5       2       0.48
5       3       0.79
6       1       1.4
6       2       0.86
6       3       0.79
7       1       1.4
7       2       0.86
7       3       0.79
8       1       1.4
8       2       1.1
8       3       0.86
9       1       0.56
9       2       0.86
9       3       0.48
```

10	1	0.56
10	2	0.86
10	3	0.48
11	1	0.56
11	2	0.86
11	3	0.48
12	1	0.56
12	2	0.86
12	3	0.48
13	1	0.48
13	2	0.48
13	3	0.48
14	1	0.48
14	2	0.48
14	3	0.48
15	1	0.48
15	2	0.56
15	3	0.48
16	1	0.48
16	2	0.56
16	3	0.48
17	1	1.3
17	2	0.56
17	3	0.48
18	1	1.3
18	2	1.0
18	3	0.48
19	1	1.3
19	2	1.0
19	3	0.48
20	1	1.3
20	2	1.0
20	3	0.48
21	1	0.48
21	2	1.4
21	3	1.0
22	1	0.86
22	2	1.4
22	3	1.0
23	1	0.86
23	2	1.4
23	3	1.0
24	1	0.56
24	2	1.4
24	3	1.0
25	1	1.0
25	2	0.48
25	3	0.56
26	1	1.0
26	2	0.48
26	3	0.56
27	1	1.0
27	2	0.48
27	3	0.56

```
28      1       1.0
28      2       0.48
28      3       0.56
29      1       0.48
29      2       0.48
29      3       0.56
30      1       0.48
30      2       0.48
30      3       0.56
31      1       0.48
31      2       0.48
31      3       0.56
32      1       0.48
32      2       0.48
32      3       0.48
;
proc glm;
class trt rep;
model mpn = trt rep;
means trt/SNK;
run;
```

Spore survivors as influenced by incubation time (see Table 4.3)

Independent variables = treatment; replication
Dependent variable (Y) = log survivor (MPN/mL)

For example: 3 min-PATP treatment in deionized water

```
option ls=120;
data FCC_HT_DIW;
input trt rep mpn;
cards;
1       1       1.8
1       2       2.2
1       3       2.3
5       1       2.1
5       2       2.7
5       3       2.3
9       1       2.3
9       2       2.7
9       3       2.3
13      1       2.3
13      2       2.7
13      3       2.3
;
proc glm;
class trt rep;
model mpn = trt rep;
means trt/SNK;
run;
```

Spore recovery in PATP-treated carrot puree (see Table 4.4)

Independent variables = treatment; replication
Dependent variable (Y) = log survivor (MPN/mL)

For example: 5 min-PATP treatment

```
option ls=120;
data FCC_BH_HT_all;
input trt rep mpn;
cards;
1       1       1.4
1       2       1.2
1       3       1.2
2       1       0.48
2       2       1.4
2       3       0.86
3       1       1.2
3       2       1.6
3       3       1.5
4       1       0.86
4       2       2.2
4       3       1.6
5       1       2.0
5       2       2.2
5       3       1.6
6       1       2.4
6       2       1.6
6       3       1.2
7       1       0.48
7       2       0.48
7       3       0.56
8       1       1.9
8       2       0.48
8       3       0.86
9       1       0.48
9       2       0.48
9       3       0.48
10      1       0.48
10      2       2.0
10      3       0.86
11      1       1.6
11      2       1.0
11      3       0.56
12      1       0.56
12      2       1.2
12      3       0.56
13      1       1.0
13      2       2.0
13      3       1.4
14      1       0.48
14      2       0.56
14      3       1.4
```

```
15      1       1.4
15      2       1.6
15      3       1.7
16      1       1.4
16      2       1.9
16      3       1.7
17      1       2.2
17      2       2.6
17      3       1.6
18      1       1.8
18      2       2.2
18      3       2.1
19      1       1.6
19      2       1.5
19      3       0.86
20      1       2.6
20      2       1.5
20      3       2.3
21      1       1.5
21      2       1.5
21      3       3.1
22      1       2.6
22      2       2.2
22      3       2.1
23      1       1.9
23      2       2.3
23      3       0.79
24      1       1.9
24      2       0.48
24      3       0.48
;
proc glm;
class trt rep;
model mpn = trt rep;
means trt/SNK;
run;
```

Appendix A3:

Spore inactivation during various stages of single- and double-pulse treatment (see Table 5.2)

Independent variables = various stages of treatment (SP-CUT, SP-3 min, DP-CUT, DP-1.5 min, etc.); sporulation media (TSAYE, NAYE)
Dependent variable (Y) = log reduction (CFU/mL)

```
data pulsing_logreduction;;
input treatment media @;
do i=1 to 5;
input y @;
output;
end;
datalines;
1       1       0.08   -0.02 0.66   0.10   0.20
1       2       0.30   0.30   0.26   0.35   0.42
2       1       -3.4   -3.2   -3.0   -3.3   -3.2
2       2       -2.5   -2.5   -2.2   -2.5   -2.8
3       1       -1.6   -1.7   -1.5   -1.7   -1.2
3       2       -1.1   -1.1   -1.0   -1.2   -1.2
4       1       -1.7   -1.9   -1.6   -1.6   -1.4
4       2       -1.2   -1.2   -1.1   -1.3   -1.2
5       1       -2.3   -2.6   -1.8   -2.4   -1.9
5       2       -1.6   -1.6   -1.3   -1.7   -1.9
6       1       -6.0   -5.8   -5.9   -6.0   -5.4
6       2       -5.1   -5.1   -4.9   -5.4   -4.9
;
proc print; run;
proc glm;;
class treatment media;
model Y = treatment media treatment*media;
means treatment media treatment*media/tukey;
run;
```

Mean comparison during various stages of single- and double-pulse treatment (see Table 5.2)

```
data pulsing_logreduction;;
input treatment @;
do i=1 to 5;
input y @;
output;
end;
datalines;
1       0.08   -0.02 0.66   0.10   0.20
2       0.30   0.30   0.26   0.35   0.42
3       -3.4   -3.2   -3.0   -3.3   -3.2
4       -2.5   -2.5   -2.2   -2.5   -2.8
5       -1.6   -1.7   -1.5   -1.7   -1.2
```

```
6       -1.1   -1.1   -1.0   -1.2   -1.2
7       -1.7   -1.9   -1.6   -1.6   -1.4
8       -1.2   -1.2   -1.1   -1.3   -1.2
9       -2.3   -2.6   -1.8   -2.4   -1.9
10      -1.6   -1.6   -1.3   -1.7   -1.9
11      -6.0   -5.8   -5.9   -6.0   -5.4
12      -5.1   -5.1   -4.9   -5.4   -4.9
;
proc print; run;
proc glm;;
class treatment;
model Y = treatment;
means treatment/tukey;
run;
```

Influence of treatment conditions on spore inactivation (see Table 5.3)

Independent variables = treatment condition (PATP, TP, HPP); treatment time (0 to 240 min); sporulation media (TSAYE, NAYE)
Dependent variable (Y) = log reduction (CFU/mL)

```
data process_logreduction;;
input process time media @;
do i=1 to 2;
input y @;
output;
end;
datalines;
1       1       1       0.00   -0.20
1       1       2       0.20   0.00
1       2       1       -0.50 -0.80
1       2       2       0.00   -0.20
1       3       1       -1.7   -1.4
1       3       2       -0.90 -0.90
1       4       1       -2.3   -2.7
1       4       2       -1.7   -1.6
1       5       1       -4.8   -5.3
1       5       2       -3.6   -3.3
1       6       1       -5.6   -6.1
1       6       2       -5.1   -5.2
2       1       1       0.30   0.00
2       1       2       0.20   0.40
2       4       1       0.20   0.10
2       4       2       0.20   0.40
2       5       1       0.30   0.00
2       5       2       0.20   0.40
2       7       1       -1.1   -1.0
2       7       2       -0.20 -0.10
2       8       1       -2.5   -2.6
2       8       2       -1.4   -0.90
2       9       1       -4.0   -3.9
2       9       2       -2.9   -2.2
2       10      1       -7.3   -7.4
```

```
2       10      2       -4.4    -4.9
2       11      1       -7.3    -7.4
2       11      2       -5.2    -5.0
3       1       1       -0.10   0.00
3       1       2       0.00    0.00
3       5       1       0.20    0.10
3       5       2       0.00    0.20
3       7       1       0.10    0.20
3       7       2       0.20    0.30
3       8       1       0.00    0.10
3       8       2       0.20    0.40
;
proc print; run;
proc glm;;
class process time media;
model Y = process time media process*time process*media time*media process*time*media;
means process time media/tukey;
run;
```

Mean comparison (see Table 5.3)

```
data process_logreduction;;
input treatment @;
do i=1 to 2;
input y @;
output;
end;
datalines;
1       0.00    -0.20
2       0.20    0.00
3       -0.50   -0.80
4       0.00    -0.20
5       -1.7    -1.4
6       -0.90   -0.90
7       -2.3    -2.7
8       -1.7    -1.6
9       -4.8    -5.3
10      -3.6    -3.3
11      -5.6    -6.1
12      -5.1    -5.2
13      0.30    0.00
14      0.20    0.40
15      0.20    0.10
16      0.20    0.40
17      0.30    0.00
18      0.20    0.40
19      -1.1    -1.0
20      -0.20   -0.10
21      -2.5    -2.6
22      -1.4    -0.90
23      -4.0    -3.9
24      -2.9    -2.2
25      -7.3    -7.4
```

```
26     -4.4   -4.9
27     -7.3   -7.4
28     -5.2   -5.0
29     -0.10  0.00
30     0.00   0.00
31     0.20   0.10
32     0.00   0.20
33     0.10   0.20
34     0.20   0.30
35     0.00   0.10
36     0.20   0.40
;
proc print; run;
proc glm;;
class treatment;
model Y = treatment;
means treatment/tukey;
run;
```

Appendix B: MATLAB commands for pressure and thermal dosages

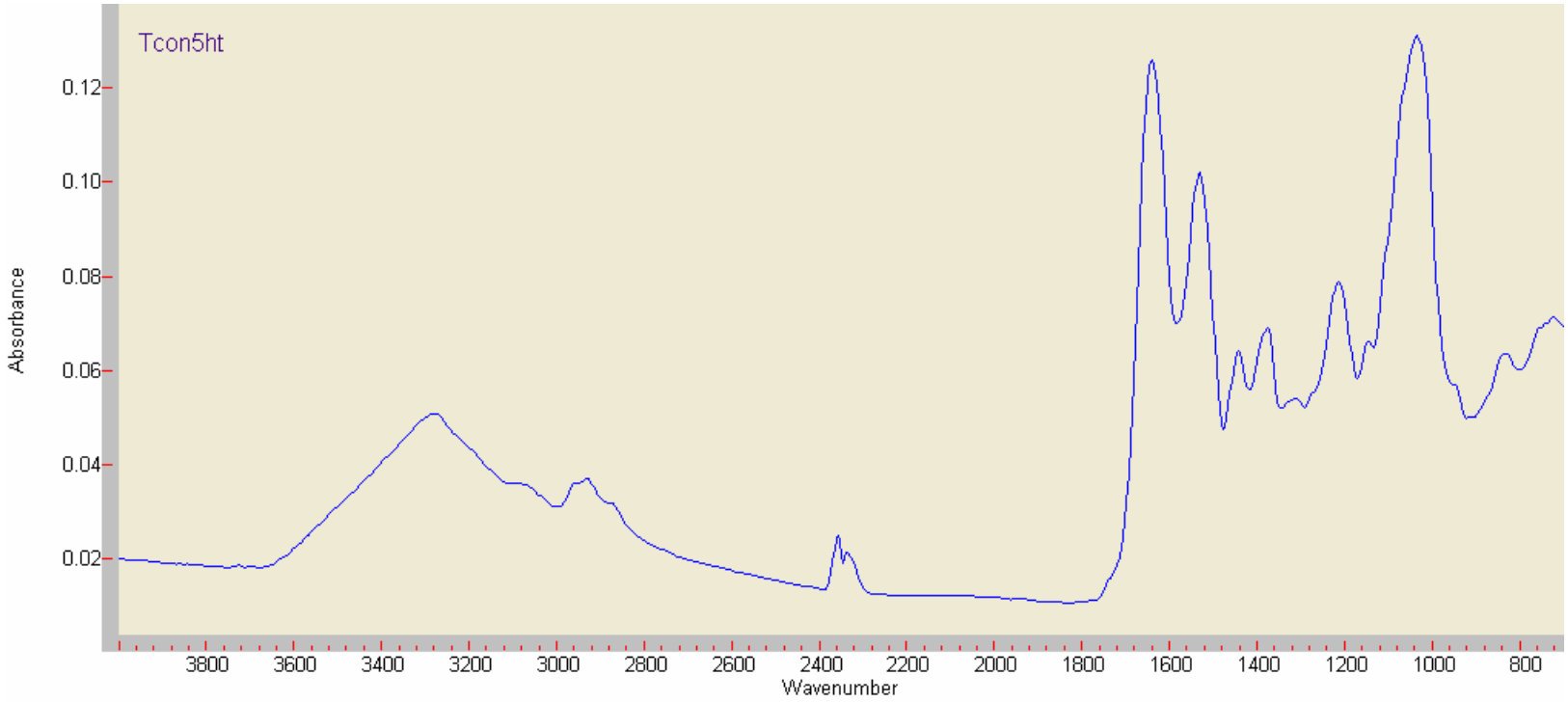
MainPT.m

```
clc
clear

TP{1}='S1-rep1';
TP{2}='S2-rep1';
TP{3}='S3-rep1';
TP{4}='S4-rep1';
TP{5}='S5-rep1';
TP{6}='S6-rep1';
TP{7}='S7-rep1';
TP{8}='S8-rep1';
TP{9}='L1-rep1';
TP{10}='L2-rep1';
TP{11}='L3-rep1';
TP{12}='L4-rep1';
TP{13}='L5-rep1';
TP{14}='L6-rep1';
TP{15}='L7-rep1';
TP{16}='L8-rep1';


      result{1,1}='Treatment';
      result{1,2}='St';
      result{1,3}='Sp';

for i=1:16;
      [St, Sp]=IntegPT(TP{i});
      St(i)=St; Sp(i)=Sp;
      fprintf(1,TP{i});
      fprintf(1,'     %2.5f\t',St(i));
      fprintf(1,'  %2.5f\t\n',Sp(i));

      result{i+1,1}=TP{i};
      result{i+1,2}=St(i);
      result{i+1,3}=Sp(i);
end

xlswrite('resultTP', result);
```

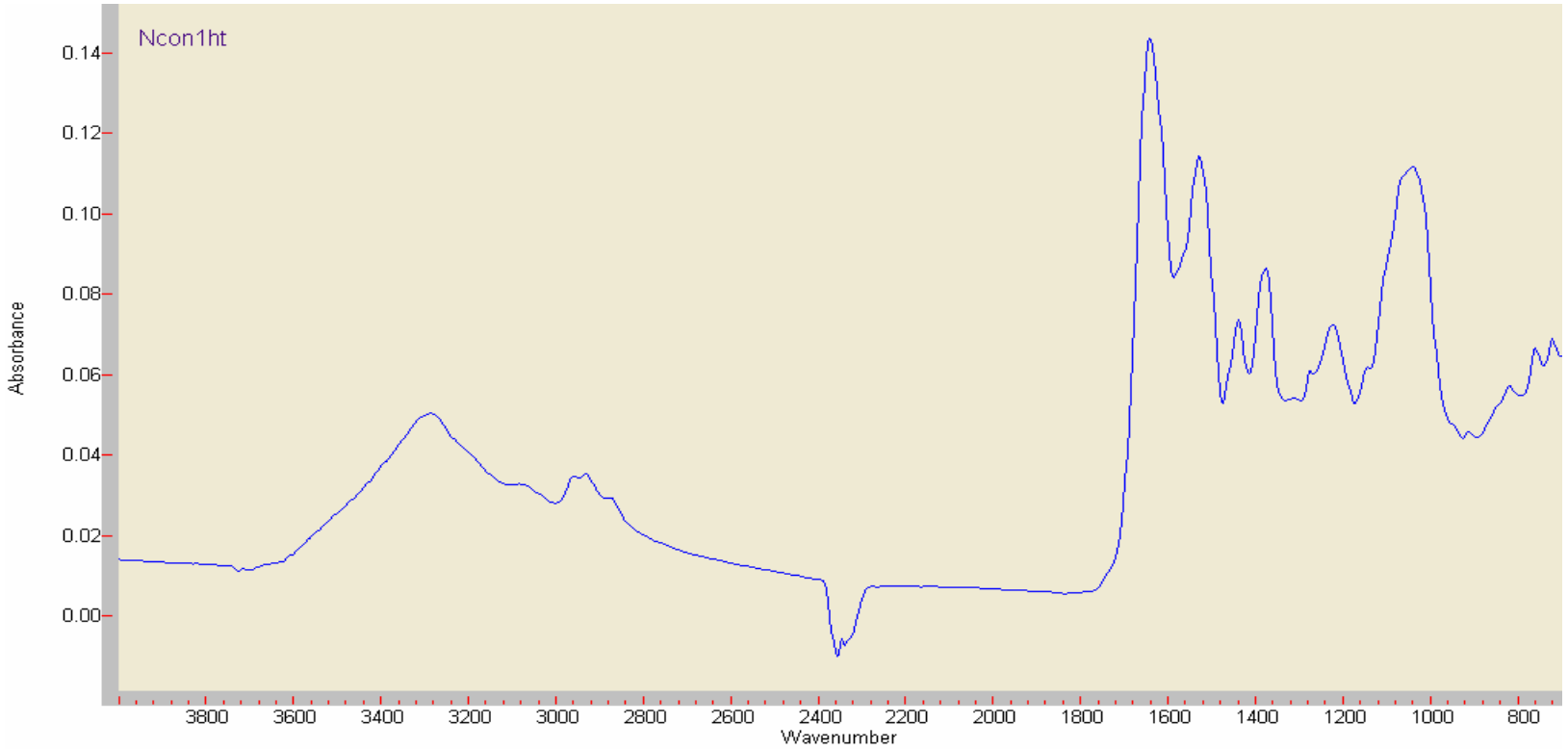
IntegPT.m

```
function [St, Sp]=IntegPT(x)

% Integration function is used to calculate area under the temperature and pressure
profile by using trapezoidal rule. The method was selected based on the properties of
tabulated data which has equal spacing and almost linear relationship within short time
interval. The surface area is counted from beginning to end of treatment including the
elapsed period between two "PULSES".

% The program is capable of loading data from excel files, calculating the desired areas
and when called by the main program, write the data to an excel file named "result".


% Loading Temperature and pressure profile data from Spread sheet


TP = xlsread(x);

% Locate the ending point of the T-P profile

[N,n]=size(TP);

St=0; Sp=0;

for i=1:N-1;
   if TP(i,3)~=0 | TP(i+1,3)~=0
      It=0.5*(TP(i,2)+TP(i+1,2));
      St=St+It;
   end
      Ip=0.5*(TP(i,3)+TP(i+1,3));
      Sp=Sp+Ip;
end

end
```

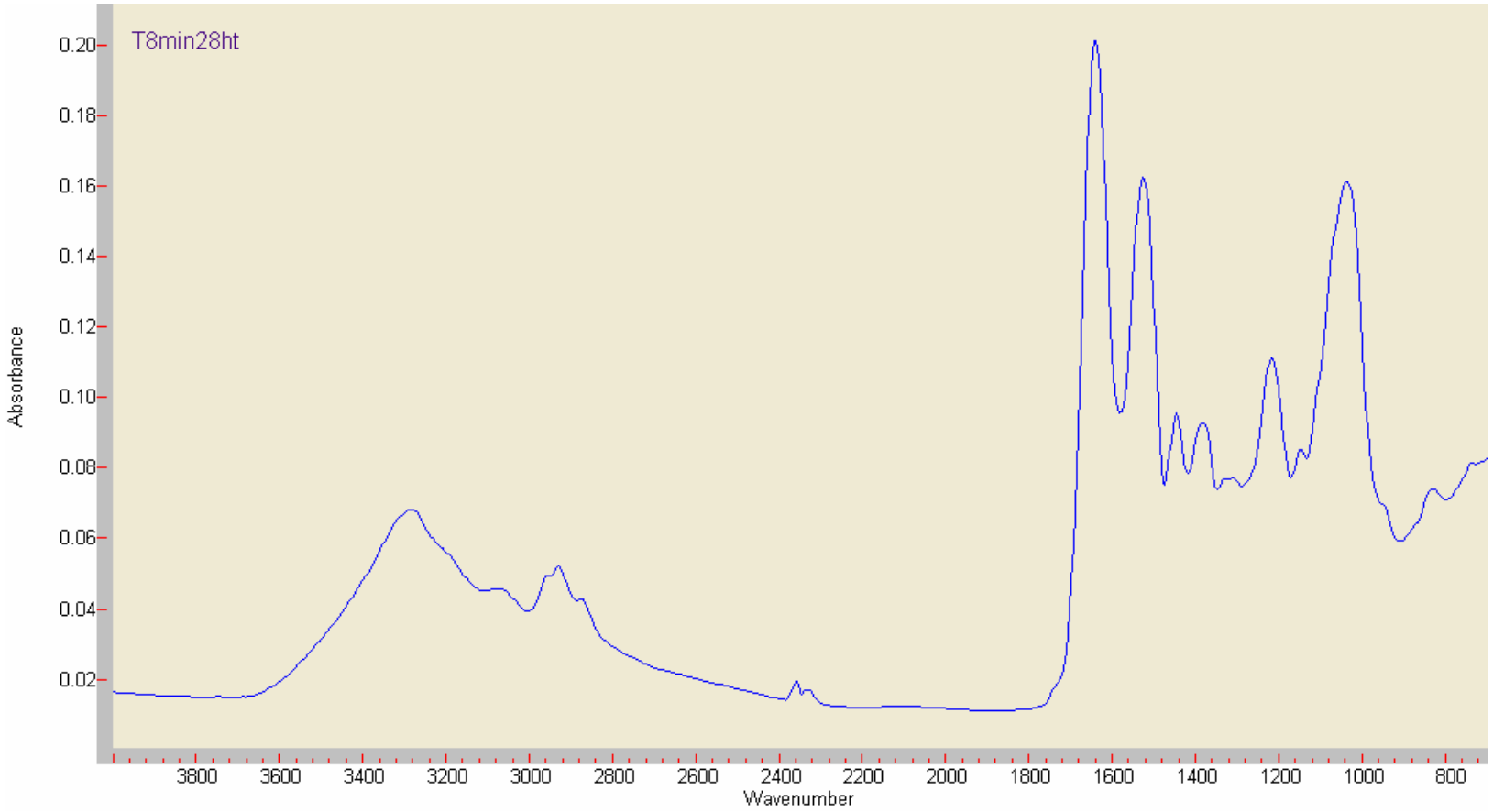
Appendix C: FT-IR spectra of *Bacillus amyloliquefaciens* TMW 2.479 spores



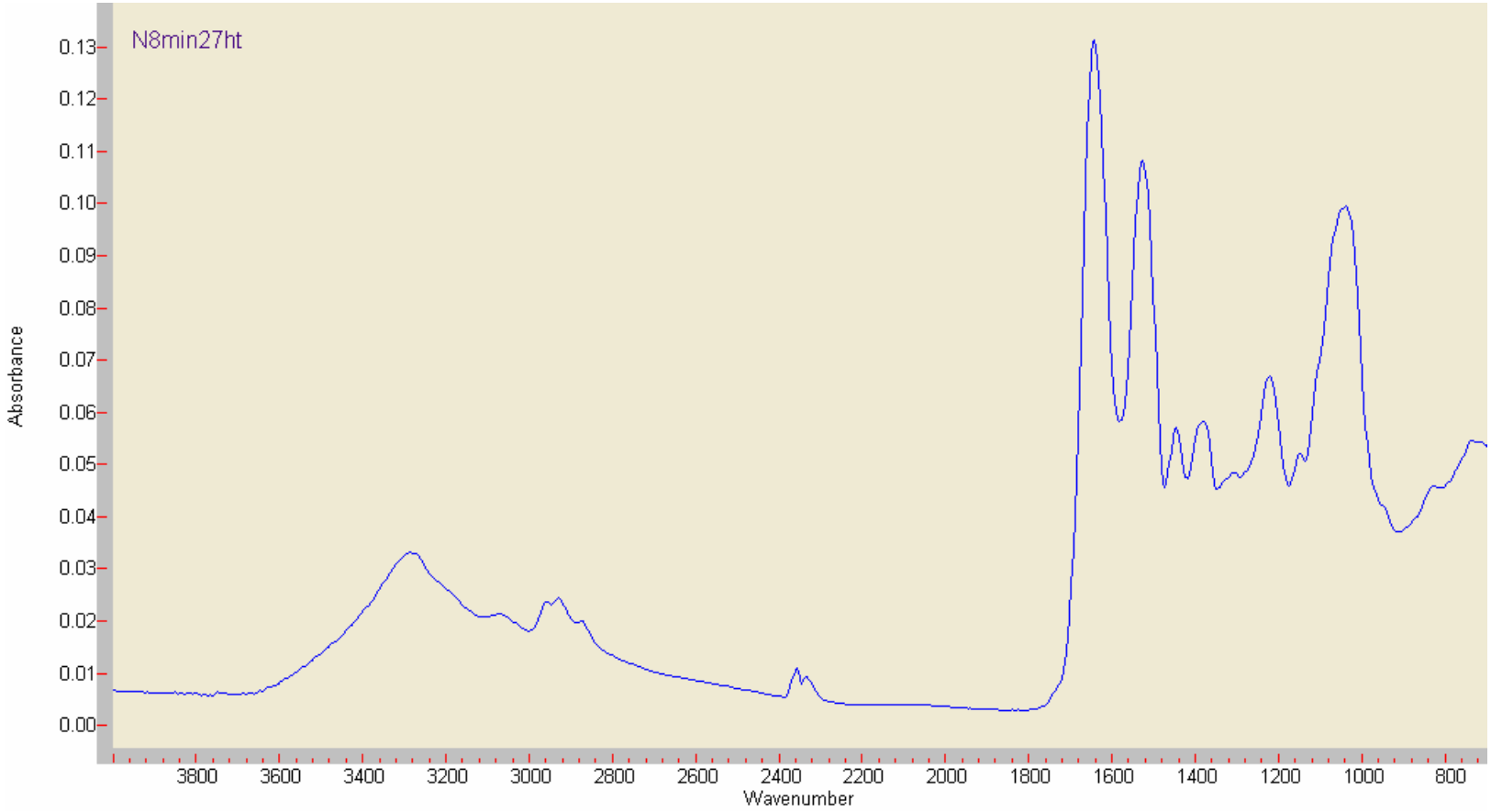
Raw spectra of TSAYE grown spores (untreated control)



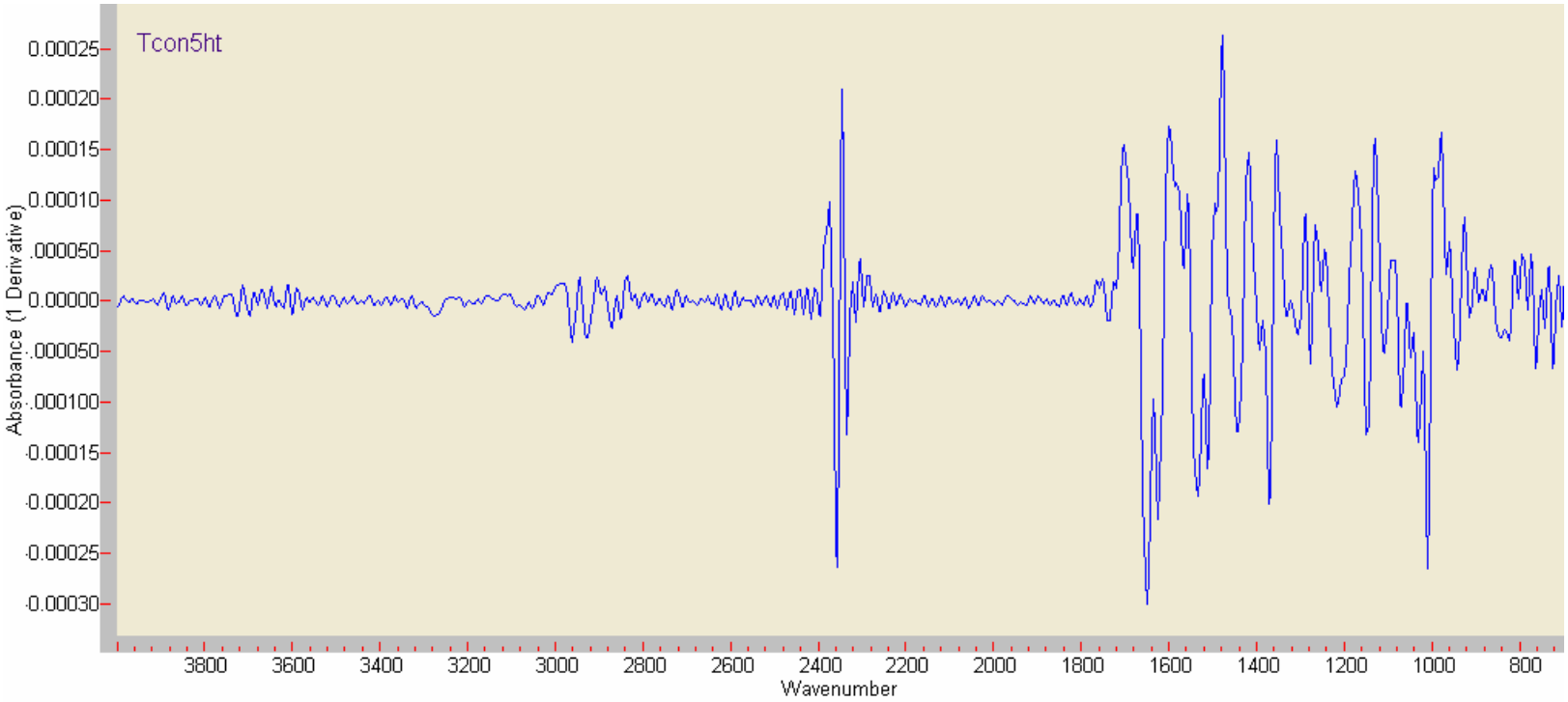
Raw spectra of NAYE grown spores (untreated control)



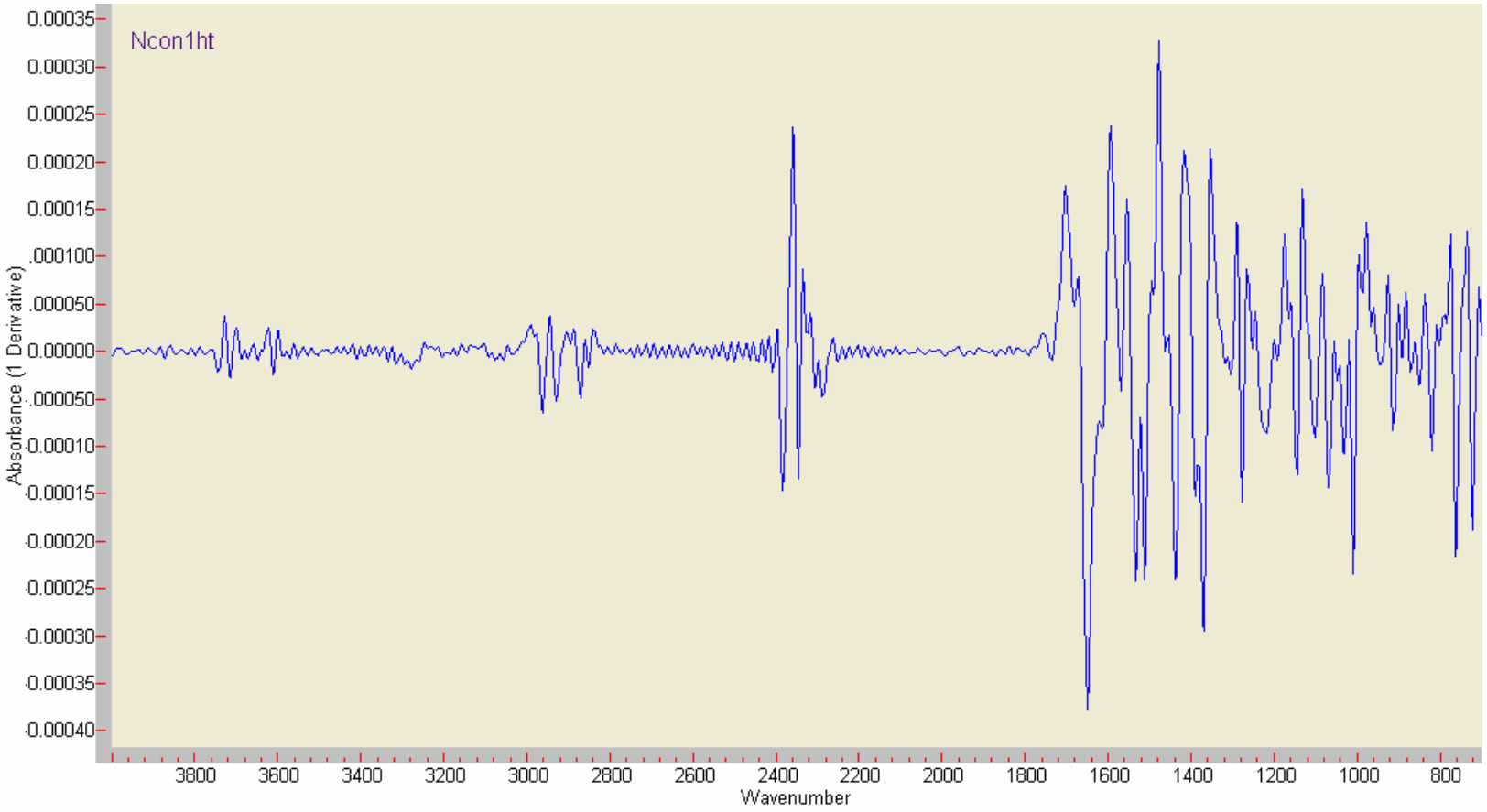
Raw spectra of TSAYE grown spores (8min-PATP treatment)



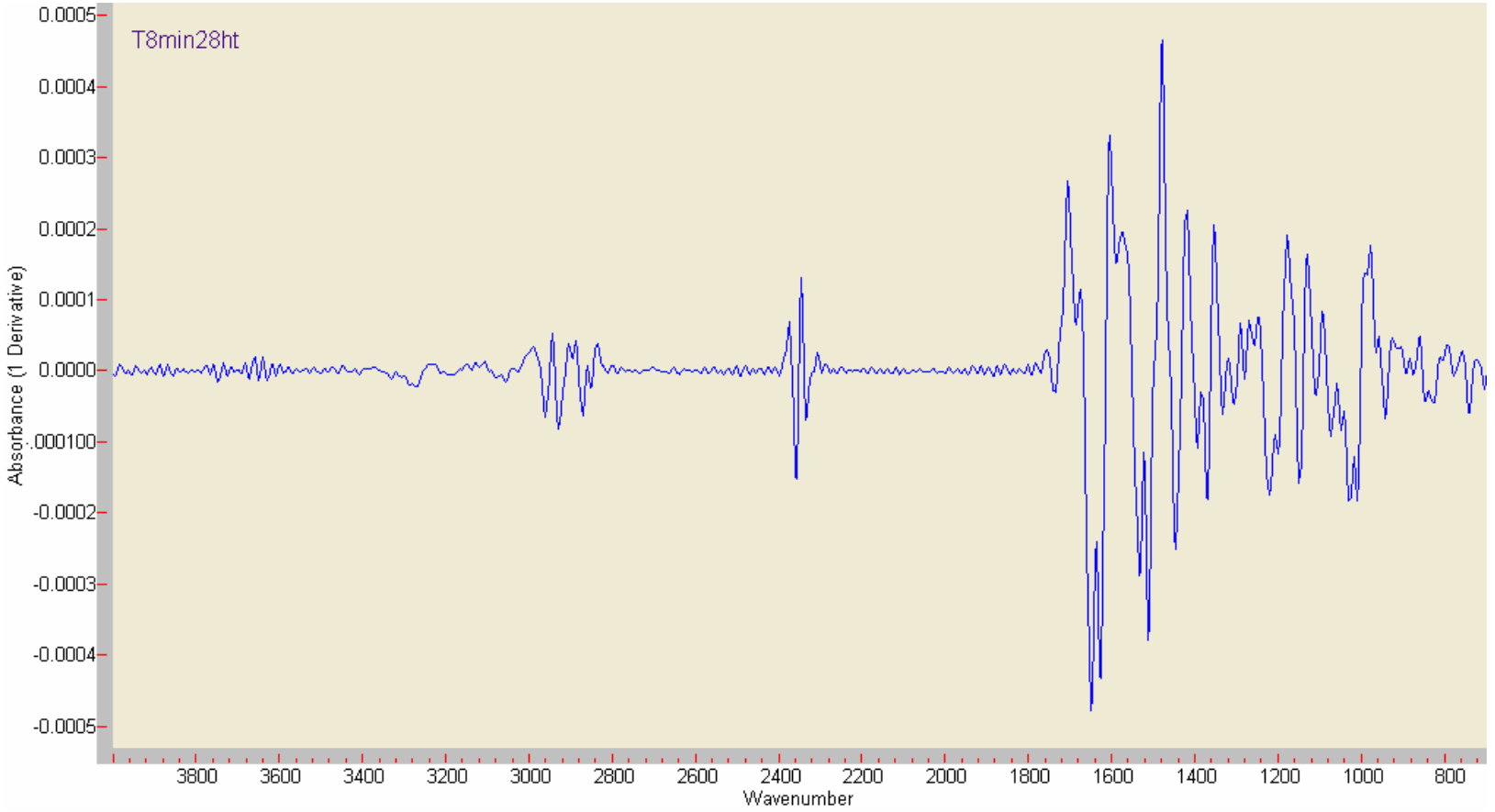
Raw spectra of NAYE grown spores (8min-PATP treatment)



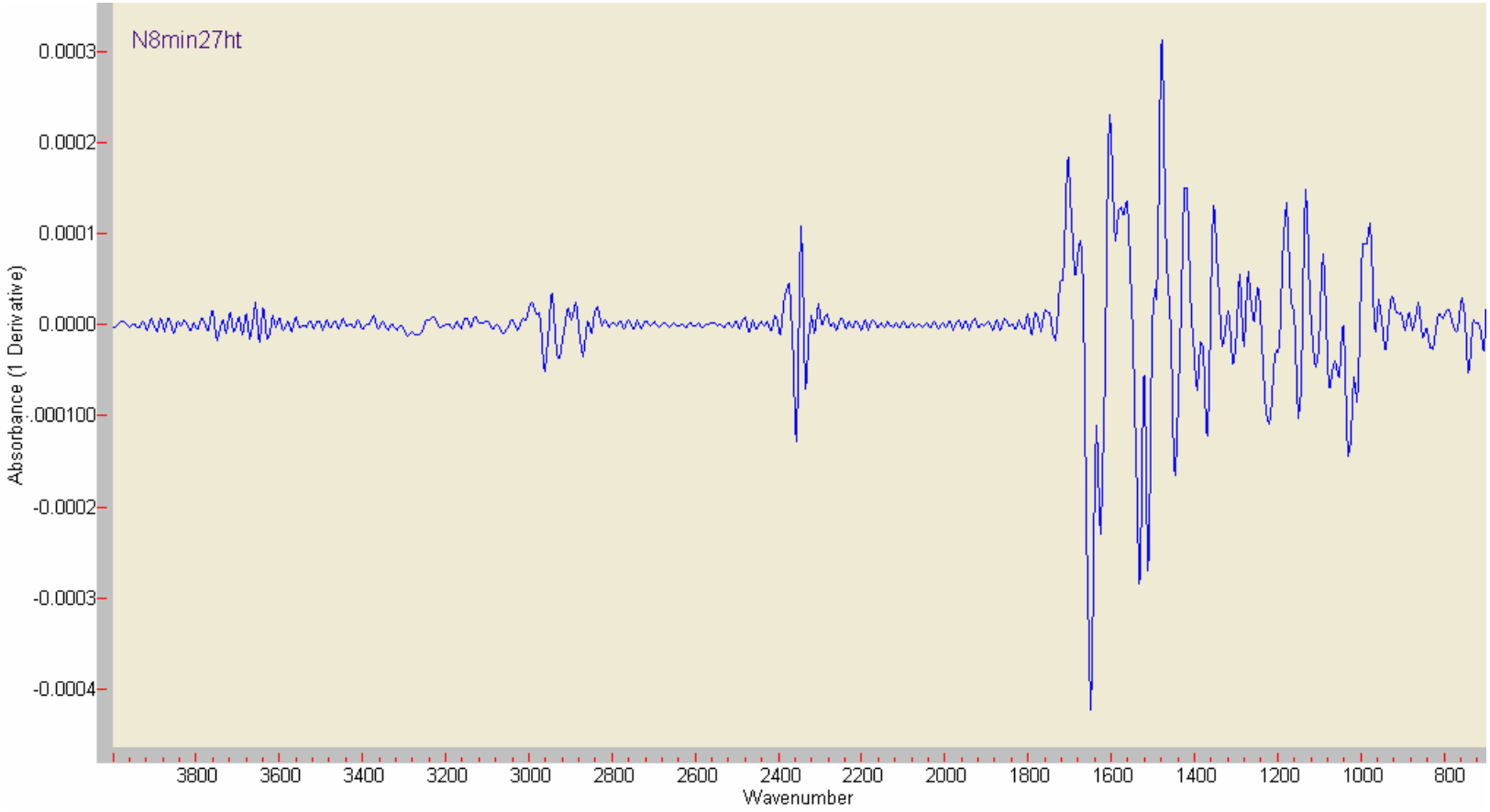
Second derivative spectra of TSAYE grown spores (untreated control)



Second derivative spectra of NAYE grown spores (untreated control)



Second derivative spectra of TSAYE grown spores (8min-PATP treatment)



Second derivative spectra of NAYE grown spores (8min-PATP treatment)