INACTIVATION KINETICS OF BACTERIAL SPORES IN EGG PATTIES BY PRESSURE-ASSISTED THERMAL PROCESSING

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ABSTRACT

The use of pressure-assisted thermal processing (PATP) to inactivate bacterial spores in shelf-stable low-acid foods, without diminishing product quality, has received widespread industry interest. Egg products subjected to prolong periods of heat exposure during thermal retorting show unacceptable changes in quality. Thus, PATP can provide an alternative method for sterilizing egg products due to shorter periods of heat exposure. The purpose of this study was to evaluate the efficacy of the PATP treatment against two process resistant bacterial spores, namely, *Bacillus stearothermophilus* and *Bacillus amyloliquefaciens*. In two separate experiments, egg patties inoculated with *Bacillus stearothermophilus* and *Bacillus amyloliquefaciens* spores were PATP treated over a range of pressure and temperature conditions. Thermal inactivation of the spores served as the control. In general, application of pressures accelerated the inactivation of both the spores when compared to the thermal-only treatment. An increase in process pressure (at constant temperature) within the 400-700 MPa range increased the inactivation rates significantly. Similarly, an increase in temperature (at constant pressure) from 95°C-121°C also enhanced the lethality of the process against the spores. While thermal inactivation of spores followed a log-linear relationship, the PATP inactivation curves were mostly biphasic, with a rapid initial inactivation region followed by a characteristic
tailing during extended pressure-hold times. There appears to be no correlation between thermal and pressure resistance. Among the non-linear models tested, the Weibull model was most suitable for describing PATP inactivation of *B. stearothermophilus* and *B. amyloliquefaciens* spores in the egg matrix.
DEDICATION

I dedicate this work to my parents Rajan Babu and Akila Rajan whose blessings and prayers were the driving force for the successful completion of this thesis.
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CHAPTER I

INTRODUCTION

1.1 Introduction

One of the major food processing methods that has been long used to extent shelf life of low-acid food products is thermal sterilization. It involves the application of high temperatures for a long time in order to completely inactivate harmful bacterial spores of public safety concern, especially that of Clostridium botulinum. Though severe heat treatment produces microbiologically safe food, it degrades the nutritional and quality attributes of the food. In the past few decades, there has been an increased consumer demand for high quality minimally processed, fresh-like and microbiologically safe low-acid shelf stable food products. In response to these needs several new alternate food processing technologies such as pressure assisted thermal processing (PATP), ohmic heating, irradiation, radio frequency and microwave heating are being investigated by the food industry. These technologies have been developed with emphasis on lowering thermal abuse to products. Among the emerging technologies, PATP is of interest because of its ability to inactivate bacterial spores at moderate pressure-temperature conditions. PATP involves simultaneous application of elevated pressures (up to 900 MPa) and sub-retorting temperatures (90-120 °C) to a preheated food for sterilization.
Some of the advantages of PATP over traditional thermal processing include reduced process times, retention of freshness, flavor, texture, color, minimal vitamin losses, no or minimal thermally induced off-flavors and changes in the functionality of food.

1.2 High pressure processing

Since late 1800 high pressure processing has been known to inactivate yeasts, molds and bacteria. However, due to the lack of practical, economical and reliable HPP equipment till early 1980s, the application of high pressure for food preservation had not been commercialized. The choice of processing pressure and temperature is dependent upon the product to be treated and treatment desired (pasteurization or sterilization). For pasteurization, moderate pressures (up to 600 MPa) with or without heat is sufficient, since moderate pressure at room temperature can inactivate most of the vegetative microorganisms. On the other hand, for sterilization, combination of elevated pressures (up to 900 MPa) and temperatures (90-120°C) are required. Product characteristics such as composition, pH, and water activity may affect the physical PATP sterilization process and the microbial inactivation kinetics.

1.3 Applications of high pressure processing

Currently, HPP is primarily used for the pasteurization of high-value and superior heat sensitive products. Commercially available pressure pasteurized products include guacamole, oysters, ham, fruit jellies and jams, fruit juices, pourable salad dressings, salsa, poultry and rice products. Typical processing costs are estimated to be 3-10 cents/pound more than thermal processing. The quality of pressure pasteurized
food is similar to that of fresh food products and the quality degradation is primarily influenced by subsequent storage and distribution rather than the pressure treatment itself. PATP processed low-acid shelf stable foods are not commercially available as yet due to lack of sufficient kinetic data on the inactivation of bacterial spores during PATP. Value added ready to eat meals such as shelf-stable omelets, mashed potatoes and soups are examples of some of the low-acid food products that may benefit from PATP treatment.

1.4 Problem Statement

There is insufficient research to predict the inactivation of different types of spores suspended in food products under various pressure-thermal conditions. Food products could have a protective effect on the spores during PATP. Further, process non-uniformity issues (i.e. presence of thermal gradient within processed volume) may also significantly influence spore inactivation in food products during PATP. Thus, in order to produce a shelf-stable food product using PATP technology, it is necessary to investigate spore inactivation in the desired food product under controlled process conditions. Preservation of eggs by traditional thermal processing leads to unacceptable changes in color, texture and flavor. Recently, the food industry has revealed interest in using PATP to produce shelf-stable egg products. For commercialization of PATP sterilized egg products, it is necessary to demonstrate that PATP can inactivate highly resistant bacterial spores in the food matrix.

Due to difference in the mechanism of inactivation, combined pressure-thermal resistance of bacterial spores may be different from that of thermal resistance. Limited studies have compared the thermal versus combined pressure-thermal resistance of bacterial spores
under controlled process conditions. Further, most of the prior PATP spore inactivation studies did not determine spore inactivation over a range of pressure and temperature conditions. Thus, the pressure and temperature coefficients (zp and zT) of bacterial spores during PATP are yet to be characterized.

While, linear kinetic model parameters such as “D and Z values” have been used to describe thermal resistance of spores, non-linear kinetic models may best describe combined pressure-thermal resistance of spores. Studies comparing linear and non-linear models under similar process conditions are limited. The interpretation of “D and z values” in terms of process lethality is well understood. However, the influence of combined pressure-thermal treatment on non-linear kinetic model parameters is largely unknown.

The most pressure resistant bacterial spore known till this date is that of the Clostridium botulinum. Since these spores are pathogenic there is an immediate research need to identify highly baro-resistant non-pathogenic surrogate organisms for industrial PATP validation studies.

1.5 Objective

The research is undertaken with the following objectives:

(1) To study the inactivation of Bacillus stearothermophilus ATCC 7953 spores in egg patties by pressure assisted-thermal processing (PATP).
(2) To compare the efficacy of PATP and conventional thermal processing against Bacillus stearothermophilus ATCC 7953 spores inoculated in egg patties or suspended in deionized water.

(3) To determine kinetic inactivation parameters of Bacillus amyloliquefaciens Fad 82 spores in egg patties over a range of PATP conditions and to document the pressure ($z_p$) and temperature ($z_T$) coefficients of B. amyloliquefaciens Fad 82 spores.

(4) To evaluate the acceptability of selected non-linear models to describe PATP spore inactivation.
CHAPTER 2

REVIEW OF LITERATURE: MICROBIAL EFFICACY OF PRESSURE ASSISTED THERMAL PROCESSING

Application of high pressures to preserve foods is gaining popularity in the food industry because of its ability to inactivate pathogenic microorganisms with reduced heat treatment, thereby preserving the nutritional and sensory characteristics of the food without sacrificing shelf-life. Though this technology has been widely used for years in material and process-engineering industry (Hoover, Metrick, Papineau, Farkas, & Knorr, 1989), the food industry has not been able to exploit this technology until recently due to limitations in process control and lack of suitable instrumentation (Rastogi, Raghavarao, Balasubramaniam, Niranjan, & Knorr, 2005). Both liquid foods and solid foods with sufficient moisture content can be subjected to HPP. However, food products containing large quantity of air pockets such as marshmallows and strawberries are not suitable candidates for this type of processing. Vegetative microorganisms can be inactivated by application of high pressures (200-600 MPa) at room temperatures (Cheftel, 1995; Smelt, 1998), whereas bacterial spores are highly pressure resistant, and therefore, requires high pressures in combination with temperatures ranging from 90-120°C for inactivation (Malladis & Drizou, 1991; Cheftel, 1995; Miglioli, Gola, Maggi, Rovere, Carpi, Scaramuzza, & Aglio, 1997; Ting, Balasubramaniam, & Raghubeer, 2002).
2.1 Principles Involved

The following principles generally govern pressure-assisted thermal processing (PATP) process:

(1) **Le Chatelier’s principle**: Application of pressure results in decrease in volume (and vice versa). Thus any reaction that involves decrease in volume is accelerated during pressurization.

(2) **Isostatic principle**: The applied pressure is uniformly transmitted throughout the sample. Hence any pressure process is independent of volume, and pressure gradients do not exist.

(3) **Arrhenius relationship**: This describes the effect of temperature on the rate of a reaction. Accordingly, rate of a reaction increases with increasing temperature. Application of pressure with temperature may have a synergistic or antagonistic effect on increasing the rate of reaction.

(4) Pressure leads to increased ionization. This results in a negative and reversible pH shift, depending on the chemical nature of the buffer or food product.

2.2 Pressure-assisted thermal processing equipment

The basic parts of high pressure food processing equipment are shown in the figure 2.1. Typically, the equipment consists of 1) pressure vessel; 2) two end closures; 3) high pressure pump and intensifier for generating targeted pressures; and 5) system control and instrumentation. The pressure vessel can be of monobloc (<600 MPa) or wire wound design (for pressures up to 1000 MPa) and it can come in various volumes depending on
Figure 2.1. Typical components of a high pressure food processor.
the requirement. Currently, both batch systems and semi-continuous systems are available. While the semi-continuous system can process only liquid products, batch systems can process both liquid and solid products. In a typical batch process, the pre-packaged food material is loaded into the vessel and filled with a pressure transmitting liquid (water or glycol). The vessel is then closed and the desired through compression of the pressure transmitting fluid. After a specified hold time at target pressure the vessel is depressurized, and the product is unloaded. Holding time at pressure may vary between 5 to 10 min depending on the food material to be processed.

2.3 Pressure-assisted thermal process

A schematic PATP process flow diagram for producing shelf-stable low acid foods is shown in Figure 2.2. The food material is vacuum packaged in a flexible pouch, preheated to a certain initial temperature and then loaded into a high pressure chamber filled with a pressure transmitting fluid (water is the pressure transmitting fluid of commercial choice). The chamber is then pressurized, during which the product temperature further increases as a function of the target pressure and its heat of compression. Product temperature under pressure is maintained, provided there is no heat loss to the environment. After a specified hold time the chamber is depressurized and the temperature of the food product drops back close to its initial temperature. A typical time-temperature curve for a PATP treatment is shown in Figure 2.3.
Food sample is then preheated to a certain initial temperature (75-90°C) in a water bath.

Preheated food is loaded into the pressure chamber.

Food sample cooled in a water bath.

Food placed inside high barrier plastic pouch and vacuum packaged.

Food sample is then preheated to a certain initial temperature (75-90°C) in a water bath.

Image: Figure 2.2. Steps and conditions involved in a typical batch PATP process for producing sterilized shelf-stable products.
Figure 2.3. Typical pressure-temperature curve during batch PATP process.
2.4 Microbial Inactivation

2.4.1 Inactivation of vegetative microorganisms

Most of the vegetative bacteria can be inactivated by the application of moderate pressures (200-600 MPa) at ambient temperatures (Cheftel, 1995; Smelt, 1998). Gram-positive bacteria such as *Listeria monocytogenes* and *Staphylococcus aureus* are more resistant to pressure than some of the gram-negative bacteria such as *Yersinia enterocolitica, Aeromonas hydrophila* and *Campylobacter* (Smelt, 1998). Significant strain-to-strain variation in resistance is also possible (Alpas, Kalchayanand, Bozoglu, & Ray, 2000; Simpson & Gilmor, 1997; Tay, Shellhammer, Yousef, & Chism, 2003). Apart from type of vegetative microorganism, the pressure resistance also depends up on the growth phase of the organism. Mackey, Forestiere, & Isaacs (1995) observed that vegetative cells in the stationary and dormant phases of growth are much more pressure resistant than those in the early growth phase. These researchers observed that stationary phase *Listeria monocytogenes* cells were reduced by 1.3 logs when treated at 400 MPa for 10 minutes, whereas early growth phase cells were reduced by almost 7 logs when subjected to the same processing conditions.

Various morphological changes such as cell lengthening, separation of cell membrane from the cell wall, compression of vacuoles, coagulation of proteins, release of intracellular constituents, etc, have been observed due to application of high pressures to microorganisms. However, the precise mechanism of action of high pressure on microorganisms is not completely known (Hoover, Metrick, Papineau, Farkas, & Knorr, 1989). Some researchers (Cheftel, 1995) suggested that high pressure inactivation could be due to denaturation of bacterial membrane bound ATPase (F₀F₁ ATPase). The
denatured ATPase disrupts the transport of protons across the membrane and thus the cell dies due to acidification (Smelt, Rijke, & Hayhurst, 1995). Other researchers (Thakur & Nelson, 1998) suggested that changes in the structure of the cell membrane during pressurization could alter its permeability, which in turn could affect the fluid transport and respiration of the microbes. Thus, the microbes eventually die because of starvation of proper nutrients.

2.4.2 Inactivation of bacterial spores

The extremely high resistance of bacterial spores to pressure treatment at ambient temperatures was reported as early as in 1918 (Larson, Hartzell, & Diehl, 1918). Subsequent research in the latter half of the century revealed that pressure alone (up to 1000 MPa) is not sufficient to inactivate spores at ambient temperature. Nakayama, Yano, Kobayashi, Ishikawa, & Sakai (1996) reported that pressure treatments of 981 MPa for 40 min did not inactivate spores of *Bacillus stearothermophilus*, *Bacillus subtilis* and *Bacillus licheniformis* at 10°C. Similarly, no significant reduction of *Clostridium sporogenes* spores was observed even when treated with pressures over 1500 MPa at 20°C (Maggi, Gola, Rovere, Miglioli, Aglio, & Lonneborg, 1996). Thus, it was concluded that pressure alone would not provide a practical method for sterilizing low-acid food. However, further research revealed that application of high pressure at elevated temperature could result in a lethal reduction of spores (Seyderhelm & Knorr, 1992; Cheftel 1995; Miglioli, Gola, Maggi, Rovere, Carpi, Scaramuzza, & Aglio, 1997; Ting, Balasubramaniam, & Raghubeer, 2002)
Knorr & Seyderhelm (1992) demonstrated that log reduction of *B. stearothermophilus* spores was higher when subjected to pressures of 200 MPa at 90°C than when conducted at lower temperatures (70°C or 80°C). Similarly, an increase in process temperatures from 35°C to 55°C at 827 MPa increased *Clostridium botulinum* Type E (Alaska and Beluga) spore inactivation (Reddy, Solomon, Fingerhut, Rhodehamel, Balasubramaniam, & Palaniappan, 1999). Rovere, Lonnerborg, Gola, Miglioli, Scaramuzza, & Sgriccia (1999) showed that inactivation of *C. sporogenes* spores is possible by applying pressures of 600-800 MPa at temperatures above 100°C during the pressure treatment. Thus, temperature plays a crucial role in high pressure inactivation of bacterial spores. Although, combinations of high pressures and elevated temperatures result in lethal reduction of spores during PATP, there seems to be no correlation between pressure and thermal resistance of spores. Nakayama et al. (1996) reported that spores of *Bacillus megaterium* were 3.4 times more resistant than those of *B. stearothermophilus* at 600 MPa. Conversely, the spores of *B. stearothermophilus* were 246 times more heat resistant (100°C) than those of *B. megaterium*. This suggests that the mechanism of PATP inactivation of bacterial spores could be different from that of thermal inactivation of spores.

### 2.5 Factors influencing microbial resistance

#### 2.5.1 Influence of process conditions

The extent of PATP spore inactivation is a function of processing conditions such as target pressure, initial product temperature, process temperature and treatment time. For each spore there appears to be a threshold pressure and temperature below which no
significant reduction occurs irrespective of the treatment time. Miglioli et al. (1997) reported that PATP treatment at 1500 MPa and 20°C for 5 min did not kill any spores of *C. sporogenes*. However, by increasing the process temperature to 90°C, they were able to achieve 5 log reductions at 800 MPa with the same hold time. Similarly, *B. stearothermophilus* spores showed no significant inactivation when treated at 1000 MPa and 10°C for 40 min (Nakayama et al., 1996). However, almost 3 log reductions were observed when treated at 350 MPa and 80°C for 30 min (Knorr & Seyderhelm, 1992). Similar results have been reported in other *Bacillus* and *Clostridium* species (Taki, Awao, Toba, & Mitsuura, 1991; Reddy et al., 1999; Okazaki, Kakugawa, Yoneda, & Suzuki, 2000).

The mechanism of spore inactivation under PATP is not well understood. At lower pressure and temperature ranges (50-400 MPa and 35-60°C) spores are believed to germinate and then the germinated cells are subsequently inactivated (Heinz & Knorr, 1998; Wuytack, Boven, & Michiels, 1998; Wuytack, Soons, Poschet, & Michiels, 2000; Knorr 1999; Ananta, Heinz, Schuttler, & Knorr, 2001). Under these conditions there is not only a decreased reduction, but also an extremely long processing time is required to achieve the reduction. However, it is generally accepted that pressure treatment above 600 MPa and temperature greater than 70°C cause direct inactivation of the spores without germination. Malladis & Drizou (1991) speculated that at higher temperature and pressure, the relative effect of pressure in reducing heat resistance was diminished. This was confirmed by the work of Ananta et al. (2001), who observed that at 105°C and 121°C, varying the pressure between 300 and 600 MPa did not significantly affect the
inactivation of *B. stearothermophilus* in mashed broccoli. Similar observations were also made by De heij, Ludo, Moezelaar, Hoogland, Matser, & Van de Berg (2003).

### 2.5.2 Influence of food matrix

Inactivation of bacterial spores by PATP could be significantly affected by food composition. Reddy, Solomon, Tetzloff, & Rhodehamel (2003) observed that *Clostridium botulinum* type A strain 62-A spores showed similar inactivation trends in both phosphate buffer and crabmeat blend during PATP. Similarly, Raso, Gongora-Nieto, Barbosa-Canovas, & Swanson (1998) reported that the inactivation of *Bacillus cereus* spores was unaffected by the presence of milk fats. On the other hand, Ananta et al. (2001) observed that cocoa mass had a protective effect on the inactivation of *B. stearothermophilus* ATCC 7953 spores during PATP. The authors concluded that protective effect of cocoa mass was not due to the presence of fat by itself, but due to the lower water activity in the system.

These results suggest that bacterial spore inactivation in buffers or laboratory media cannot always be extrapolated to real food situations (Smelt, 1998). Since limited PATP spores inactivation studies are available in food products, further investigation is required to determine the effect of different foods (containing different food components) on PATP spore inactivation.

### 2.5.3 Influence of pH

Application of pressure enhances the ionic dissociation of water, with a corresponding decrease in pH by 0.73 units for every 100 MPa of pressure applied at 25 °C (Cheftel, 16
Thus most food materials and buffers undergo a transient and reversible pH change during PATP, and the magnitude of the shift will depend on the pressure and temperature levels used. In addition, the pH shift also depends on the characteristics of the buffer. Results of previous studies have shown that the pH of food system or buffer significantly influences the inactivation of spores subjected to PATP (Roberts & Hoover, 1996; Raso et al., 1998; Stewart, Dunne, Sikes, & Hoover, 2000; Balasubramanian & Balasubramaniam, 2000). Stewart et al. (2000) reported that counts of C. sporogenes PA3679 in phosphate buffer at pH 7 were reduced by less than 0.5 log units when exposed to 404 MPa at 25°C for 30 min. However, decreasing the pH of the phosphate buffer to 3, increased the inactivation by almost 2 log units under the same process conditions. Robert & Hoover (1996) found an additional 1.5 log reduction of B. coagulans 7050 spores when the pH was lowered form 7 to 4 during pressurization at 400 MPa and 45°C for 30 min. Similarly, a change in pH of citrate-phosphate buffer from 7 to 3 increased the inactivation of B. subtilis spores when treated at 827 MPa and 75°C for 5 min. Thus it is clear that a decrease in the pH of the medium from neutrality enhances the inactivation of spores when subjected to PATP.

2.5.4 Influence of water activity

Limited studies investigated the effect of water activity (aw) on PATP inactivation of bacterial spores. In general, reducing aw of the food medium seems to protect spores against PATP inactivation. Ananta et al. (2001) reported that a lower aw in cocoa mass resulted in a marked reduction in the inactivation of B. stearothermophilus spores at 600 MPa and temperatures of 70, 80 and 90°C. Furukawa & Hayakawa (2000) observed that
addition of glucose solution, sodium chloride and ethanol to *B. stearothermophilus* spores suspended in phosphate buffer (12%, 6% and 20% w/v respectively) reduced the inactivation rates during PATP at 600 MPa and 95°C. The authors concluded that the addition of food additives decreased the water activity of the phosphate buffer, which in turn decreased the inactivation of the spores. Lowering the water activity of the spore solution by addition of sucrose also protected *B. cereus* spores against the inactivation effect of PATP (Raso et al., 1998). Similarly, Sale, Gould, & Hamilton (1970) observed that the inactivation of *B. coagulans* decreased due to the addition of sodium chloride and calcium chloride to phosphate buffer. They attributed this reduction to the higher concentrations of ions. However, this protective effect on spores could have been due to lower water activity of phosphate buffer.

Though the results of several studies indicate that reducing the water activity of the suspending medium directly protect the spores against PATP inactivation, it can also be expected, that the recovery of spores sub-lethally injured by PATP could be inhibited by the lower water activity of the medium (Smelt, 1998). Further research is required to confirm this hypothesis.

### 2.5.5 Role of process non-uniformities on spore inactivation

During PATP, pressure is instantaneously and uniformly distributed at all points within the pressure chamber. However, deviations from process uniformity can occur due to temperature gradients between the test samples, pressure transmitting fluid and pressure vessel after compression (Ting et al., 2002, Rasanayagam, Balasubramaniam, Ting, Sizer, Bush, & Anderson, 2003, Balasubramaniam, Ting, Stewart, & Robbins, 2004). Thermal
gradients within the pressure vessel during the pressure-hold could significantly influence spore inactivation.

All compressible substances change temperature during physical compression. The magnitude of the temperature increase depends in part, on the compressibility of the substance and its specific heat (Balasubramanian and Balasubramaniam, 2003). Water, for example, undergoes a temperature rise of 2-3°C per 100 MPa and this rise is dependant on the initial temperature of the sample. Fats and oils have much higher compression heating values and they do not show dependence on the initial temperature (Rasanayagam et al., 2003). Often, oil-based solutions such as glycol, vegetable oil and silicone oil are used as pressure-transmitting fluids in lab scale high pressure equipments. On the other hand, water is the pressure-transmitting fluid of choice in commercial high pressure systems. Due to difference in compressibilities between water and oil based systems, comparing spore inactivation data in these systems could lead to errors due to unintended thermal effects (Balasubramaniam, 1999; Ting et al., 2002). Balasubramanian and Balasubramaniam (2003) reported that use of pressure transmitting liquids with different compression heating characteristics significantly influenced the inactivation of \textit{B. subtilis} spores (2-3 log difference under similar processing conditions). The effects of process non-uniformities on spore inactivation require further investigation in order to commercialize PATP technology for producing shelf stable low-acid foods.

2.6. \textbf{Surrogate organism for validating pressure-assisted thermal processing}

One of the main challenges in using PATP for producing low acid shelf-stable foods is to demonstrate the effectiveness of the process in eliminating highly baro-resistant
pathogenic bacterial spores. Microbial studies to date indicate that pathogenic *C. botulinum* type A and B spores are the most resistant to pressure (Reddy, Solomon, Tetzloff, Balasubramaniam, Rhodehammel, & Ting, 2001, Reddy et al., 2003). It is highly undesirable to inoculate food, equipment or the production environment with high levels of pathogenic spores while trying to assess the efficacy of the PATP process. Hence, it is important to identify a surrogate organism for PATP process validation studies. Generally, surrogates should be non-pathogenic, should respond to a particular treatment in a similar manner to the target pathogen and its resistance to a particular treatment should be equivalent or greater than that of the pathogen (FDA, 2001). A surrogate microorganism should also have stable and consistent growth characteristics, should be easily prepared to yield high-density populations and it should be easily enumerated using rapid and inexpensive detection systems (Busta, Suslow, Parish, Beuchat, Farber, Garrett, & Harris, 2003). It is also important that the surrogate organism is easily distinguishable from other microflora and it will not establish itself as a spoilage organism on the equipment or production areas (Busta et al, 2003).

In order to identify a suitable surrogate for the PATP treatment, pressure resistances of *Clostridium sporogenes* and several other non-pathogenic spores belonging to the *Bacillus* species have been studied (Hayakawa, Kanno, Yoshiyama, & Fujio, 1994; Crawford, Murano, Olson, & Shenoy, 1996; Miglioli et al., 1997; Rovere, Gola, Maggi, Scaramuzza, & Miglioli, 1998; Stewart et al., 2000; Margosch, Ehrmann, Ganzle, & Vogel, 2004). However, it is difficult to compare their pressure resistances since treatment conditions were often different and some of the studies lacked measures to control the temperature achieved during PATP. Reddy et al. (2003) observed that spores
of *C. sporogenes* PA 3679 are more pressure resistant than *C. botulinum* type E spores, but less pressure resistant than *C. botulinum* type A spores. Recently, Margosch et al. (2004) reported that spores of *Bacillus amyloliquefaciens* are more pressure resistant than all other spores of *Bacillus* or *Clostridium* species, including strains of *C. botulinum* type A, and hence they could be used as surrogate spores for validating PATP studies. Their study revealed that pressure treatment at 800 MPa for 16 min (process temperature varied between 70°C and 100°C) did not reduce the spore count of *B.amyloliquefaciens* in mashed carrots. However, it is important to note that the deviation in process temperature values reported by Margosch et al. (2004) was extremely high when compared to the values reported by Reddy et al. (2003). Further, the high pressure equipment used by former had a much slower compression rate (2 MPa/s) when compared to the latter (6 MPa/s). It is possible that a slow compression rate could induce a stress response and hence make the spores more process resistant (Smelt, 1998). Since the above inactivation studies were carried out using different high pressure machines under different conditions, it is not possible to make a fair comparison of the pressure resistance of *B.amyloliquefaciens* and *C. botulinum* type A spores.

Once a suitable surrogate for PATP has been established, the next step is to design a safe process that would reproducibly produce safe products under defined conditions (Sizer, Balasubramaniam, & Ting, 2002). Proposed validation approaches include, considering PATP as a conventional thermal process, demonstrating a 12- D process using inactivation kinetics of *C. botulinum*, demonstrating a 12-D process using a surrogate spore, and mathematical modeling.
2.7. Modeling pressure-assisted thermal processing inactivation curves

Very few studies have reported that the inactivation of bacterial spores by PATP followed first order kinetics (Nakayama et al., 1996; Furukawa & Hayakawa, 2000), which assumes that all spores in a given population have an equal resistance to a combined pressure-thermal treatment. Most researchers reported that PATP spore inactivation curves appear to be concave upwards with a first-order inactivation (linear) period followed by a characteristic tailing (Okazaki et al., 2000; Ananta et al., 2001; Reddy et al., 2003; Margosch et al., 2004).

Predicting PATP spore inactivation curves using the modeling approach can be extremely useful in the development of a safe PATP preservation process. The foundation of any predictive modeling is the estimation of inactivation kinetics over time by use of a mathematical equation. Traditionally, linear survival models have been used to describe microbial survival curves. However, such models cannot accurately predict PATP spore inactivation because of their inability to account for tailing or regions of non-log linear inactivation. Thus, using the D value (time required for a inactivation curve to transverse one log cycle when subjected to a lethal treatment) concept of a linear model to predict PATP spore inactivation curves are not desirable, as they would provide an underestimate of the organism’s resistance (Peleg, 1998).

Over the years several other primary models (describe the change in microbial population over time) have been proposed to predict non-linear survivor curves (Table 2.1). As early as in 1977, Cerf proposed a two parallel-reactions model for describing biphasic curves. In 1992, Whiting & Buchanan proposed a two-phase logistic model to describe non-linear inactivation. However, these kinetic models were not able to fully describe the
<table>
<thead>
<tr>
<th>Model</th>
<th>Formula</th>
<th>Reference</th>
</tr>
</thead>
<tbody>
<tr>
<td>Cerf</td>
<td>( \frac{N}{N_0} = F_1 e^{k_1 t} + (1 - F_1) e^{-k_2 t} )</td>
<td>Cerf (1977)</td>
</tr>
<tr>
<td>Whiting &amp;</td>
<td>[ \log \left( \frac{N}{N_0} \right) = \log \left( \frac{F_1 \left(1 + e^{-t/l_1}\right) + (1 - F_1) \left(1 + e^{-t/l_2}\right)}{1 + e^{t/l_1}} \right) ]</td>
<td>Whiting and Buchanan (1992)</td>
</tr>
<tr>
<td>Buchanan</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Baranyi</td>
<td>[ \log \left( \frac{N}{N_0} \right) = \log \left( q_b + (1 - q_b) e^{-k_{\max} (t - B(t))} \right) ]</td>
<td>Baranyi and Roberts (1994)</td>
</tr>
<tr>
<td>Modified</td>
<td>[ \log \left( \frac{N}{N_0} \right) = Ce^{-e^{BM}} - Ce^{-e^{-B (t - M)}} ]</td>
<td>Bhanduri et al. (1996)</td>
</tr>
<tr>
<td>Gompertz</td>
<td></td>
<td></td>
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<tr>
<td>Log-Logistic</td>
<td>[ \log N = \alpha + \frac{\omega - \alpha}{1 + e^{4\sigma (\tau - \log t)/(\omega - \alpha)}} ]</td>
<td>Cole et al. (1993)</td>
</tr>
<tr>
<td>Weibull</td>
<td>[ \log \left( \frac{N}{N_0} \right) = -bt^n ]</td>
<td>Peleg and Cole (1998)</td>
</tr>
<tr>
<td>Eyring-Arrhenius</td>
<td>[ k = k_e \exp \left{ - \left[ \frac{\Delta V}{RT (P - P_o)} \right] + \frac{E_a}{R} \left( \frac{1}{T} - \frac{1}{T_o} \right) \right} ]</td>
<td>Rodriguez (2002)</td>
</tr>
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</table>

Table 2.1 Models for microbial survivor curves (Refer Appendix for explanation of terms).
tailing behavior due to the assumption of near flat rate of inactivation within the tailing region (Guentert, Mohtar, Linton, Tamplin, & Luchansky, 2005). The model developed by Baranyi & Roberts (1994) could accommodate the description of shoulders and/or tails in addition to the potential linear inactivation, but it used classical log-linear kinetics to conservatively estimate inactivation rate. Thus, this approach prevented adequate description of non-log linear inactivation and did not address tailing. The modified Gompertz equation was used by Bhanduri, Smith, Palumbo, Turner-Jones, Marmer, Buchanan, Zaika, & Williams (1991) to model the non-linear survival curves of *L. monocytogenes* heated in liver sausage slurry. However, Xiong, Xie, Edmondsonm, Linton, & Sheard (1999) described improved prediction performance by Baranyi model over modified Gompertz equation. Cole, Davies, Munro, Holyoak, & Kilsby (1993) proposed the log-logistic equation to describe the non-linear thermal destruction kinetics of microorganisms. Subsequently, this equation has been used to describe the effect of temperature on the non-linear inactivation of *L. monocytogenes* (Stephens, Cole, & Jones, 1994), *Salmonella Typhimurium* (Ellison, Anderson, Cole, & Stewart, 1994) and *C. botulinum* spores (Anderson, McClure, Baird-Parker, & Cole, 1996). In 1998, Peleg and Cole used an alternative approach to describe different shapes of non-linear survival curves. According to this approach, microorganisms in a population have different resistances and the survival curve is a cumulative form of a Weibull distribution of lethal events. This (Weibull) model enables the calculation of time needed to achieve any degree of microbial reduction numerically, without the need to assume any kinetics. Thus, the death time could be calculated from the Weibull model parameters using the equation proposed by Peleg (1998).
\[ t_{\text{lethal}} = (LR/b)^{1/n} \] (1)

where \( b \) and \( n \) are the parameters obtained from the Weibull model and \( LR \) is the desired level of log reductions of a microbial population.

Among all the primary models developed till date, the Weibull and Log-logistic model seem to be most successful in predicting non-linear thermal inactivation of microorganisms under various experimental conditions (Peleg & Cole, 1998, 2000; Mattick, Jorgensen, Wang, Pound, Vandeven, Ward, Legan Lappin-Scott, & Humphrey, 2001; Corradini & Peleg, 2004). Chen & Hoover (2003) evaluated the applicability of the above models to describe the combined effect of pressure and mild heat on the inactivation of \( L. \) monocytogenes in milk. They reported that both the Weibull and the Log-logistic model gave accurate predictions of the high pressure inactivation of \( L. \) monocytogenes at different temperature levels. Since non-linear models for describing PATP spore inactivation curves is not available, it is worthwhile to evaluate the applicability of the above-mentioned non-linear models to predict PATP spore inactivation. The parameters of such non-linear models fitted to PATP inactivation curves would give a more accurate description of the data, since the both the linear and tailing portion of the curves can be incorporated.

Apart from the primary models mentioned above, secondary models that describe the relationship between the primary model parameters and various processing conditions (temperature, pressure, pH and water activity) can be used to predict PATP spore inactivation. Secondary models are generally based on Arrhenius relationships and they use polynomial regression analysis to describe the effects of individual and combined
processing conditions on microbial inactivation rates. Rodriguez (2002) and De Heij et al. (2003) demonstrated that a standard thermodynamic formula (modified Eyring-Arrhenius equation) could be used to describe the relationship between temperature, pressure and microbial inactivation rate. Development of secondary mathematical models to predict pressure inactivation kinetics could aid in the improvement, trouble-shooting and optimization of PATP processes, and it would be an invaluable tool in the validation of high pressure sterilization process for commercial use.
CHAPTER 3

INACTIVATION OF Bacillus stearothermophilus SPORES IN EGG PATTIES BY PRESSURE-ASSISTED THERMAL PROCESSING

3.1 Abstract

The use of pressure-assisted thermal processing (PATP) to inactivate bacterial spores in shelf-stable low-acid foods, without diminishing product quality, has received widespread industry interest. Egg patties were inoculated with Bacillus stearothermophilus spores (10^6 spores/g) and the product was packaged in sterile pouches by heat sealing. Test samples were preheated and then PATP-treated at 105°C at various pressures and pressure-holding times. Thermal inactivation of spores was studied at 121°C using custom-fabricated aluminum tubes; this treatment served as a control. Application of PATP at 700 MPa and 105°C inactivated B. stearothermophilus spores, suspended in egg matrix rapidly, (4 log reductions in 5 min) when compared to thermal treatment at 121°C (1.5 log reduction in 15 min). Spore inactivation by PATP progressed rapidly (3 log reductions at 700 MPa and 105°C) during pressure-hold for up to 100 s, but greater holding times (up to 5 min) had comparatively limited effect. When PATP was applied to spores in water suspension or egg patties, D values were not significantly different. While thermal inactivation of spores followed first-order kinetics, PATP inactivation exhibited non-linear inactivation kinetics. Among the non-linear models
tested, the Weibull model best described PATP inactivation of *B. stearothermophilus* spores in the egg product.

### 3.2 Introduction

Traditionally, thermal processing has been used as the primary method of food sterilization through the application of intense heat, which impairs the quality and nutritional content of the food. Preservation of eggs by traditional thermal sterilization has major challenges. Egg products are normally processed for extended periods under excessive retort temperatures to ensure that harmful bacterial spores are eliminated. Consequently, the final processed product may show rubberiness in texture, greenish color, and poor flavor (Barbosa-Canovas et al., 2004). Recent advances in pressure-assisted thermal processing (PATP) provide an alternative method for sterilization of egg products. The process, in general, involves simultaneous application of elevated pressures (up to 700 MPa) and sub-retorting temperatures (90-120°C) to a preheated food (Balasubramanian & Balasubramaniam, 2003; Matser, Krebbers, Van-de-berg, & Bartels, 2004). One of the unique advantages of the technology is the ability to increase the temperature of the test samples instantaneously; this is attributed to heat of compression, resulting from the rapid pressurization of the sample. This compression heating helps to reduce the severity of thermal effects encountered with conventional processing techniques.

The efficacy of high-pressure treatments against non-sporeforming bacteria has been demonstrated (e.g., Cheftel, 1995; Farkas & Hoover, 2000; Smelt, 1998); however, there are limited studies on the efficacy of combined pressure-temperature treatments on spore
inactivation (e.g., Gola, Foman, Carpi, Maggi, Cassara, & Rovere, 1996; Reddy, Solomon, Fingerhut, Rhodehamel, Balasubramaniam, & Palaniappan, 1999). Pressure alone is not sufficient to inactivate bacterial spores (Crawford, Murano, Olson, & Shenoy, 1996; Farkas & Hoover, 2000; Rovere, Gola, Maggi, Scaramuzza, & Miglioli, 1998). Knorr & Seyderhelm (1992) demonstrated that pressure inactivation of *B. stearothermophilus* spores was greater with treatment at 90°C than at lower temperatures (50°C or 60°C). Similarly, greater inactivation of *Clostridium botulinum* Type E and A spores was reported when the temperature was increased from 35°C to 75°C at 827 MPa (Reddy et al., 1999; Reddy, Solomon, Tetzloff, & Rhodehamel, 2003). According to Ananta, Heinz, Schuttler & Knorr (2001), many of the prior studies investigating the effects of PATP treatments on bacterial spores have been carried out in buffer solutions and thus the knowledge of PATP spore inactivation in real foods is still very limited. Rasanayagam, Balasubramaniam, Ting, Sizer, Bush & Anderson (2003) reported that thermal effects under pressure are also often overlooked. An IFT expert panel further identified the lack of kinetic data for various bacterial spores suspended in food products under various pressure-thermal conditions (IFT, 2000). While, log-linear kinetic models have been traditionally used for thermal processing, recent literature (Peleg & Cole, 1998) suggests that non-log-linear models are better suited for alternative technologies such as high pressure processing. Often, researchers choose to evaluate microbial data using either linear or non-linear models. Studies comparing linear and non-linear models under similar process conditions are limited. The interpretation of “D and z values” in terms of process lethality is well understood. However, the influence of combined pressure-thermal treatment on non-linear kinetic model parameters is largely unknown.
Thus, the objectives of the study were (1) to compare the efficacy of PATP and thermal processing against *B. stearothermophilus* spores, inoculated in egg patties or suspended in deionized water and (2) to evaluate the applicability of selected linear and non-linear models to describe PATP spore inactivation in egg products. Spores of the nonpathogenic flat-sour thermophilic organism, *B. stearothermophilus*, have been used as one of the surrogate organisms for *Clostridium botulinum* in thermal processing (IFT 2000; Tucker, 2001) and its PATP resistance under controlled process conditions in egg matrix was evaluated.

### 3.3 Materials and Methods

#### 3.3.1 Preparation of *Bacillus stearothermophilus* spore suspensions

Spore suspensions of *B. stearothermophilus* ATCC 7953 were prepared as described by Sala, Ibraz, Palop, Raso & Condon (1995). Stock cultures of *B. stearothermophilus* were spread-plated onto a nutrient agar (BD Difco; Becton, Dickinson and Co., Sparks, MD, U.S.A.) that was supplemented with 500 mg/Kg dextrose (BD Difco) and 3 mg/Kg manganese sulfate (Fisher Scientific, Pittsburgh, PA, U.S.A.). The inoculated plates were incubated for 10 d at 55°C before harvesting the spores. The plates were packaged during incubation to prevent drying. The level of sporulation was verified by inspection of the sample colony using a phase contrast microscope. At > 97% sporulation, the plates were flooded with water and the growth was scraped, washed 6-7 times by centrifugation (8000 x g for 20 min at 4°C), and resuspended in deionized water. The suspension was treated with lysozyme (100 μg/ml for 30 min) and trypsin (200 μg/ml for 2 h) to minimize interfering cell debris. After enzyme treatment, the spore suspension was again
washed 4-5 times and finally the spore pellet was diluted to approximately $10^8$ spores/ml using deionized water. The spore suspension was stored at 4°C until use.

### 3.3.2. Inoculation of egg patties for thermal inactivation studies

Frozen egg patties were obtained from a commercial egg processor (Michael Foods, Minnetonka, MN, U.S.A.). The key ingredients of the patties were whole eggs, water, soybean oil, modified food starch, whey solids, salt, natural and artificial flavors, nonfat dry milk, xanthan gum, citric acid and EDTA. The round-shaped (8.9 ± 0.6 cm dia) patties weighed 42.5 ± 7.0 g. A day prior to use, the egg patties were thawed in a refrigerator (4°C), mashed manually in sterile stomacher bags (Fisher Scientific) and then 10.0 g of the mashed patty was transferred to another stomacher bag. Spore suspension (1ml; ~$10^8$ spores/ml) was added to the mashed patties and thoroughly mixed to get a final concentration of approximately $10^6$ spores/g egg patty.

### 3.3.3 Thermal inactivation of spores

The thermal resistance of *B. stearothermophilus* spores, suspended in deionized water, was determined at 121°C using custom-fabricated aluminum tubes (Luechapattanaporn, Wang, Al-Holy, Kang, Tang, & Hallberg, 2004). Spore suspension (1 ml in deionized water) was transferred into each of the six aluminum tubes. The tubes were then submerged simultaneously into a 28-litre circulating oil bath (Fisher Scientific), which was maintained at 121 ± 0.1°C. The come-up time required to reach the desired process temperature of 121°C was monitored using a K-type thermocouple (Omega Engineering, Stamford, CT, U.S.A.). The temperature was recorded using a data logger (IOtech, 31
Cleveland, OH, U.S.A.). The heating time was recorded when the temperature reached 121°C. The first aluminum tube was removed from the oil bath at the end of come-up time (3.33 min). The other aluminum tubes were subjected to five different heating times ranging from 2.5 min to 12.5 min. After the thermal treatments, the samples were immersed promptly into an ice bath to avoid further inactivation. Spores surviving thermal treatment were enumerated as described later. The untreated control samples were heated at 80°C for 30 min to activate the spores before enumeration. For the thermal inactivation experiments using egg patties, 1 g of the mashed egg patties was inoculated with the spore suspension, and transferred into one of the six aluminum tubes. The thermal inactivation of spores in egg patties was conducted using the same procedure as described earlier for deionized water.

3.3.4. Pressure-assisted thermal processing of spores

3.3.4.1 Preparation of samples containing aqueous spore suspensions

Aliquots (5 ml) of *B. stearothermophilus* spore suspensions (10^8 spores/ml) in deionized water were transferred into stomacher bags (10 x 15 cm; Fisher Scientific), which were sealed using an Impulse heat sealer (American International Electric, Whittier, CA, U.S.A.). Each stomacher bag was then placed inside a larger high-barrier plastic pouch and vacuum packaged. The sealed pouches were kept under refrigerated conditions until PATP-treated.

3.3.4.2 Preparation of spore-inoculated egg patties

Frozen egg patties were thawed under refrigeration (4°C). Each egg patty was inoculated with one ml of spore suspension, which was uniformly spread on the patty using a sterile
glass spreader. The inoculated egg patties had a final spore concentration of ~10^6 spores/g. The egg patties were then transferred into stomacher bags (18 x 30 cm; Fisher Scientific), which were then sealed under a vacuum setting of 20 kPa and sealing time of 0.1 s (Sipromac Vacuum Sealer, St-Germain, Quebec, Canada). The bags were further packaged and refrigerated until PATP treatment, as described earlier.

3.3.4.3 Pressure-assisted thermal processing treatment

Pouches containing spore suspensions or spore-inoculated egg patties were preheated to the desired initial temperature using a water bath (Isotemp 928; Fisher Scientific). The preheating time for the spore suspension to achieve the desired initial temperature was 4.5 min, as compared with 27 min for egg patties. Sets of preheated spore samples were enumerated for the spore count (N_0') to determine the effects of preheating on the initial spore population (N_0). No significant difference was observed between (N_0) and (N_0') (data not shown). In order to achieve a maximum final process temperature of 105°C, the initial temperatures of the spore suspension or egg patties were adjusted as a function of final target pressure. The initial temperature was estimated based on the heat of compression of glycol and egg patties (Balasubramaniam, Ting, Stewart, & Robbins, 2004). Preheated samples were then immediately loaded into a high-pressure food processor (QFP-6, Flow Autoclave Systems, Columbus, OH, U.S.A) and subjected to the desired pressure level (400-700 MPa) over different hold times (0-300 s). PATP come-up times were 1.9 min, 2.2 min and 2.4 min for 400, 600 and 700 MPa respectively. The process hold-time did not include the pressure come-up or the de-pressurization times. The initial temperature of the pressure-transmitting fluid (equal volumes of water and glycol) (Houghton Safe-620TY, Houghton International Inc., Valley Forge, PA, U.S.A.)
was also adjusted to a temperature close to that of the samples, taking the heat of compression of glycol-water mix into account. The pressure and temperature data were recorded at regular intervals during the pressure come-up, holding and depressurization times (Figure 3.1). After depressurization, the samples were cooled immediately in an ice bath. The untreated control samples (non-pressure treated spore suspension or inoculated egg patties) were heated at 80°C for 30 min, to activate the spores for enumeration purposes. The contents of the pouches containing spore suspension (control and pressure-treated) were directly used for enumeration. Pouches containing inoculated egg patties (control and pressure-treated) were opened aseptically, their contents were mixed with an equal volume of peptone water (1 g/L) and homogenized for 2 min in a stomacher (Seward Lab Stomacher Norfolk, UK) to release the surviving spores in the aqueous solution. The aqueous solution was then used for the enumeration of survivors.

3.3.5. Enumeration of survivors

Heat and pressure-treated samples were serially diluted in peptone water (1 g/L). Dilutions were pour-plated using plate count agar (PCA; BD Difco), and the plates were incubated at 55°C for 48 h before enumeration. The enumeration of colonies was done using a dark-field Quebec colony counter (Leica Microsystems, Ontario, Canada.)

3.3.6 Data analysis

Multiple analysis of variance using the General Linear Model (GLM) procedure of Statistical Analysis Software (SAS, release 9.2.1, SAS Institute Inc, Cary, NC, U.S.A) was done to analyze the effect of pressure level, holding time, and pressure-time
Figure 3.1 Sample pressure-temperature profile observed during pressure-assisted thermal processing. Data presented are mean values of two independent trials.
interactions on spore inactivation. A similar analysis was done using thermal treatments data. Significant mean differences among pressure treatments and holding times were calculated by Fisher's least significant difference at 5% of significance level ($P < 0.05$). All the pressure experiments were replicated on separate dates while thermal inactivation studies were done in triplicate.

3.3.7 Modeling of inactivation curves

Modeling parameters were calculated using spore count ($N_0$) after process (thermal or pressure) come-up times as the initial point of the survivor plots.

3.3.7.1 First-order kinetics

The decimal reduction time (D value) was calculated based on the reduction in microbial population at a constant pressure (or temperature) from the linear portion of the survivor curve using the equation

$$\log \frac{N}{N_0} = \frac{-t}{D}$$

(1)

where $N_0$ = Spore count after process (thermal or pressure) come-up time, $N$ = number of survivors after being exposed to a lethal treatment for a specific time (t).

3.3.7.2 Log-logistic model

Cole, Davies, Munro, Holyoak, & Kilsby (1993) described non-linear thermal inactivation of microorganism using the following equation:

$$\log N = a + \frac{\omega - a}{1 + e^{4a(t-\log t)/(\omega-a)}}$$

(2)

where $a$= upper asymptote (log CFU/ml)
ω = lower asymptote (log CFU/ml)

σ = the maximum rate of inactivation (log (CFU/ml)/log min)

τ = the log time to the maximum rate of inactivation (log min)

Since log t at 0 min is not defined, a small value of t (t = 10^(-6)) was used to approximate t = 0. Log N_0 was calculated from Eq. (2) and substituted back into Eq. (2) to avoid the direct use of different initial numbers (Chen & Hoover, 2003):

\[
\log \frac{N}{N_0} = \log N - \log N_0 = -\frac{\omega - \alpha}{1 + e^{4\sigma(\tau - \log t)/(\omega - \alpha)}} - \frac{\omega - \alpha}{1 + e^{4\sigma(\tau + 6)/(\omega - \alpha)}}
\]  

(3)

By taking A = ω - α, the number of parameters were reduced from 4 to 3:

\[
\log \frac{N}{N_0} = -\frac{A}{1 + e^{4\sigma(\tau - \log t)/A}} - \frac{A}{1 + e^{4\sigma(\tau + 6)/A}}
\]  

(4)

3.3.7.3 Weibull model

The following equation is a cumulative form of the Weibull distribution and it was used by Peleg & Cole (1998) to describe microbial survival.

\[
\log \frac{N}{N_0} = -bt^n
\]  

(5)

where b and n are the scale and shape factors (Peleg & Cole, 1998).

3.3.7.4 Model comparison and curve fitting

Mean square error (MSE), R^2 and Accuracy factor (A_f) values were used to compare the above models. Better fitting models produce smaller MSE values (Neter, Kutner, Nachtsheim, & Wasserman, 1996). Higher R^2 values means that the model is adequate to describe the data (Neter et al., 1996) and an A_f value closer to 1 indicates that the model produces a perfect fit to the data. The curve fitting was done using the non-linear (PROG
NLIN) procedure of the statistical program (SAS) and the MSE and $R^2$ values were obtained from the analysis. The $A_f$ values were calculated using the following equation:

$$A_f = 10 \sum \frac{\log(\text{predicted} / \text{observed})}{n}$$

(6)

### 3.4 Results and Discussions

*Bacillus stearothermophilus* spores exhibited different inactivation patterns during thermal and PATP treatment. While thermal inactivation of spores follows first-order kinetics, PATP- treated spores clearly showed divergence from this trend and exhibited a non-linear behavior beyond 100-s pressure-holding time (Figure 3.2). This non-linear survivor curves may indicate that HPP has multiple targets of action in bacterial spore. The observed tailing phenomenon could also be attributed to cell clumping, stress adaptation, or the genetic heterogeneity of the microbial population (Tay, Shellhammer, Yousef, & Chism, 2003). At the initial stages of the pressure treatment (pressure-holding time of less than 100 s), there was rapid inactivation, but for pressure-holding time beyond 100 s microbial inactivation rates decreased at all the pressure levels tested. Similar trends in inactivation have been observed in other work involving HPP treatments of *Listeria monocytogenes*, *Escherichia coli* and spores of *B. stearothermophilus* suspended in mashed broccoli (Ananta et al., 2001; Hoover, Metrick, Papineau, Farkas, & Knorr, 1989).

Decrease in inactivation rate seems to coincide with the decline in process temperature during the PATP treatment (Figures 3.1 and 3.2). While the temperature increase (due to
Figure 3.2 Log survivor fraction of *Bacillus stearothermophilus* spores, in different media, during thermal treatment and pressure-assisted thermal processing (PATP). Come-up time for the thermal treatment was 3.33 min and that for PATP was 2.4 min. Media: (A) egg patties and (B) deionized water. Process: (■) heating at 121°C, and (●) PATP at 700 MPa and 105°C.
heat of compression) in polar compounds such as water readily follow pressurization rate, non-polar compounds such as fats and oils exhibit a shouldering effect, and they maximum process temperature 30-60 s after the targeted pressure is attained (Rasanayagam et al., 2003). Due to differences in heat of compression between test sample, pressure-transmitting fluid and pressure vessel, a temperature gradient develops during PATP of food samples. In the absence of insulation in the pressure vessel, cooling of the sample medium by conventional heat transfer occurs. During the first 100 s of pressure-hold, the heat of compression generated within the food sample and pressure-transmitting medium negate the convectional loss of heat to the surroundings. However, beyond the 100-s pressure-hold time, heat loss to the surroundings becomes more dominant than the temperature increase due to heat of compression, causing the temperature of the sample to decline steadily from the process temperature of 105°C to 95 ± 1°C at the end of the 5 min hold (Table 3.1). The survivor plots also showed deviation from linearity beyond the 100 s hold period (Figure 3.2). Statistical analysis of the data also revealed that microbial inactivation during longer pressure-hold times (beyond 3 min) were not significantly different (P< 0.05), highlighting a tailing effect.

With the maximum processing-temperature at 105°C, an increase in the pressure level from 400 MPa to 600 MPa considerably decreased spore viability (Figure 3.3). However, an increase in pressure from 600 MPa to 700 MPa (keeping processing temperature constant at 105°C) did not affect the spore viability significantly (P < 0.05). Similar observations have been made by Gola et al. (1996). Further, higher inactivation was achieved during pressure come-up times of 600-and 700-MPa treatments than at 400 MPa. Though the final process temperature was kept at 105°C, depending upon the target
Figure 3.3 Observed and predicted (as fitted by Weibull model) survivor fraction of *Bacillus stearothermophilus* spores in egg patty by pressure-assisted thermal processing at different pressure levels. Observed: (◆) 400 MPa, (○) 600 MPa and (▲) 700 MPa. Predicted: (—) 400 MPa, (---) 600 MPa and (----) 700 MPa.
Sample | Preheating time (min) | Target pressure (MPa) | Conditions within the high pressure processor

<table>
<thead>
<tr>
<th>Water Jacket temperature (°C)</th>
<th>Initial</th>
<th>Maximum</th>
<th>Final</th>
<th>After depressurization</th>
</tr>
</thead>
<tbody>
<tr>
<td>Egg patty</td>
<td>27</td>
<td>700</td>
<td>80</td>
<td>71.6 ± 1.2</td>
</tr>
<tr>
<td>Egg patty</td>
<td>27</td>
<td>600</td>
<td>80</td>
<td>74.5 ± 1.2</td>
</tr>
<tr>
<td>Egg patty</td>
<td>27</td>
<td>400</td>
<td>80</td>
<td>84.9 ± 0.7</td>
</tr>
<tr>
<td>Deionized water</td>
<td>4.5</td>
<td>700</td>
<td>80</td>
<td>73.5 ± 0.6</td>
</tr>
</tbody>
</table>

\(a\) Maximum temperature at target pressure (at the end of come-up time).

\(b\) Temperature just before depressurization.

Table 3.1 Typical pressure and temperature conditions for inactivation of *Bacillus stearothermophilus* spores in egg patties (QFP-6).
pressure, the initial temperature of the test samples was different (Table 3.1). The microbial lethality attributed to non-isothermal effects during combined pressure-thermal treatment is not well characterized and this is a topic of current research interest (Ting, Balasubramaniam, & Raghubeer, 2002). Thermal treatment of *B. stearothermophilus* spores in egg patties at 105°C and atmospheric pressure, for up to 20 min, did not yield any significant lethality (data not shown). Therefore, a thermal process with measurable spore lethality (i.e., 121°C for up to 15 min) was compared with the investigated PATP. When the process resistance was compared using D values (calculated from the linear segments of survivor plots), *B. stearothermophilus* spores exhibited significantly greater resistance (*P* < 0.05) to the thermal treatment than to PATP (Table 3.2). Accordingly, the combined pressure-thermal treatment, compared to temperature alone, accelerated the inactivation of *B. stearothermophilus* spores in egg patties. Gola et al. (1996) found that the application of high pressures caused morphological changes in the outer structure of bacterial spores. The visible changes in spore coats may serve as evidence that other sub-lethal changes in the inner structures may have occurred due to the pressure treatment. Pressure-induced changes in the inner membrane, for example, may sensitize spores to the concurrent thermal treatment. Resistance of spores to PATP is greater in *B. stearothermophilus* than in many other *Bacillus* spp. such as *B. polymyxa* and *B. subtilis* (data not shown), but it is smaller than that in *C. botulinum* type A and B (Reddy et al. 2003). Recently *B. amyloliquefaciens* spores have been suggested as a possible surrogate for *C. botulinum* spores in PATP treatments (Margosh, Ehrmann, Ganzle, & Vogel, 2004).
<table>
<thead>
<tr>
<th>Process</th>
<th>Media</th>
<th>Temperature (°C)</th>
<th>Pressure (MPa)</th>
<th>Linear</th>
<th>Weibull</th>
<th>Log-logistic</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td></td>
<td></td>
<td>D (min)</td>
<td>MSE ($R^2$)</td>
<td>$A_f$</td>
</tr>
<tr>
<td>PATP</td>
<td>Egg</td>
<td>105</td>
<td>400</td>
<td>0.72 ± 0.17</td>
<td>0.04</td>
<td>0.95</td>
</tr>
<tr>
<td>PATP</td>
<td>Egg</td>
<td>105</td>
<td>600</td>
<td>0.41 ± 0.12</td>
<td>0.17</td>
<td>0.93</td>
</tr>
<tr>
<td>PATP</td>
<td>Egg</td>
<td>105</td>
<td>700</td>
<td>0.53 ± 0.17</td>
<td>0.12</td>
<td>0.92</td>
</tr>
<tr>
<td>PATP</td>
<td>Water</td>
<td>105</td>
<td>700</td>
<td>0.41 ± 0.12</td>
<td>0.66</td>
<td>0.93</td>
</tr>
<tr>
<td>Heat</td>
<td>Egg</td>
<td>121</td>
<td>0.1</td>
<td>8.50 ± 1.39</td>
<td>0.01</td>
<td>0.98</td>
</tr>
<tr>
<td>Heat</td>
<td>Water</td>
<td>121</td>
<td>0.1</td>
<td>6.95 ± 1.66</td>
<td>0.03</td>
<td>0.95</td>
</tr>
</tbody>
</table>

Non-Linear models were not applied for thermal inactivation curves

**Table 3.2** Model parameters of linear, Weibull and log-logistic models for pressure-assisted thermal inactivation of *Bacillus stearothermophilus* spores in egg patties under various treatment conditions.

*a* The smaller the MSE (Mean Square Error) values, the better the model fit the data.

*b* The higher the $R^2$ values, the better is the adequacy of the model to fit the data.

*c* $A_f$ value closer to 1 indicates that the model produces the closest fit.
4.1 Influence of egg matrix on *Bacillus stearothermophilus* inactivation

The D values for PATP inactivation of *B. stearothermophilus* in egg patties and deionized water were not significantly different (Table 3.2). The $D_{p(105^\circ C, 700 \text{ MPa})}$ value for spore inactivation in egg patties was $0.53 \pm 0.17$ min as compared $0.41 \pm 0.12$ min in deionized water. This is consistent with the observations of Reddy et al. (2003), who reported that a crabmeat blend (pH 7.2-7.4) did not protect *C. botulinum* BS-A and 62-A spores against inactivation by high-pressure processing. The decimal reduction values for thermal inactivation (121°C) in egg patties and deionized water also showed no significant difference ($P < 0.05$) (Table 3.2). Despite the fact that the egg patties tested had a fat content of approximately 11 g/100 g of patty, the product does not seem to provide any protective effect against PATP treatments. Ananta et al. (2001) suggested that the protective effect in a fat rich medium is not due to the high fat content itself, but to the lower water activity in a fat-rich system. The egg patties tested had a high water activity (~0.99). This could explain why the solid constituents of egg patties (fats, proteins and minerals molecules) did not reduce the lethality of the combined pressure-heat treatment.

3.4.2 Modeling of inactivation curves

For the modeling of inactivation data, $N_0$" (spore count after pressure come-up time) was chosen as the initial point of the log survivor fraction curve. Thus the inactivation achieved during pressure come-up time (0.75, 1.35 and 1.50 log reductions for 400, 600 and 700 MPa curves respectively) was not considered in modeling. Among the non-linear models tested, the Weibull model described best the PATP inactivation curves of *B.*
*B. stearothermophilus* in egg patties (Figure 3.3) followed by the Log-logistic model (Table 3.2). Though the Weibull and Log-logistic model had similar MSE and $R^2$ values, the $A_f$ (1.04 for 400 MPa, 1.05 for 600 MPa and 1.10 for 700 MPa) values for the Weibull model were considerably closer to 1, when compared to the $A_f$ (1.33 for 400 MPa, 1.17 for 600 MPa and 1.22 for 700 MPa) values for the Log-logistic model. This indicates that the Weibull model produces a better fit to the data obtained within the range of experimental conditions of the present study. The value of the parameters ($b$ and $n$) of the Weibull model for different pressure levels is shown in Table 3.2. For all the pressure levels tested, it was observed that $n$ is less than 1, indicating that log survivor fraction curve has an upward concavity (Peleg et al., 1998).

It is evident that the D value and the n value of the Weibull model (Table 3.2) change in a similar manner when the pressure level is changed. Accordingly, the pressure levels that yielded higher D values also resulted in higher n values. For example, at a pressure level of 600 MPa, the D value was 0.41 and the n value in the fitted Weibull model was 0.30. A decrease in the pressure level to 400 MPa resulted in a higher D value (0.72) and also a higher n (0.53) value in the Weibull model. The parameter $b$ of the Weibull model increased for a treatment that yielded a greater log reduction. For example, treating egg with PATP for 5 min at 700 MPa inactivated 4.1 log *B. stearothermophilus* spores but a similar treatment for the spores suspended in water caused 6.1-log reductions. The b values for the curves fitted to these two sets of data were 1.6 and 3.2, respectively. Therefore, the Weibull model not only fit the PATP data closely, but also the parameters of this model are good indicators of inactivation kinetics.
3.5 Conclusions

Combinations of high pressures and moderate temperatures are potentially useful alternative to retorting for the production of shelf-stable egg products. These combinations not only accelerated the inactivation of *B. stearothermophilus* spores but also reduced the temperature required for inactivation. The egg patties did not offer any protective effect for *B. stearothermophilus* spores. Although PATP treatments at 600 and 700 MPa produced similar inactivations, the higher pressure should be preferred, since the desired final temperature can be achieved starting at a lower initial temperature. Among the nonlinear models tested, the Weibull model described best PATP inactivation of *B. stearothermophilus* spores in egg. The use of such mathematical models to predict PATP inactivation of bacterial spores could allow processors and manufactures to predict and control the safety and shelf-life of foods at a design stage.

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CHAPTER 4

COMBINED PRESSURE-THERMAL INACTIVATION KINETICS OF *Bacillus amyloliquefaciens* SPORES IN MASHED EGG PATTIES

4.1 Abstract

*Bacillus amyloliquefaciens* is a potential surrogate for *Clostridium botulinum* in validation studies involving spore inactivation by pressure-assisted thermal processing (PATP). Spores of *B. amyloliquefaciens* Fad 82 were inoculated into mashed egg patties (10⁸ spore/g), and the product was treated by combinations of pressure (0.1 MPa-700 MPa) and heat (95°C-121°C) in a custom-made high pressure kinetic tester. Inactivation kinetic parameter (D), temperature coefficient (zT) and pressure coefficient (zp) values were determined using a linear model. Additionally, inactivation parameters from the non-linear, Weibull model were estimated. The increase in process pressure decreased the D value at 95°C, 105°C and 110°C considerably; however, at 121°C, the contribution of pressure to spore lethality was less pronounced. zp value increased from 170 MPa at 95°C to 332 MPa at 121°C, suggesting that *B. amyloliquefaciens* spores became less responsive to pressure changes at higher temperatures. Similarly, zT value increased from 8.2°C at 0.1 MPa to 26.8°C at 700 MPa, indicating that, at elevated pressures the spores were less responsive to changes in temperature. The non-linear Weibull model parameter, b,
increased with increasing pressure or temperature and had an inverse relationship with D value. The test product (egg patties) is a suitable food matrix for PATP application in food processing.

4.2 Introduction

Pressure assisted thermal processing (PATP) offers new opportunities for the food industry to respond to the growing consumer demand for high-quality, minimally processed low-acid shelf-stable products. During a typical PATP process, the food is subjected to a combination of elevated pressures (600-900 MPa) and moderate heat (90-120°C) for 1 to 3 min. One of the unique advantages of PATP is its ability to provide a rapid and uniform increase in the temperature of treated food samples. Uniform compression heating and expansion cooling on decompression help to reduce the severity of thermal effects encountered with conventional processing techniques. Although, shelf-stable low-acid foods processed by this technology are not commercially available yet, the technology can be used for processing heat sensitive products such as soups, egg products, coffee, tea and mashed potato (Meyer, Cooper, Knorr, & Lelievald, 2000).

Thermal sterilization of egg products has severe limitations. Such a treatment results in the development of thermally-induced off-flavors, syneresis, and a green-gray discoloration of the egg products due the formation of iron-sulfur compounds (Cotterill, 1995). When conventional thermal retorting and PATP treatment were compared, the latter produced a more acceptable egg product due to a shorter exposure to heat (Barbosa-Canovas et al., 2005). Establishing a safe and efficient PATP sterilization process requires a proper definition of inactivation kinetic parameters for various target
pathogenic and spoilage microbes, under pressure, heat, and their combinations. Limited studies addressed PATP spore inactivation kinetics over a range of pressure and temperature conditions (Miglioli, Gola, Maggi, Rovere, Carpi, Scaramuzza, & Aglio, 1997; Reddy, Solomon, Fingerhut, Rhodehamel, Balasubramaniam, & Palaniappan, 1999; Rovere et al, 1996). Hence, the pressure and temperature coefficients for inactivation of bacterial spores during PATP are yet to be characterized. This knowledge would be extremely useful in developing a safe PATP process for a specific application. In contrast to the typical log-linear spore inactivation behavior during thermal processing, many researchers observed that PATP survivor curves appear to concave upwards with a linear inactivation period followed by characteristic tailing (Okazaki, Kakugawa, Yoneda, & Suzuki, 2000; Ananta, Heinz, Schuttler, & Knorr, 2001; Reddy, Solomon, Tetzloff, & Rhodehamel, 2003; Rajan, Pandrangi, Balasubramaniam, & Yousef, 2005). Therefore, the use of non-linear, in lieu of linear, kinetic models to describe PATP spore inactivation may be more appropriate (Peleg & Cole, 1998). However, the influence of combined pressure-thermal treatment on the non-linear kinetic model parameters is largely unknown (Rajan et al., 2005). Thus, the objectives of the study were (1) to determine the linear and non-linear PATP kinetic inactivation parameters of Bacillus amyloliquefaciens spores in mashed egg patties over a range of pressure and temperature conditions, (2) to evaluate the pressure and temperature coefficients ($z_p$ and $z_T$, respectively) of $B. \text{ amyloliquefaciens}$ spores. Since Bacillus amyloliquefaciens Fad 82 produces PATP-resistant spores, researchers proposed using this bacterium as a surrogate for Clostridium botulinum spores (Margosch, Ehrmann, Ganzle, & Vogel, 2004). Therefore, spores of this bacterium were chosen in this investigation.
4.3 Materials and Methods

4.3.1 Bacillus amyloliquefaciens spore preparation

*B. amyloliquefaciens* Fad 82 strain, a strain initially isolated from ropy bread, was kindly provided by M. Gänzle, Lehrstuhl für Technische Mikrobiologie, Technische Universität München (Freising, Germany). The bacterium was grown aerobically at 32°C for 24 h in trypticase soy broth supplemented with 0.1% yeast extract (TSBYE; BD Difco, Becton, Dickinson and Co., Sparks, MD, USA). Preparation of spore suspension was adapted from Margosch et al. 2004. Freshly prepared cultures (100µl) of *B.amyloliquefaciens* were spread-plated onto trypticase soy agar (TSA; BD Difco) that was supplemented with 10 mg/Kg of MnSO₄ (Fisher Scientific, Pittsburgh, PA, USA). The inoculated plates were incubated at 32°C until more than 95% sporulation was observed by microscopic examination. Spores were then collected by flooding the surface of the plates with 10 ml of sterile distilled water and scraping the colonies with a sterile glass spreader. The collected spore suspension was washed five times by differential centrifugation (from 2,000 to 8,000 × g for 20 min each at 4°C), sonicated, and then heated at 80°C for 10 min to destroy any remaining vegetative cells. Finally, the spore pellet was resuspended in deionized water, to obtain 10⁹ spores/ml, and stored at 4°C until use.

4.3.2 Preparation of egg samples

Frozen egg patties were obtained from a commercial egg processor (code 03-1426-9, Michael Foods, Minnetonka, MN, U.S.A.). The egg patties are used in the fast food industry and are also available in the retail food market. The key ingredients of the patties were whole eggs, water, soybean oil, modified food starch, whey solids, salt, natural and
artificial flavors, nonfat dry milk, xanthan gum, citric acid and EDTA. According to the manufacturer, the egg patties were made by mixing the whole eggs with dry and liquid ingredients, and then the mix was pumped in a mold within a flat cooking belt. Egg mix portions were cooked and preformed in a convection oven at 180 and 250 °C for a predetermined time, and then frozen and packaged (Knipper & Beam, 2002). The round-shaped (8.9 ± 0.6 cm diam) patties weighed 42.5 ± 7.0 g and their pH and water activity values were 7.25 and 0.99 respectively. A day prior to use, the egg patties were thawed in a refrigerator at 4°C.

4.3.3 Thermal inactivation of spores

The thermal inactivation of B. amyloliquefaciens spores in egg patties was determined at 95°C, 105°C, 110°C and 121°C using custom-fabricated aluminum tubes (Luechapattnaporn, Wang, Al-Holy, Kang, Tang, & Hallberg, 2004). A sample of a mashed egg patties (0.9 g) was transferred into each aluminum tube and 0.1ml (~10⁹ spores/ml) of the spore suspension was added to get a final spore concentration of approximately 10⁸ spores/g egg patty. Six tubes were then submerged simultaneously into a 28-liter circulating oil bath (Fisher Scientific), which was maintained at the desired target temperature. The sample temperature was monitored and recorded using a K-type thermocouple (Omega Engineering, Stamford, CT, U.S.A.) attached to a data logger (IOTech, Cleveland, OH, U.S.A.). The heating time (come-up time) was recorded when the target temperature of the sample was reached. The process come-up times were 3.58 min for 95°C, 3.33 min for 105°C, 3.25 min for 110°C and 3.30 min for 121°C. The first aluminum tube was removed from the oil bath at the end of come-up time. The other
aluminum tubes were subjected to five different hold times; the hold time intervals being different for each target temperature. After the thermal treatments, the sample-containing tubes were immersed promptly into an ice-water bath to avoid further inactivation. Spores surviving the thermal treatment were enumerated as described later. The untreated control samples were heated at 80°C for 30 min to activate the spores before enumeration.

4.3.4 Pressure-assisted thermal processing of spores

4.3.4.1 High pressure microbial kinetic tester

A custom-made high pressure kinetic tester (PT-1, Avure Tecnologies Inc, Kent, WA, U.S.A) was used in the study (Figure 4.1). The unit is rated for operation up to a maximum pressure of 700 MPa and a maximum process temperature of 130°C. It has a 54 ml stainless steel pressure chamber immersed in a temperature-controlled bath, and the system is pressurized by an intensifier (M-340 A, Flow International, Kent, WA). An external bath surrounding the pressure chamber was maintained at a suitable temperature (Table 4.1) so that isothermal-process conditions could be maintained throughout the pressure holding time. Propylene glycol (57-55-6, Avatar Corporation, University Park, IL) was used as the heating medium in the bath. Figure 4.2 shows a sample pressure-temperature profile at 700 MPa and 105°C. While the pressure come-up time depended upon the target pressure (Table 4.1) the depressurization occurred in less than 4 s, regardless of the pressurization level. The sample temperature and chamber pressure were recorded every 2 s during the entire treatment cycle using a K-type thermocouple sensors (Model KMQSS-04U-7; Omega Engineering, Stamford, CT, U.S.A) and pressure
Figure 4.1 Cross section of high pressure kinetic tester, PT1 (Adapted from the equipment operation manual, Avure technologies, Kent, WA).
Figure 4.2 Pressure and temperature history, experienced by the spore sample during pre-heating and pressure-assisted thermal treatment at 700 MPa and 105°C. Data presented are mean values of two independent trials. Time scale include (A) Spore sample preheating time (min) in water bath prior to loading into pressure machine, (B) Time (min) for the sample in the pressure chamber to reach desired initial temperature before the commencement of pressurization, (C) Pressure come-up time (min), and (D) Pressure hold time (min). Depressurization time (< 2 s) is not shown. Glycol bath is maintained at 105°C.
Table 4.1 Typical pressure-assisted thermal processing settings used in this study.
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 7-Channel thermocouple expansion card, DBK 203-expansion card; IOtech, Cleveland, OH, U.S.A.) and software (DasyLab 7.00.04; National Instruments Corp., Austin, TX, U.S.A.), was used to record the data.

4.3.4.2 Preparing microbial test samples for pressure-assisted thermal processing

High barrier pouches (5 x 2.5 cm each) made from sterile filter bags (01-002-57, Fisher Scientific) were used as sample holders. A sample of mashed egg patties (0.9g) and 0.1ml of the spore suspension (~10^9 spores/ml) were placed inside these high barrier pouches to get a final spore concentration of approximately 10^8 spores/g egg patty. The pouches were then heat-sealed (Impulse Food Sealer, American International Electric, Whittier, CA, U.S.A) and the contents of the pouch were mixed thoroughly. The packaged samples were then placed in a sample carrier consisting of a 10-ml capacity polypropylene syringe (Model 309604; Becton, Dickinson and Company) covered with two layers of insulating material (Model 09-356; Fisher Scientific). Water was used as the pressure transmitting fluid within the syringe. Prior to pressurization experiments, the sample carrier containing the spore-inoculated egg patty was preheated in a water bath (Isotemp 928; Fisher Scientific) to a suitable preprocessing temperature, T_1 (Table 4.1 and Figure 4.2).

4.3.4.3 Pressure-assisted thermal processing of egg patties

Preheated sample carrier was then immediately loaded into the chamber of the pressure kinetic tester and the pressurization was started when the sample temperature reached a predetermined value, T_2 (Table 4.1 and Figure 4.2). This temperature (T_2) was estimated based on the following equation
\[ T_2 = T_3 - \left( \frac{C}{H} \cdot \Delta P + \Delta T_H \right) \]

where \( T_3 \) is the desired process temperature in °C, \( CH \) is the compression heating value of the test sample, \( \Delta P \) is the pressure applied in MPa and \( \Delta T_H \) is the heat absorbed from the surrounding glycol bath during pressurization. Depending up on the target process temperature (\( T_3 \)) and process pressure, the value of \( \Delta T_H \) was estimated on a trial and error basis. Compression heating values of egg samples were experimentally determined (data not shown) and were found to be similar to that of the published value of water (Balasubramaniam, Ting, Stewart, & Robbins, 2004). The test samples were subjected to a combination of process temperatures (95°C, 105°C, 110°C, and 121°C) and pressures (500, 600 and 700 MPa) for upto 15 min. The process hold-time intervals were adjusted for each combination of process pressure and temperature so that adequate data points were collected for subsequent data analysis. The process hold-times did not include the pressure come-up or the de-pressurization times. After depressurization, the samples were cooled immediately in an ice-water bath. The untreated control samples (non-pressure treated inoculated egg patties) were heated at 80°C for 30 min, to activate the spores for enumeration purposes. Pouches containing the inoculated egg product (control and pressure-treated) were opened aseptically, and their contents were used for determining the total viable spore count as indicated later.

4.3.5 Enumeration of survivors

Heat- or PATP-treated sample (1g) was mixed with 9 ml of peptone water (1 g/liter) and homogenized for 2 min in a stomacher, (Seward Lab Stomacher, Norfolk, UK). The
homogenized sample was further serially diluted in peptone water (1 g/liter) and the dilutions (1 ml) were pour-plated using duplicate trypticase soy agar (TSA; BD Difco) plates. The plates were then incubated at 32°C for 48 h before enumeration. The enumeration of colonies was done using a dark-field Quebec colony counter (Leica Microsystems, Ontario, Canada.). The minimum detection limit for the enumeration procedure was $10^1$ spore/g egg product.

4.3.6. Data analysis

All data were analyzed using the Statistical Analysis System software (SAS, release 9.2.1, SAS Institute Inc, Cary, NC, U.S.A). The General Linear Model (GLM) and least significant difference (LSD) procedures were used to compare means. Significant mean differences among PATP treatments and holding times were calculated by Fisher's least significant difference at 5% of significance level ($P < 0.05$). Two independent repeats of thermal and PATP experiments were carried out.

4.3.7.1 Determination of kinetic inactivation parameters

The decimal reduction time (D value) at different pressure and temperature combinations was calculated from the linear portion of the survivor curve, observed immediately after the come-up time, using the equation

$$\log \frac{N}{N_0} = -\frac{t}{D}$$

(2)

where $N_0$ = Spore count after process (thermal or pressure) come-up time, $N$ = number of survivors after being exposed to a lethal treatment for a specific time ($t$). The pressure
coefficient, $z_p$ (MPa), at constant temperature (i.e., the pressure in MPa required at constant temperature to achieve a 10-fold change in the D value) was estimated as a negative reciprocal of the slope resulting from plotting log D against pressure. Similarly, the temperature coefficient, $z_T$ (°C), at constant pressure (i.e., temperature change at constant pressure, required to achieve 10-fold change in the D value) was estimated as a negative reciprocal of the slope of the log D versus temperature plots. The reaction rate constant, $k$ (min\(^{-1}\)), is inversely related to the D value and determined using the relationship

$$k = \frac{2.303}{D}$$

The volume change of activation $\Delta V$ (m\(^3\) mole\(^{-1}\)), which is a measure of the net effect of pressure reactions causing physiological changes at constant temperature, was estimated using the following equation (Cheftel, 1995; Mussa, Ramaswamy, & Smith, 1999)

$$\Delta V = -RT \left[ \frac{\Delta \ln k}{\Delta P} \right]_T$$

where $P$ is the pressure (MPa), $T$ is the absolute temperature (°K) and $R$ is the universal gas constant ($8.314 \times 10^{-6}$ m\(^3\) mole\(^{-1}\) MPa K\(^{-1}\)). The energy of activation $E_a$ (J mole\(^{-1}\)), which describes the effect of temperature changes on the reaction rate (at constant pressure), was obtained from the following equation

$$E_a = -R \left[ \frac{\Delta \ln k}{\Delta T} \right]_P$$

where $R$ is the universal gas constant ($8.314$ J mole\(^{-1}\) K\(^{-1}\)$).
4.3.7.2 Weibull model parameter estimation and curve fitting

This model was initially proposed by Peleg & Cole (1998) to describe microbial curves. The Weibull model is described by the following equation:

\[
\log \left( \frac{N}{N_0} \right) = -bt^n
\]  

(6)

where b and n are the scale and shape factors, respectively (Peleg & Cole, 1998).

The curve fitting and model parameter estimation were done using the non-linear (PROG NLIN) procedure of the statistical program (SAS). Mean square error (MSE), regression coefficient (R^2) and accuracy factor (Ar) values were used to evaluate the goodness of fit. A relatively small MSE value and large R^2 value is indicative of good fitting models (Neter, Kutner, Nachtsheim, & Wasserman, 1996), and an Ar value of one indicates that the model produces a perfect fit to the data (Ross, 1996). While MSE and R^2 were obtained from the analysis, the Ar values were calculated using the following equation:

\[
Ar = 10 \left[ \frac{\sum |\log(\text{predicted} / \text{observed})|}{n} \right]^{-1}
\]  

(7)

4.4. Results and Discussions

Figure 4.3 compares survivor curves of B. amyloliquefaciens during treatment of the spores in egg patties by combinations of pressure (0.1 MPa - 700 MPa) and heat (95°C - 121°C). In general, the PATP inactivation of B. amyloliquefaciens spores exhibited a bi-phasic behavior through rapid initial inactivation immediately after pressure-come-up time, followed by a characteristic tailing during extended pressure-holding times. Similar bi-phasic PATP inactivation behavior was observed for other spores such as those of B.
Figure 4.3 Log survivor fraction of *B. amyloliquefaciens* spores in mashed egg patties subjected to pressures of 0.1-700 MPa at temperatures of 95°C-121°C. (+) 95°C, (■) 105°C, (▲) 110°C and (●) 121°C. * Number of survivors was an estimate (<20 CFU/g).
*stearothermophilus* (Okazaki et al., 2000; Ananta et al., 2001; Rajan et al., 2005), *Clostridium botulinum* (Reddy et al., 2003) and *Clostridium thermosaccharolyticum* (Margosch et al., 2004).

The reduction in spore count during pressure-come up time varied between 0.1 and 1.2 log spore/g egg product, depending on the treatment (Table 4.2). Within the range of conditions tested in the present study, lower pressure-thermal combination (e.g., 500 MPa at 95°C-105°C) resulted in no significant reduction in the spore count during the pressure come-up time; but application of elevated pressures (> 600 MPa) and temperatures (121°C) inactivated up to 1.2 log spore population during that period (Figure 4.3). Margosch et al. (2004) also reported a reduction in the population (< 0.5 log-reduction) of *B. amyloliquefaciens* spores during a 5-min pressure come-up time of the PATP treatment at 600 MPa and 80°C. These authors also reported reduction in population for other spores tested, such as *C. botulinum* TMW 2.357 (1.5 log), *C. thermosaccharolyticum* (3 log) and *Bacillus subtilis* (>5 log) during a similar come-up time. These observations suggest that different spores are likely to have different resistances during the pressure come-up time, and highlights the importance of documenting the PATP come-up time and corresponding spore inactivation.

The PATP lethality against *B. amyloliquefaciens* spores increased with increase in process pressure at a given temperature (Figure 4.3). For example, the D value for PATP treatment at 105°C decreased with pressure increase from 500 to 700 MPa (Table 4.3). Similar changes in the D values with pressure were reported for the PATP treatment of *C. sporogenes* spores in meat broth (Rovere, Gola, Maggi, Scaramuzza, & Miglioli, 1998) and *B. stearothermophilus* spores in egg patties (Rajan et al., 2005).
<table>
<thead>
<tr>
<th>Process pressure (MPa)</th>
<th>Process temperature (°C)</th>
<th>Come-up time (min)</th>
<th>Log reduction at the end of process come-up time</th>
<th>Time for tailing to occur (min)</th>
<th>Log reduction before commencement of tailing</th>
</tr>
</thead>
<tbody>
<tr>
<td>0.1 Thermal treatment</td>
<td>95</td>
<td>3.6 ± 0.1</td>
<td>0.04 ± 0.03</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td></td>
<td>105</td>
<td>3.3 ± 0.1</td>
<td>0.12 ± 0.15</td>
<td>140.00</td>
<td>5.62 ± 0.25</td>
</tr>
<tr>
<td></td>
<td>110</td>
<td>3.3 ± 0.1</td>
<td>0.45 ± 0.09</td>
<td>35.00</td>
<td>5.87 ± 0.07</td>
</tr>
<tr>
<td></td>
<td>121</td>
<td>3.3 ± 0.1</td>
<td>1.23 ± 0.31</td>
<td>0.75</td>
<td>4.78 ± 0.20</td>
</tr>
<tr>
<td>500</td>
<td>95</td>
<td>0.5 ± 0.1</td>
<td>0.01 ± 0.04</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td></td>
<td>105</td>
<td>0.5 ± 0.1</td>
<td>0.05 ± 0.04</td>
<td>10.00</td>
<td>3.60 ± 0.15</td>
</tr>
<tr>
<td></td>
<td>110</td>
<td>0.5 ± 0.1</td>
<td>0.04 ± 0.01</td>
<td>10.00</td>
<td>6.04 ± 0.06</td>
</tr>
<tr>
<td></td>
<td>121</td>
<td>0.5 ± 0.1</td>
<td>0.32 ± 0.14</td>
<td>1.50</td>
<td>4.61 ± 0.12</td>
</tr>
<tr>
<td>600</td>
<td>95</td>
<td>0.6 ± 0.1</td>
<td>0.04 ± 0.22</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td></td>
<td>105</td>
<td>0.6 ± 0.1</td>
<td>0.09 ± 0.04</td>
<td>3.00</td>
<td>3.87 ± 0.49</td>
</tr>
<tr>
<td></td>
<td>110</td>
<td>0.6 ± 0.1</td>
<td>0.35 ± 0.06</td>
<td>2.00</td>
<td>4.67 ± 0.17</td>
</tr>
<tr>
<td></td>
<td>121</td>
<td>0.6 ± 0.1</td>
<td>1.10 ± 0.20</td>
<td>0.50</td>
<td>5.72 ± 0.08</td>
</tr>
<tr>
<td>700</td>
<td>95</td>
<td>0.7 ± 0.1</td>
<td>0.08 ± 0.01</td>
<td>3.00</td>
<td>3.74 ± 0.08</td>
</tr>
<tr>
<td></td>
<td>105</td>
<td>0.7 ± 0.1</td>
<td>0.14 ± 0.07</td>
<td>1.50</td>
<td>4.18 ± 0.26</td>
</tr>
<tr>
<td></td>
<td>110</td>
<td>0.7 ± 0.1</td>
<td>0.21 ± 0.01</td>
<td>1.00</td>
<td>4.94 ± 0.10</td>
</tr>
<tr>
<td></td>
<td>121</td>
<td>0.7 ± 0.1</td>
<td>1.19 ± 0.10</td>
<td>0.42</td>
<td>5.80 ± 0.07</td>
</tr>
</tbody>
</table>

Table 4.2 Reduction in *B. amyloliquefaciens* spore population during pressure-come time and before the commencement of tailing.

64
<table>
<thead>
<tr>
<th>Pressure (MPa)</th>
<th>Temperature (°C)</th>
<th>Linear</th>
<th>Weibull</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td>D (min)</td>
<td>b (95% CI)</td>
</tr>
<tr>
<td>500</td>
<td>95</td>
<td>11.58±0.39</td>
<td>0.15 (0.12-0.18)</td>
</tr>
<tr>
<td></td>
<td>105</td>
<td>2.9±0.16</td>
<td>0.31 (0.23-0.38)</td>
</tr>
<tr>
<td></td>
<td>110</td>
<td>1.66±0.01</td>
<td>0.66 (0.43-0.71)</td>
</tr>
<tr>
<td></td>
<td>121</td>
<td>0.32±0.03</td>
<td>2.83 (2.60-3.07)</td>
</tr>
<tr>
<td>600</td>
<td>95</td>
<td>1.79±0.10</td>
<td>0.91 (0.79-1.04)</td>
</tr>
<tr>
<td></td>
<td>105</td>
<td>0.72±0.01</td>
<td>1.60 (1.24-1.97)</td>
</tr>
<tr>
<td></td>
<td>110</td>
<td>0.46±0.01</td>
<td>2.30 (1.76-2.84)</td>
</tr>
<tr>
<td></td>
<td>121</td>
<td>0.11±0.02</td>
<td>4.92 (4.33-5.52)</td>
</tr>
<tr>
<td>700</td>
<td>95</td>
<td>0.80±0.01</td>
<td>1.63 (1.35-1.92)</td>
</tr>
<tr>
<td></td>
<td>105</td>
<td>0.31±0.01</td>
<td>2.83 (2.49-3.17)</td>
</tr>
<tr>
<td></td>
<td>110</td>
<td>0.21±0.02</td>
<td>3.94 (3.51-4.36)</td>
</tr>
<tr>
<td></td>
<td>121</td>
<td>0.08±0.01</td>
<td>5.83 (5.01-6.65)</td>
</tr>
<tr>
<td>0.1</td>
<td>95</td>
<td>349±49</td>
<td></td>
</tr>
<tr>
<td></td>
<td>105</td>
<td>24±3.4</td>
<td></td>
</tr>
<tr>
<td></td>
<td>110</td>
<td>5.90±0.87</td>
<td></td>
</tr>
<tr>
<td></td>
<td>121</td>
<td>0.25±0.01</td>
<td></td>
</tr>
</tbody>
</table>

Table 4.3 Linear and Weibull model kinetic parameters of Bacillus amyloliquefaciens spores during thermal or pressure-assisted thermal processing in mashed egg patties.

Weibull model was not applied for thermal inactivation curves.

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Comparison of survivor curves (Figure 4.3) indicates that an increase in process temperature (at constant pressure) also decreased the spore viability considerably. Accordingly, a temperature rise from 95°C to 121°C at all the pressure levels tested decreased the D values significantly ($P < 0.05$) (Table 4.3). The effect of rising process temperatures on increasing the PATP inactivation rates have also been observed for spores of *B. stearothermophilus* in mashed broccoli (Ananta et al., 2001) and *C. sporogenes* in meat broth (Miglioli, Gola, Maggi, Rovere, Carpi, Scaramuzza, & Aglio, 1997). Further, different combinations of pressure and temperature can bring about a similar level of *B. amyloliquefaciens* spore inactivation. For example, a PATP treatment at 600 MPa and 105°C produces similar log survivor curves as a PATP treatment at 700 MPa and 95°C. Researchers hypothesized that the non-isothermal conditions inside the pressure vessel could also influence the spore inactivation (Ting, Balasubramaniam, & Raghubeer, 2002; De heij, Ludo, Moezelaar, Hoogland, Matser, & Van de berg, 2003; Rajan et al., 2005). Figure 4.4 shows the effect of temperature decline (non-isothermal conditions) during PATP treatment on the survivor curves of *B. amyloliquefaciens* spores. The decline in temperature was simulated in the pressure kinetic tester by maintaining the temperature of glycol bath below that achieved in the pressure chamber by compression heating. When spore survivor curves, obtained under isothermal and non-isothermal conditions, were compared, the latter exhibited less overall lethality (Figure 4.4). This illustrates the importance of maintaining uniform process conditions during the PATP. The time interval between the commencement of iso-baric-thermal process (i.e., when the targeted pressure and temperature are achieved) and the tailing of spore population,
Figure 4.4 (a) Time-temperature profile and the corresponding inactivation of *Bacillus amyloliquefaciens* spores in mashed egg patties, when subjected to a pressure-assisted thermal treatment at 600 MPa and 105°C. (a) Time-temperature profile. (b) Survivor plot. (-----) isothermal treatment at 105°C. (------) non-isothermal treatment in the range of 105°C to 85°C.
appeared to be function of target process conditions (Table 4.2 and Figure 4.3). Visual examination of Figure 4.3 suggest that at elevated process pressures (700 MPa) and temperatures (121°C), tailing occur early, and these processing conditions were effective in decreasing spore population up to 4.6-5.8 log/g egg product. Extended treatment appears to be ineffective in causing additional lethality. More research is needed to understand bacterial tailing, ways to minimize it, and enhance process lethality.

4.4.1. Spore resistance during thermal or pressure-assisted thermal processing

Resistance of *B. amyloliquefaciens* spores to PATP (as measured by kinetic parameter D value) was significantly smaller than that to thermal processing, at an equivalent process temperature (Table 4.3). Similar results were reported for other PATP target spores such as *C. tyrobutyricum, C. thermosaccharolyticum, C. sporogenes* and *B. stearothermophilus* (Rajan et al, 2005, Rovere et al., 1998; Margosch et al., 2004). However, synergy between heat and pressure diminishes at elevated process temperature and heat becomes the dominant contributor to the lethality at 121°C. In Figure 4.5a, thermal treatment at 121°C (6.04 log reduction after 3.3 min come-up time and 2 min process hold time) appears to be more lethal than the PATP treatment at 500 MPa and 121°C (4.38 log reduction after 0.5 min come-up time and 2 min pressure hold time). However, a considerable part of heat treatment lethality (1.23 Δ log spore/g egg product) occurred during the longer come-up time (3.3 min), whereas the shorter PATP come-up time (0.5 min) resulted in only 0.32 Δ log spore/g reduction. If spore lethality only during process treatment (excluding come-up time) is considered, a similar spore inactivation was observed for both the thermal-only treatment (121°C) and PATP treatment (500 MPa).
Figure 4.5 Log survivor fraction of *B. amyloliquefaciens* spores in mashed egg patties subjected to a process temperature of 121°C at pressures of 0.1-700 MPa. (♦) 0.1 MPa, (■) 500 MPa, (▲) 600 MPa and (●) 700 MPa. (a) Total treatment time (come-up time --- and process hold time ---), and (b) Process hold time only. * Number of survivors was an estimate (<20 CFU/g).
and 121°C (Figure 4.5b). The come-up time in the pressure kinetic tester used in this study cannot be varied and thus attempts were not made to match the come-up times of PATP and thermal treatments. In the absence of such comparable come-up times, it may not be possible to make meaningful conclusions about the presumed pressure protective effects at elevated temperatures.

### 4.4.2 Pressure and thermal coefficients of *Bacillus amyloliquefaciens* spores

The $z_p$ values of *B. amyloliquefaciens* spores subjected to PATP treatments increased from 170.2 MPa at 95°C to 332.2 MPa at 121°C (Table 4.4). At 121°C, a similar $z_p$ (370 MPa) values were reported for *B. stearothermophilus* spores (Rodriguez et al., 2004). Our results suggest that the spores were becoming less responsive to pressure changes as processing temperatures increased. Therefore, contribution of the pressure to spore lethality was less pronounced at elevated process temperatures (121°C), than at lower process temperatures (95°C or 105°C or 110°C). This observation is consistent with that of Miglioli et al. (1997) who estimated $z_p$ values for *C. sporogenes* at different PATP process temperatures. Values of activation volume ($\Delta V$) at all temperatures (Table 4.4) were negative, indicating that pressure has a lethal effect on *B. amyloliquefaciens* spores; this result is consistent with the Le Chatelier’s principle. In general, a less negative $\Delta V$ value also signifies a lower responsiveness of the spores to changes in pressure (Mussa et al., 1999). In the current study, the $\Delta V$ value becomes less negative with an increase in process temperature ($-4.4 \times 10^{-5}$ m$^3$/mole at 95°C to $-2.3 \times 10^{-5}$ m$^3$/mole at 121°C); therefore, the activation volume concept further confirms that the spores became less responsive to pressure changes when the processing temperature increased.
<table>
<thead>
<tr>
<th>Process temperature (°C)</th>
<th>$Z_p$ (MPa)$^a$</th>
<th>$\Delta V$ (x $10^{-5}$ m$^3$/mole)$^b$</th>
</tr>
</thead>
<tbody>
<tr>
<td>95</td>
<td>170 ± 1</td>
<td>-4.4</td>
</tr>
<tr>
<td>105</td>
<td>206 ± 2</td>
<td>-3.7</td>
</tr>
<tr>
<td>110</td>
<td>220 ± 6</td>
<td>-3.3</td>
</tr>
<tr>
<td>121</td>
<td>332 ± 42</td>
<td>-2.2</td>
</tr>
</tbody>
</table>

$^a$ A higher $Z_p$ value implies lower responsiveness to pressure change

$^b$ A less negative $\Delta V$ value implies lower responsiveness to pressure change

Table 4.4 Pressure coefficients ($Z_p$ and $\Delta V$) for *B. amyloliquefaciens* spores suspended in egg matrix, at different temperatures during pressure-assisted thermal processing.
Bacillus amyloliquefaciens spores were more responsive to temperature changes at atmospheric pressure \( z_T = 8.2^\circ C \) than at elevated pressure \( z_T = 26.8^\circ C \) at 700 MPa (Table 4.5). Similar observations were made by Miglioli et al. (1997) who reported \( z_T \) values of 11.8°C and 17.1°C for C. sporogenes at 0.1 and 700 MPa respectively. The calculated activation energy for B. amyloliquefaciens spores at ambient pressures was similar to published value of 2.5-3.4 \( \times 10^5 \) J/mole for spore destruction (Heldman & Hartel, 1998). Activation energy decreased with increasing pressure (from 3.4 \( \times 10^5 \) J/mole at 0.1 MPa to 1.1 \( \times 10^5 \) J/mole at 700 MPa), suggesting that pressure synergistically contributed to spore lethality within the range of conditions studied.

4.4.4 Influence of pressure and temperature on inactivation parameters of Weibull model

For the model parameter estimation and curve fitting, the inactivation achieved during the pressure come-up time (Table 4.2) was not considered. Preliminary model fitting was done using both the Weibull and Log-logistic models; the Weibull model was chosen to describe the inactivation curves because it resulted in a better fit of the raw data (data not shown). Many researchers (Mattick, Jorgensen, Wang, Pound, Vandevein, Ward, Legan, Lappin-Scott, & Humphrey, 2001; Von Boekel, 2002; Hassani et al., 2005; Rajan et al., 2005) also preferred the Weibull model to describe non-linear log survivor curves, due to its mathematical simplicity and ability to address the microbial tailing.

The Weibull model parameter \( b \), increased with increase in pressure and temperature (Table 4.3). A higher \( b \) value corresponds to a steeper slope of the log survivor curve, which in turn implies that the spore inactivation occurred at a faster rate. Consequently,
<table>
<thead>
<tr>
<th>Process pressure (MPa)</th>
<th>$z_T$ ($^\circ$C)$^a$</th>
<th>$E_a$ (x $10^5$ J/mole)$^b$</th>
</tr>
</thead>
<tbody>
<tr>
<td>0.1</td>
<td>8.2 ± 0.2</td>
<td>3.4</td>
</tr>
<tr>
<td>500</td>
<td>16.7 ± 0.4</td>
<td>1.7</td>
</tr>
<tr>
<td>600</td>
<td>21.5 ± 1.5</td>
<td>1.3</td>
</tr>
<tr>
<td>700</td>
<td>26.8 ± 1.2</td>
<td>1.1</td>
</tr>
</tbody>
</table>

$^a$ A higher $z_T$ value implies lower responsiveness to temperature change

$^b$ A lower $E_a$ value implies lower responsiveness to temperature change

Table 4.5 Temperature coefficients ($z_T$ and $E_a$) for *B. amyloliquefaciens* spores suspended in egg matrix, at different pressures during pressure-assisted thermal processing.
there is an inverse relationship between \( b \) and \( D \) values (Table 4.3), which can be described by the following equation:

\[
b = \frac{t^{1-n}}{D}
\]  

(8)

Thus when \( n = 1 \) (indicating a linear inactivation) \( b \) and \( D \) are inversely proportional to each other.

A value of \( n < 1 \) indicates that the survivor curve is concave upward with presence of tailing (Peleg & Cole, 1998). In general, in our study, \( n \) was less than 1 at all combinations of pressure and temperature (Table 4.3). Further, an increase in pressure at constant process temperature resulted in curves with distinct linear slope region followed by a near-horizontal flat region (i.e., sudden tailing). Correspondingly, the value of the parameter \( n \) also decreased. Thus, curves in which tailing occurs suddenly have a lower \( n \) value when compared to curves in which tailing is gradual.

4.4.5 Comparison of pressure-assisted thermal resistance of *Bacillus amyloliquefaciens* spores with other surrogate spores

The PATP resistance of *B. amyloliquefaciens* Fad 82 spores was compared to the PATP resistance of *B. stearothermophilus* ATCC 7953 spores in mashed egg patties under similar process conditions (700 MPa, 105°C using PT-1 equipment). The D value for *B. amyloliquefaciens* spores was \( 0.31 \pm 0.01 \) min as compared to \( 0.38 \pm 0.08 \) min for *B. stearothermophilus* spores. Though the D values suggest similar PATP resistance at 700 MPa and 105°C, the log survivor curves (Figure 4.6) clearly indicate that there were more
Figure 4.6 Log survivor fraction of *Bacillus* spores in egg patties subjected to PATP treatment at 700 MPa and 105°C in PT-1. (■) *Bacillus amyloliquefaciens* FAD 82 spores, and (♦) *Bacillus stearothermophilus* ATCC 7953 spores.
log-survivors of *B. amyloliquefaciens* spores than *B. stearothermophilus* spores at each time point during the PATP treatment. Thus, *B. amyloliquefaciens* spores when compared to *B. stearothermophilus* spores, exhibited a greater PATP resistance with extensive tailing under the conditions of our study. This spore-resistance comparison study clearly illustrates the importance of considering the entire inactivation curves (both come-up time and hold time inactivation) when comparing the PATP resistances of different surrogate spores.

Other spore-resistance comparison studies in deionized water (CAPPS report) showed that *B. amyloliquefaciens* spores exhibited a greater PATP resistance when compared to spores of *Bacillus sphaericus*, *C. tyrobutyricum*, *C. sporogenes* and *C. thermosaccharolyticum*. Further, Margosch et al. (2004) have reported that the combined pressure-thermal resistance of *B. amyloliquefaciens* Fad 82 spores (at 800 MPa and 80°C) was greater than that of selected strains of *Bacillus licheniformis*, *Bacillus subtilis*, *Thermoanaerobacterium thermosaccharolyticum* and *C.botulinum* spores in mashed carrots. Thus, on the basis of the data currently available, *B. amyloliquefaciens* spores can be suggested as a target surrogate organism for validating PATP sterilization of egg products.

4.5 Conclusions

Different combination of pressure and temperature can be used to bring about similar levels of *B. amyloliquefaciens* Fad 82 during pressure-assisted thermal processing. D values decreased with increase in pressure or temperature and was inversely related to Weibull parameter, b value. The processing resistance parameter, D value, for *B.
*amyloliquefaciens* spores in egg patties was lower in PATP than in thermal processing at an equivalent temperature. However, *B. amyloliquefaciens* spores became less responsive to pressure changes at 121°C (indicated by the higher \( z_p \) and less negative \( \Delta V \) value) than at lower temperatures. Furthermore, the responsiveness of *B. amyloliquefaciens* spores to temperature changes, was less at 700 MPa (indicated by the higher \( z_T \) and lower \( E_a \) value) than at lower pressures.

This manuscript has been submitted to Journal of Food Protection for publication.
CHAPTER 5

CONCLUSIONS AND FUTURE RECOMMENDATIONS

5.1 Summary and Conclusions

Combinations of elevated pressures and moderate temperatures accelerated the inactivation of *B. stearothermophilus* and *B. amyloliquefaciens* spores in the egg products when compared to thermal sterilization. There appears no correlation between pressure resistance and thermal resistance. The egg patties did not offer a protective effect to the spores, possibly due to the high water activity of the egg matrix. For a PATP process, an increase in process pressure (at constant temperature) within the 400-700 MPa range increased the inactivation rates significantly. Similarly, an increase in temperature (at constant pressure) from 95°C-121°C also enhanced the lethality of the process against the spores. For *B. amyloliquefaciens* spores, the pressure coefficient (zp) values increased from 170 MPa at 95°C to 333 MPa at 121°C, suggesting that the spores became less responsive to temperature changes at high pressures. Similarly, the temperature coefficients (zT) values increased from 8.2°C at 0.1 MPa to 26.8°C at 700 MPa, indicating that at elevated pressures the spores were less responsive to temperature changes. These results suggest that the contribution of the pressure component to PATP lethality is most effective when the PATP treatments are conducted under moderate temperature conditions (105°C-110°C).
For all the pressure and temperature combinations tested, the PATP survivor curves exhibited a biphasic pattern, with a rapid initial inactivation region followed by a characteristic tailing. Researchers in the past hypothesized that non-isothermal conditions (temperature drop during the pressure-hold time) inside the pressure vessel could be a factor contributing to the tailing effect. Our experiments with *B. amyloliquefaciens* spores at 600 MPa and 105°C under both isothermal and non-isothermal conditions, confirmed that a temperature drop inside the pressure vessel affects the inactivation rates and also the onset and extent of tailing. Thus, the tailing effect observed under isothermal conditions could be due to other uncontrollable changes in the PATP process or biological variability among a spore population.

Among the non-linear models tested, the Weibull model was most suitable for describing PATP inactivation of *B. stearothermophilus* and *B. amyloliquefaciens* spores in the egg matrix. The parameter b of the Weibull model showed clear trends in change with increasing pressure or temperature and was inversely related to D value. Thus the parameters of Weibull model are good indicators of PATP inactivation kinetics.

PATP technology can be used as a safe alternative to retorting for the production of shelf-stable egg products. The kinetic parameters determined during this study could enable processors and manufactures to predict and control the safety and shelf-life of low-acid foods at a design stage.

### 5.2 Future recommendations

It would be interesting and beneficial to focus research efforts on bacterial spore inactivation by PATP to the following:
- Effect of different pressurization rates (come-up time) on bacterial spore inactivation needs to be investigated
- Effect of different food matrices on the PATP inactivation kinetics need to be studied
- Pressure and temperature coefficients for other bacterial spores subjected to PATP treatments need to be determined
- More research is required to understand bacterial tailing, minimize it and enhance the lethality of the PATP process. Further, mechanistic studies are needed to understand underlying biochemical changes in spores
- Although \textit{B. amyloliquefaciens} spores are suggested as a suitable target organism for PATP sterilization of low acid foods, additional combined pressure-thermal death time data for a larger number of strains is required to establish a suitable surrogate
APPENDIX A

NOMENCLATURE OF TERMS USED IN DIFFERENT NON-LINEAR MODELS FOR MICROBIAL SURVIVOR CURVES
NOMENCLATURE OF TERMS USED IN DIFFERENT NON-LINEAR MODELS FOR MICROBIAL SURVIVOR CURVES

(Non-linear models are listed on page 23)

A. Cerf Model

N₀ = Initial number of microbes

N = number of surviving microbes after being exposed to a lethal treatment for a specific time (t)

F₁ and (1 - F₁) = two fraction of bacteria differing in resistance

k₁ and k₂ = two fraction of bacteria having different killing rates

B. Whiting and Buchanan

N₀ = Initial number of microbes

N = number of surviving microbes after being exposed to a lethal treatment for a specific time (t)

F₁ and (1 - F₁) = two fraction of bacteria differing in resistance

B₁ and B₂ = two fraction of bacteria having different killing rates

t₀ = time for tailing to occur

C. Baranyi

N₀ = Initial number of microbes

N = number of surviving microbes after being exposed to a lethal treatment for a specific time (t)

kₘₐₓ = maximum relative death rate (log CFU/ml)/min)
\( B_t \) = lag time function (min)

\( q_b = (N_{\text{min}} / N_0) \) is the tailing ratio

**D. Modified Gompertz**

\( N_0 \) = Initial number of microbes

\( N \) = number of surviving microbes after being exposed to a lethal treatment for a specific time (t)

\( M \) is the time at which the absolute death rate is maximum

\( B \) is the relative death rate at \( M \)

\( C \) is the difference in value of the upper and lower asymptotes

**E. Log-logistic**

\( N_0 \) = Initial number of microbes

\( N \) = number of surviving microbes after being exposed to a lethal treatment for a specific time (t)

\( \alpha \) = upper asymptote (log CFU/ml)

\( \omega \) = lower asymptote (log CFU/ml)

\( \sigma \) = the maximum rate of inactivation (log (CFU/ml)/log min)

\( \tau \) = the log time to the maximum rate of inactivation (log min)

**F. Weibull**

\( N_0 \) = Initial number of microbes

\( N \) = number of surviving microbes after being exposed to a lethal treatment for a specific
time (t)

b = Scale factor of log survivor curve

n = Shape factor of log survivor curve

**G. Eyring-Arrhenius**

k = rate constant (min$^{-1}$) at a given condition

$k_o$ = rate constant (min$^{-1}$) at reference condition

$\Delta V$ = the activation volume (m$^3$ mole$^{-1}$)

P = pressure (MPa)

$P_o$ = reference pressure (MPa)

$E_a$ = the energy of activation (J mole$^{-1}$)

T = the absolute temperature (K)

$T_o$ = the absolute temperature (K) at reference condition

R = the gas constant (8.314 J mole$^{-1}$ K$^{-1}$)
APPENDIX B

SAS SYNTAX
SAS SYNTAX FOR DETERMINING SIGNIFICANT MEAN DIFFERENCES IN SPORE INACTIVATION AT SPECIFIC TIME POINTS AMONG DIFFERENT TREATMENT CONDITIONS

```sas
option ls=120;
data egg;
input trt $ time $ rep cfu;
cards;

[DATA]

proc glm;
class trt time rep;
model cfu = trt time trt*time rep;
means trt*time;
run;

proc sort data=egg out=new1;
by trt;

proc glm data=new1;
by trt;
class trt time rep;
model cfu=time;
means time/lsd;
run;

proc sort data=egg out=new2;
by time;

proc glm data=new2;
by time;
class trt time rep;
model cfu=trt;
means trt/lsd;
run;
```

**KEY**
trt -refers to treatment condition such as 700MPa-105°C
time- refers to the process hold time such as 1 min
rep- refers to the number of replicates
cfu- refers to log reduction in the number of spores (Log N/No)
SAS SYNTAX FOR DETERMINING SIGNIFICANT MEAN DIFFERENCES IN SPORE INACTIVATION AMONG DIFFERENT TREATMENT CONDITIONS

data egg;
input trt $ time $ rep cfu;
cards;

[DATA]

proc glm;
class trt time rep;
model trt time trt*time rep;
means rep time trt/lsd;
run;

KEY
trt - refers to treatment condition such as 700MPa-105°C
time - refers to the process hold time such as 1 min
rep - refers to the number of replicates
cfu - refers to log reduction in the number of spores (Log N/No)
BIBLIOGRAPHY


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