HIGH PRESSURE DESTRUCTION KINETICS OF BACTERAL SPORES IN LOW ACID FOOD AT ELEVATED TEMPERATURES

By

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Abstract

High pressure (HP) sterilization of low acid foods is a process involving HP at elevated temperatures. Data available on HP destruction kinetics of pressure resistant pathogenic, spoilage-causing and surrogate bacterial spores are limited, but essential for establishing such a process. While compression heating could be employed to provide the elevated temperature, accurate data gathering under such conditions is difficult due to rapid heat loss during the treatment. The objectives of this research were to first standardize the set up carrying out HP tests at elevated and stabilized temperatures, and subsequently to evaluate HP destruction kinetics of selected bacterial spores under HP elevated temperature processing conditions.

The set-up consisted of a thick insulated chamber for holding samples during the test. A relationship was established for the adiabatic temperature rise milk which was used as the main low acid food medium: $\Delta T_P = -0.306 + 0.0224T_i + 0.0423P + 4.49x10^{-4}T_i^2 + 1.31x10^{-4}T_iP - 1.24x10^{-5}P^2$ (R^2 =0.999, n = 50, SE = 0.20°C, $p<0.05$). Initial temperatures at 83.5, 91.7, 95.8°C and 80.7, 88.9, 93.0°C provided operating process temperature 121, 130, 135°C, at 800, 900MPa, respectively.

Destruction kinetics tests were carried with two strains of *Clostridium sporogenes* (11437, 7955) and *Geobacillus stearothermophilus* 10149 spores suspended in milk at 700-900 MPa and 70-100°C. These strains were selected for their relatively high pressue and thermal resistance. The survival counts were well fitted by first order linear models. The D values *C. sporogenes* 11437 varied from 0.73 min at 900 MPa 100°C to 17.0 min at 700 MPa 80°C HP treatments while they ranged from 6.0 to 833 min at 80-100°C under thermal processing conditions. The D values associated with of *C. sporogenes* 7955 spores were higher and varied from 1.3 min at 900 MPa 100°C to 38.2 min at 700 MPa 80°C HP treatments, and from 12.1 to 156 min at 80-100°C during thermal treatments. The D values of *Geobacillus stearothermophilus* 10149 spores varied from 0.6 min at 900 MPa 90C to 20.9 min at 500 MPa 70°C HP treatments with 6.3 to 49.4 min for thermal treatments at 110-120°C. Hence *C. sporogenes* 7955 spores were the most resistant among those studied. The HP destruction kinetics of *C. sporogenes* 7955 spores were also studied in salmon and were lower than in milk.

Pressure resistance screening of selected group I *C. botulinum* spores was carried out at 800-900 MPa at 90-100°C. Nominal D values of each strain were evaluated which demonstrated that PA9508B, HO9504A and CK2-A were the more resistant spores (PA9508B > HO9504A > CK2-A). HP destruction kinetics of *C. botulinum* PA9508B spores in milk were evaluated in detail at 700-900 MPa at 90-100°C and parallel thermal treatment at 90-100°C. The survival counts were again described by first order linear models (R^2 >0.86). The D values varied from 0.35 min at 900 MPa 110°C to 38.9 min at 700 MPa 90°C for HP treatments and 14.4 to 273 min at 90-100°C for thermal treatments. These demonstrated that HP processing combined with elevated temperatures will accelerate the spore destruction rate. However, the associated D values of *C. botulinum* PA9508B spores in milk were more resistant than those of *C. sporogenes* 7955 at 90, 100°C, which indicated that the surrogate may not be very effective for using as a target for verification HP at elevated temperature processing conditions.

Overall, this work has demonstrated several findings. The non-pathogenic *C. sporogenes* 7955 spore was the most resistant surrogate but the pathogenic *C. botulinum* PA9508B spore was even more resistant. D values associated with HP at elevated temperatures were higher than under conventional thermal treatments, and hence provide accelerated destruction kinetics at least for the non-pathogenic spores and hence better spoilage control. However, from safety point of view the conventional thermal sterility requirements would still persist even under HP processing conditions. Milk as a low acid food medium provided more resistance for HP destruction than fish.

Résumé

La stérilization de Haute Pression (HP) sur l'aliments peu acide est un processus impliquant la HP sous les température élevées. Les données disponibles sur HP destruction cinétique de spores bactériennes de pathogènes résistants à la pression, d'altération-provoquant et de substitution sont très limité, mais essentielles pour établir un tel processus. Bien que la compression de chauffage pourraient être utilisées pour fournir l'augmentation de la température, la collecte de données précises dans ces conditions est encore difficile à cause de la perte de chaleur rapide pendant le traitement. Les objectifs de cette étude étaient d'abord de normaliser le coup-monté sur la HP à des températures élevées et stabilisé, et ensuite d'évaluer HP cinétique de la destruction des spores bactériennes sélectionnées traitées par HP à température élevée.

Le coup-monté consistait en une chambre isotherme épais pour la tenue d'échantillons en cours d'essai. Une relation a été établie pour l'élévation de la température adiabatique de lait qui a été utilisé comme le véhicule alimentaire acide faible principal: $\Delta T_P = -0.306 + 0.0224T_i + 0.0423P + 4.49x10^{-4}T_i^2 + 1.31x10^{-4}T_iP - 1.24x10^{-5}P^2$ (R^2 =0.999, n = 50, SE = 0.20 °C, $p<0.05$). Températures initiales à 83.5, 91.7, 95.8 °C et 80.7, 88.9, 93.0 °C offraient la température du processus de traitement 121, 130, 135 °C sous 800, 900MPa respectivement.

Destruction cinétique tests ont été réalisés avec deux types de spores suspendues au laità 700-900 MPa et 70-100 °C : *Clostridium sporogenes* (11437, 7955) et *Geobacillus stearothermophilus* 10149. Ces types choisis résistent relativement a la pression élevée et l'environnement thermique. Les survivants étaient descrites par les modèles linéaires de premier ordre. Les valeurs D de *C. sporogenes* 11437 variaient de 0.73 min à 900 MPa 100 °C à 17.0 min à 700 MPa 80 °C sous le HP traitement alors qu'ils allaient de 6.0 à 833 min 80-100 °C sous des conditions de traitement thermique. Les valeurs de *C. sporogenes* 7955 étaient plus élevées et variées, passant de 1.3 min à 900 MPa 100 °C à 38.2 min à 700 MPa 80 °C sous le HP traitements, et de 12.1 à 156 min à 80-100 °C durant les traitements thermaux. Les valeurs D de spores *Geobacillus stearothermophilus* 10149

s'étendaient de 0.6 min à 900 MPa 90 °C à 20.9 min à 500 MPa à 70 °C sous le HP traitement avec 6.3 à 49.4 min pour les traitements thermiques à 110-120 °C. En conséquence, spores *C. sporogenes* 7955 sont les plus résistantes parmi ces études. La HP destruction cinétique de spores de *C. sporogenes* 7955 ont également été étudiées dans le saumon mais ils étaient plus faibles que dans le lait.

La résistance à la pression a été réalisée à 800-900 MPa à 90-100 °C sur la groupe choisie I de spores de *C. botulinum*. Les valeurs D nominal de chaque type ont été évaluées, ce qui montrent que PA9508B, HO9504A et CK2-A sont les spores les plus résistantes (PA9508B> HO9504A> CK2-A). HP destruction cinétique de PA9508B de spores *C. botulinum* dans le lait ont été évalués en détail à 700-900 MPa à 90-100 °C et parallèle au traitement thermique de 90-100 °C. Les survivants ont été de nouveau décrits par les modèles linéaires de premier ordre (R^2> 0.86). Les valeurs D variaient de 0.35 min à 900 MPa 110 °C à 38.9 min à 700 MPa à 90 °C pour les traitements de HP et de 14.4 à 273 min à 90-100 °C pour les traitements thermaux. Ceux-ci ont démontré que HP traitement combiné avec des températures élevées accélérera le rythme de destruction des spores. Toutefois, les valeurs associées D de PA9508B de spores de *C. botulinum* dans le lait étaient plus résistantes que celles de *C. sporogenes* 7955 à 90, 100 °C, ce qui a indiqué que le substitut ne pourrait être très efficace pour l'utilisation comme cible pour la vérification HP à température élevée en traitement.

Dans son ensemble, cette étude a démontré plusieurs conclusions. Le non pathogène *C. sporogene* spore 7955 était le subsitut le plus résistant mais le pathogène *C. botulinum* PA9508B litiges est encore plus résistant. Les D valeurs associées à HP à des températures élevées ont été même plus élevés que dans le cadre de traitements thermiques classiques, et donnent donc la destruction cinétique accéléré au moins pour les non pathogènes spores, et conséquemment au meilleur contrôle d'altération. Cependant, du point de vue de la sécurité les stérilité sous les thermiques classiques ont besoins de plus de persistances même dans des conditions de traitement de HP. Le lait comme un vehicule d'acide faible alimentaire peut fournir plus de résistance pour HP destruction que le poisson.

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List of publications and presentations

Part of this thesis research has been published or prepared for publication in a refereed scientific journal:

Shao, Y., Zhu, S., Ramaswamy, H.S., and Marcotte, M. (2007). Compression heating and temperature control for high pressure destruction of bacterial spores: an experimental method for kinetics evaluation. Food and Bioprocess Technologies. (accepted for publication)

Shao, Y., and Ramaswamy, H.S. Destruction kinetic studies on high-pressure of *Clostridium sporogenes* ATCC11437 and ATCC7955 spores in milk at elevated quasi-isothermal conditions (in preparation)

Shao, Y., and Ramaswamy, H.S. Destruction kinetics of *Clostridium sporogenes* ATCC7955 spores in salmon meat base by combinations of high pressure and elevated temperatures (in preparation)

Shao, Y., and Ramaswamy, H.S. Inactivation kinetics of *Geobacillus stearothermophilus* 10149 spores in milk by combination of high pressure and high temperature (in preparation)

Shao, Y., Bussey, J., Ramaswamy, H.S., and Austin, J. Resistance screening of twelve *Clostridium botulinum* (group I) spores in phosphate buffer by combinations of high pressure and high pressure (in preparation)

Shao, Y., Bussey, J., Ramaswamy, H.S., and Austin, J. Combined effects of high pressure and elevated temperature on destruction kinetics of *Clostridium botulinum* PA9508B spores in milk (in preparation)

Part of this thesis has been presented in refereed scientific conferences:

Shao, Y., Ramaswamy, H.S., and Zhu, S. (2005). Compression heating behavior of milk under high pressure, Agricutural and Biosystems Engineering Tech. Conference, Saint-Hyacinth, Quebec, Canada, March 23, 2005

Shao, Y., and Ramaswamy, H.S. (2006). Destruction kinetics of *C. sporogenes* ATCC11437 spores in milk by high pressure combined with elevated temperature, IFTPS, paper competition, Jan, 2006

Shao, Y., and Ramaswamy, H.S. (2006). High pressure high temperatures destruction kinetics of *Geobacillus stearothermophilus* 10149 spores in milk, CIFST, Montreal, May 28, 2006

Shao, Y., and Ramaswamy, H.S. (2006). High pressure destruction kinetics of *C. sporogenes* 7955 spores in milk at elevated temperatures, IFT Conference, Orlando, FL, June 25, 2006. 2006-IFTPS Paper competition, 4th place finish.

Shao, Y., and Ramaswamy, H.S. (2007). Destruction kinetics of *C. sporogenes* ATCC7955 spores in salmon meat by high pressure combined with elevated temperatures, 2007-IFTPS paper competition. 2nd place finish.

Contributions of authors

Part of the thesis research has been published and presented in conferences. Additional papers are being prepared for publication. Four other authors have contributed to this research in addition to the candidate and his supervisor, and their contributions to the various articles are as follows:

Yanwen Shao is the Ph.D candidate who planed and conducted all experiments, gathered and analyzed the results, and prepared the drafts all manuscripts for scientific publications.

Dr. H. S. Ramaswamy is the thesis supervisor, under whose guidance the overall research was carried out, and who assisted the candidate in planning and conducting the research and also in correcting, editing and reviewing the manuscripts. Dr. John Austin supervised the candidate's *C. botulinum* research work at Microbiology Research Division, Bureau of Microbial Hazards, Health, Canada, Ottawa, ON. He provided the laboratory facilities, offered scientific advice, reviewed experimental procedures and supported the candidate to carry out the experimental study on pressure resistance screening and pressure inactivation kinetics of *C. botulinum* PA9508B.

Dr. Songming Zhu helped candidate in the fabrication of the insulated test chamber, and assisted in necessary equipment modifications for the experimental study. He also reviewed the manuscript of compression heating work. Mr. Jeff Bussey helped candidate to prepare *C. botulinum* spores and enumerate survival spores at Microbiology research division, Bureau of microbial hazards, Health, Canada, Ottawa, ON.

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Nomenclature

D	Decimal reduction time (min)
D_1	Decimal reduction time (min) at holding time t_1
D_2	Decimal reduction time (min) at holding time t_2
Log D	Logarithmic decimal reduction time (min)
N	Number of surviving microorganisms after pressure treatment (CFU/g) at time t (min)
No	Initial number of microorganisms with no pressure treatment (CFU/g)
P	Pressure (MPa)
ΔT_P	Adiabatic temperature rise (°C)
t	Holding time (min)
t_1	The first treatment holding time (min)
t_2	The second treatment holding time (min)
t_{eT}	effective time (min)
t_{eq}	equivalent time (min)
Δt	Real time interval (min)
Ti	Initial temperature (°C)
Tp	Processing temperature (°C)
T_{Vi}	Initial temperature of pressure medium in the vessel (°C)
V_{In}	Outer volume of the POM insulator (m^3)
V_V	Volume of HP vessel (m^3)
V_m	Volume of pressure medium inside high pressure vessel (m^3)
z	Thermal sensitivity (°C)
z_p	Thermal resistance or thermal sensitivity (°C) at constant pressure
z_T	Pressure resistance or pressure sensitivity (MPa) at constant temperature

Abbreviations

ANOVA	Analysis of variances
ATCC	American Type Culture Collection
CFU	Colony forming unit
CSA	Campdem sporulating agar
FDA	Food and Drug Administration (USA)
HP	High pressure
HPB	Health Protection Branch
HPHT	High pressure high temperature processing
HTST	High temperature short time processing
LACF	Low acid food
MPA3679	Microbiological medium agar specific for *C. sporogenes* 7955
NA	Nutrient agar
PATP	High pressure assisted thermal processing
PDT	Pressure destruction time
POM	high polymer polyoxymethylene
RCM	Reinforced clostridial medium broth
TDT	Thermal death time
TSA	Tryptic soy agar
TSBYE	Tryptic soy broth with yeast extract
UHT	Ultra high temperature processing
YDTAS	Yeast Dextrose Tryptone Agar with enriched amidon

Chapter 1

Introduction

Consumer demand for convenient, fresh-like, safe, high-quality food products has always been growing. To meet this trend, there has been increasing interest in using high hydrostatic pressure processing as a non-thermal food preservation technique. Over the past decades, high pressure has emerged as a commercial alternative to traditional thermal processing methods for some foods, e.g., jams, fruit juices, guacamole, and fresh whole oysters. Its primary advantage is that it can inactivate microorganisms and enzymes at substantially lower treatment temperatures (as compared to conventional thermal processing) that result in processed foods possessing sensory and nutrient qualities closely resembling the original fresh or raw product (Hoover, 1993; Smelt, 1998). Generally, high pressure processing as a novel non-thermal food processing technique is widely applied in pasteurization food product industries to extend food shelf life and preserve high qualities, such as natural color, flavor and nutrients. However, several resistant microorganisms and enzymes can survive in the pasteurization. Therefore, high pressure processed foods should be kept at low temperature refrigeration conditions to prevent spoilage (Patterson, 2005).

Nowadays, sterile shelf-stable products are currently produced by thermal processing. New thermal processing techniques, such as high temperature short time (HTST) processing and aseptic processing, provide effective microbial inactivation while causing less damage to the sensory and nutritional characteristics of the products. However, the damage is not avoidable and processed food types are limited. Although there is great potential for the use of high-pressure high-temperature processing (HPHT) or high-pressure assisted thermal processing (HPTP) as an alternative method to thermal sterilization process, commercial high pressure sterilization technology has not been realized yet, because resistant bacterial spores cannot be inactivated by high pressure alone (Sale et al., 1970; Knorr, 1995; Maggi et al., 1996; Mills et al., 1998).

Some studies have shown that high pressure treatment at elevated temperatures, it is possible to achieve spore inactivation (Mills et al., 1998; Scurrah et al., 2006; Reddy et al., 1999 and 2003). High pressure (HP) as an alternative preservation technique for thermal sterilization of low acid foods (LACF) has some advantages, such as quick heating, quick cooling and shorter process time. Adiabatic compression during HP treatment will increase the temperature of foods by approximately 3-9 °C per 100 MPa, depending on the temperature and composition of the food. Adiabatic heating will benefit HP sterilization. It can be achieved as an accelerated thermal process by taking advantage of compression heating during pressure treatment or taking into account higher destruction rates at the sterilization temperatures that may be possible by the combined use of high-temperature and high-pressure. Compared with thermal processing, the advantage of HPHT sterilization is that HP treatment results in rapid heating to lethal levels during pressurization and subsequent rapid cooling rate due to the quick depressurization. This feature will minimize food quality deterioration. HP sterilized food quality should be equal to or better than that of frozen foods (Hoogland et al., 2001; Matser et al., 2004).

Currently, sterile low acid food products are produced by heat processing to destroy the heat resistant microorganisms. FDA stipulates that for sterile low acid foods regulation, commercial sterilization requires at least to achieve a 12-log reduction of target *Clostridium botulinum* spores (F_0=3min). However, canning industry usually requires a common process lethality (F_0) of 5 minute for controlling low acid product spoilage. As a pathogenic *Clostridium* species that is able to produce serious toxic neurotoxin, it not convenient work with *C. botulinum* in canning research and development. *Clostridium sporogenes*, a typical thermal resistant, mesophilic non-pathogenic spore-former, is usually used as a surrogate of *Clostridium botulinum* in establishing thermal processing specifications. *C. sporogenes* ATCC7955 (PA3679) spore has heat resistance similar to or greater than that of *C. botulinum.* Maggi et al. (1996) reported this bacterial spore has been recognized for its resistance to heat as well as pressure. In addition, one of the most heat resistant non-pathogenic thermophilic *Geobacillus stearothermophilus* spores, whose D_{121C} was 5.5 min, has been used in

validating thermal processing parameters (especially in agitation processes). For high pressure sterilization, there is an assumption that *G. stearothermophilus* spores which were resistant in thermal processing could have similar trend in HPHT processing. Thus, the high pressure inactivation of *C. botulinum, C. sporogenes* ATCC7955, *Geobacillus stearothermophilus* and other resistant bacterial spores need to be verified. High pressure sterilization processing needs to fulfill the low acid food sterilization requirements before commercialized pressure treated foods become available.

Thermal inactivation kinetics of heat resistant bacterial spores have been generally assumed to follow a first order model. Based on these models, thermal processing theory has been well developed. Similarly, in order to design an applicable HP sterilization process, it is essential to define high pressure and high temperature inactivation kinetics of target pathogenic, spoilage causing and spore forming bacteria. Therefore, it is becoming more and more important to have a good kinetic database on the high-temperature high-pressure resistance of pathogenic and spoilage-causing bacterial spores. Such data can then be used to develop and verify HP sterilization processes and subsequently assure microbial safety and optimal product quality. Although inactivation of bacterial spores using HP has been studied extensively, information on the inactivation kinetics of resistant bacterial spores under high pressure at elevated temperatures is still limited. An IFT expert panel has pointed out the lack of kinetic data for various bacterial spores suspended in food products under various pressure–thermal conditions so far (FDA, 2000). Thus, predicting the effectiveness of HPHT processing against these spores based on accurate inactivation kinetics is essential to permit establishment of safe processing conditions and critical for its effective application in low acid food HP sterilization.

The problem to measure kinetic data by high pressure processing at high temperature is the temperature rise in the pressure equipment chamber due to the adiabatic compression heat and subsequent rapid heat loss to the vessel. During HP processing, the compression heat is removed rather quickly due to the unavoidable heat transfer between food sample and equipment chamber wall which usually remains at a

relatively much lower temperature. Food temperature decreases significantly due to this heat loss. The non-isothermal condition during high pressure treatment makes the kinetic data less reliable. Continued change in temperature results in apparent deviations from the log-linear trend even for those which strictly follow the first order kinetics. So, controlling sample temperature within a narrow temperature range is the key to study microbiological destruction kinetics (Patazca et al., 2005). An internal electric heater installed in the chamber has been used to prevent the heat loss and maintain stable temperatures while in other studies, test samples have been sufficiently insulated (Koutchma et al., 2005; Patazca et al., 2006).

The overall objectives of this research were

1. To evaluate the adiabatic heating behavior of milk under HP and standardize the processing conditions to control process temperature at HP and elevated temperature processing conditions
2. To investigate HPHT destruction kinetics of resistant bacterial spores (non-pathogenic and pathogenic)
3. To compare HP sterilization with thermal sterilization for inactivation advantage

The specific objectives included:

1. To establish HP processing conditions spore destruction kinetics in milk
2. To evaluate HP destruction kinetic of spores in milk or fish meat slurry:
   Geobacillus stearothermophilus 10149 and *Clostridium sporogenes* 11437, 7955 (possible surrogates)
   C. botulinum (pathogenic studies to be carried out in Health Canada facility in Ottawa)
3. To evaluate inactivation kinetic parameters by thermal and HP treatments

Based on above research objectives, studies were carried out as indicated in the following flow chart (Figure 1.1).

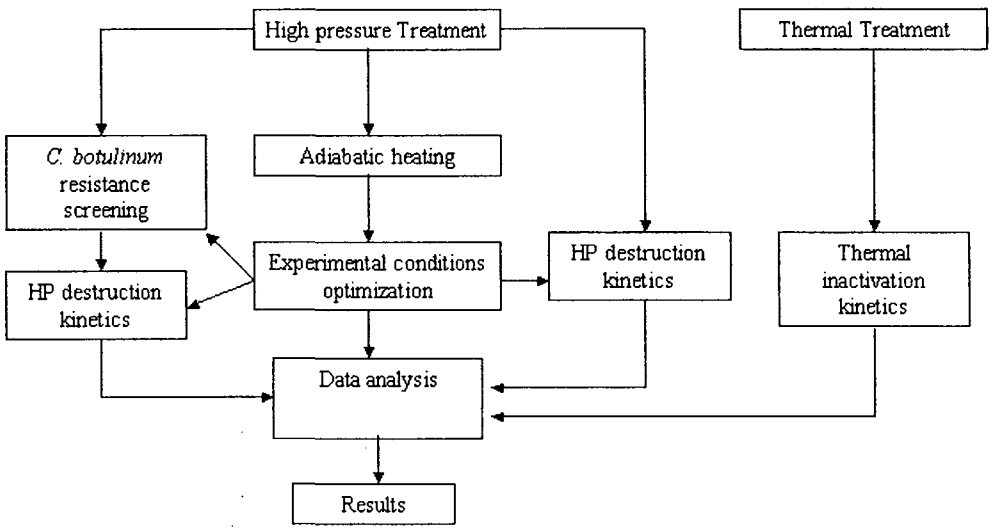


Figure 1.1 Basic flow chart of this research work

Chapter 2

Literature review

2.1 HP sterilization of low acid foods

High pressure is an alternative processing method to traditional thermal sterilization of low acid foods (LACF) with shorter process times. As per LACF regulations (21 CFR 113), in order to establish sterilization process, commercial sterility specification must be determined in terms of spoilage causing organisms and pathogens able to grow under the storage and distribution conditions. However, the process is not defined in the regulations.

In food industry, the problems with spoilage can come from different phases of production, storage and distribution and have been the biggest concern for a long time. In order to solve these problems, extensive studies have been made on some typically heat-resistant bacterial spores. For establishing a safe and sound process, bacterial spores of *Geobacillus stearothermophilus* along, a thermophilic heat resistant bacteria, as well as *Clostridium sporogenes*, a mesophilic and spore-forming organism, have been investigated as surrogates. Spores of the strain *C. sporogenes* PA 3679 are selected because their heat resistance has been found to be similar and equal to or greater than that of spores of *Clostridium botulinum.* It has also been noticed that these bacterial spores have shown resistance to pressure as well as to heat (Maggi et al., 1996).

Although the LACF regulations are presently not intended for pressure processed foods, it can be logically extended to HP sterilization of LACF, because the regulations require a strong thermal component for spore destruction, which provides room for HP processing to demonstrate that high pressure does play an important role not only in accelerating the spore inactivation kinetics, but also in achieving the process more rapidly due to the inherent adiabatic compression resulting in rapid temperature rise in the product.. Therefore, the requirements in the Regulations must be respected before a pressure-sterilized LACF can enter commercial production.

The issues of public health concerned spoilage or pathogen need to be addressed from two aspects: (1) inactivation kinetic parameters of target pathogenic and spoilage-causing spore-forming bacteria must be acquired in order to establish high pressure high temperature sterilization processes; (2) the established sterilization process needs to be verified with the process outcome to show its verification and effectiveness.

Current studies show that pressure has a complementary synergistic effect on the inactivation of microorganisms and elimination of problematic spore-formers in foods (Wilson, 1974; Rovere et al., 1996b). Thus, it is important to combine HP with high temperature (HT) during pressure processing to achieve microbial inactivation. Knowledge of the HP inactivation kinetics of pathogens is essential to design a safe sterilization process. FDA (2000) indicated that in addition to being extremely heat-resistant, bacterial spores also show highly variable levels of pressure resistance. This variability depends on the conditions of their sporulation and pressure treatment. Given these characteristics, consistency of spore crop preparation and standardization of methods are quite important for inoculated pack studies using nonpathogenic spore forming indicator organisms. *C. sporogenes* PA 3679 appears to be a good choice in that this strain has served as an indicator organism for *C. botulinum* in the canning industry over a long time. *Bacillus subtilis* may be a better choice for some applications because spore suspensions of *B. subtilis* are highly pressure resistant, and as a facultative anaerobe, easier to grow and handle. In some recent studies (Margosch et al., 2004; Ahn et al., 2007) other thermophilic spores such as *Clostridum thermosacchrolyticum* and *Bacillus amyloliquifaciens* have been shown to have good pressure resistance. *B. amyloliquefaciens* spores have the most pressure resistance than other spores. However, there are no detailed kinetic information available on these spores.

Stumbo (1973) put forward that thermal processing should achieve a 12-decimal point reduction of *C. botulinum*, but did not establish an endpoint. Pflug (1987) first defined the thermal process as the probability of survival of either a spoilage-causing organism or pathogen and proposed an alternative approach to calculate the process lethality by describing the outcome. The last but not least essential step in establishing the process is to measure the outcome in product samples to determine if desired log

reductions have been achieved and convert this to a process value. Sizer et al. (2003) recommended various validation strategies which can be used to demonstrate the safety of high-pressure-processed low acid foods.

Balasubraminiam (Balasubraminiam et al., 2003; Matser et al., 2004; Clark, 2002, 2006) described a variation he called pressure-assisted thermal treatment in which food is heated to about 90°C, then pressurized to about 690 MPa. The adiabatic heating raises the temperature to about 121°C, hot enough to kill *C. botulinum* (Figure 2.1). This permits treatment of low-acid foods and heat inactivating the enzymes. The advantage of the process is that when pressure is released, cooling is very quick. In conventional thermal processing, cooling can be slow and much of the damage to flavor and texture occurs then.

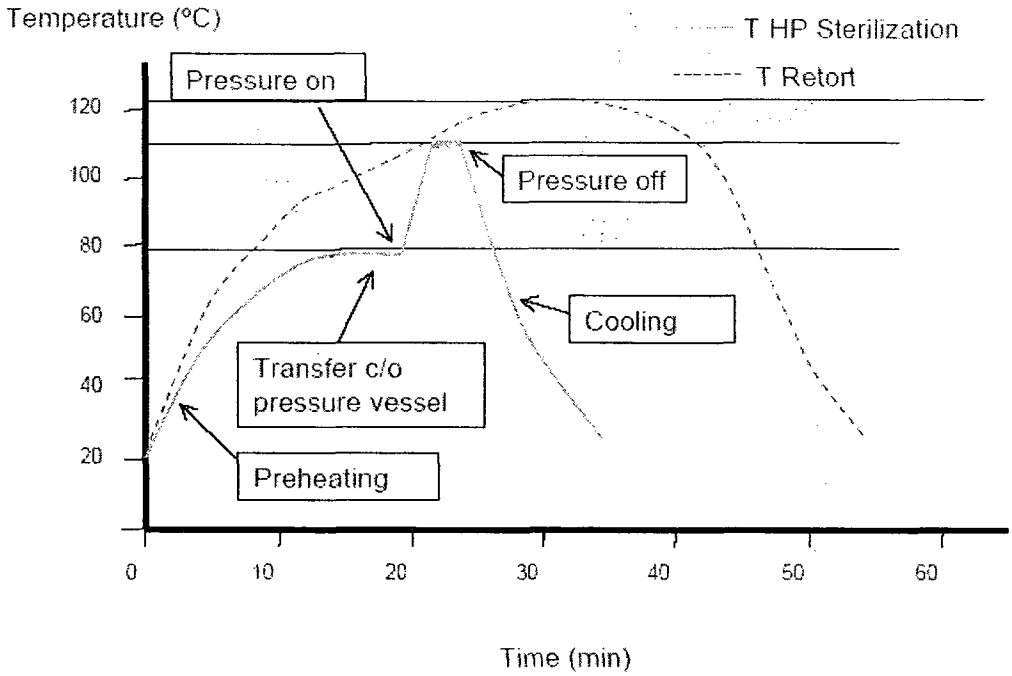


Figure 2.1 Temperature in center food package during high-pressure high-temperature processing with conventional preheat against conventional heat sterilization

2.2 Thermal effects during high-pressure processing

Compression heating is an unavoidable thermal effect during HP processing which could be desirable in HP processing. Pressure can be instantaneously and uniformly distributed at all points of foods in a pressure chamber, but temperature may not be uniform because of compression heating and subsequent heat transfer (Ting et al., 2002). Many factors can effect temperature variation and distribution of foods during HP processing, including HP medium, sample size and components, initial temperature, HP equipment (chamber volume), etc. Deviations from process uniformity are thus mainly due to variations in temperature. Compression heating of pressure could be combined with conventional heating for food sterilization (Maggi et al., 1996; Alpas et al., 1998). However, early HP laboratory equipment frequently do not include temperature sensors within the pressure chamber. As a result, the effects of compression heating under pressure were not investigated (Ting et al., 2002). So far, only few articles have shown the temperature history during HP processing of microbial inactivation. Kinetic data of thermal effects together with pressure are still very scarce (Knorr, 1999).

Since the early work of Bridgman (1912), the adiabatic compression behavior of water has been well characterized. All compressible substances change temperature during physical compression, an unavoidable thermodynamic effect. The magnitude of this change depends mainly on the compressibility of the substance and its specific heat. Water typically changes about 3°C for every 100 MPa of pressure change. Temperature increases with pressure and decreases with pressure release.

Since water is a main ingredient in most foods, the compression of most foods exhibits adiabatic temperature changes very similar to that of water (Table 2.3). Taking into account the importance of temperature and temperature control could make high-pressure sterilization economically more feasible. Maximizing the high adiabatic heating foods temperature should benefit for high-pressure sterilization (de Heij et al., 2003). Foods that are notably different are oils and alcohol. Many of the early laboratory machines were not fabricated from stainless steel and necessitated the use of oils as the

pressure medium. Solutions of castor oil, silicone oil, sodium benzoate, and glycol are sometimes used as pressure-transmitting fluids. If the pressure-medium fluid exhibits high compression heating, the inactivation data could include unintended thermal effects. For example, many oils have a maximum compression heating temperature three times that of water. There stands a chance that the pressure-medium fluid upon compression will heat the sample. This can lead to errors in the inactivation study. These errors can be large when a small sample size is used with a large quantity of medium fluid.

In reality, despite the fact that the liquid and pressure vessel may have started at the same temperature, upon pressurization the compressed liquid is hotter than the metal chamber surrounding it. The metals used in pressure vessel construction are not subjected to meaningful compression heating. This deviation from the zero-heat-transfer, adiabatic assumption, makes the adiabatic temperature a maximum possible value during HP processing. The difference in chamber wall temperature and content temperature results in the transfer of heat from the content into the chamber wall and a lowering of temperature during processing. If held under pressure for a sufficient time, the temperature of the pressurized liquid will eventually match the chamber wall temperature. In general, for microbiological inactivation that is benefited by higher temperatures, the longer that a sample is held at pressure under an uncontrolled temperature condition, the lower is the marginal benefit due to a decreasing temperature as a result of cooling. When this type of data is analyzed using first-order kinetics, a tailing effect can result.

There have been limited studies describing the change in temperature of food products during high-pressure processing: (Ting et al., 2002; Balasubramanian et al., 2003; Otero et al., 2003; Rasanayagam et al., 2003; Ardia et al., 2004; Patazca et al., 2007). The magnitude of the temperature increase depends on factors such as process pressure, initial temperature, the composition of food and pressure transmitting fluid; Knowledge of the compression heating properties of food constituents over a range of pressure-temperature combination is needed for better understanding of various HPP parameters on food safety and quality (Balasubramaniam et al., 2003). Temperature may play an important role during HP processing. Therefore, pressure, temperature, and time-

based data should be reported to characterize the studies being performed (Ting et al., 2002).

Table 2.1 Adiabatic compression for some substances (from Ting et al., 2002)

Substance (25°C)	T change per 100MPa (°C)
Water	~ 3
Mashed potato	~ 3
Orange juice	~ 3
2%-fat milk	~ 3
30%-fat Cheese	~ 5*
Salmon	~ 3.2
Water/glycol (50/50)	4.8 ~ 3.7^
Beef fat	~ 6.3
Olive oil	8.7 ~ 6.3^
Soy oil	9.1 ~ 6.2^

*Tested in our laboratory; ^Compression heating decreases at higher pressure

High temperature caused by adiabatic compression heating is good for inactivation of microorganisms and enzymes. Since temperature rise increases with increasing of pressure and initial temperature; water temperature changes about 3°C for every 100 MPa change in pressure at 25°C and would change 4.8°C for every 100MPa of pressure change at 60°C (Otero et al., 2003; Kalichevsky et al., 1995). So, pressure results in higher temperature at elevated temperature. It is possible that water temperature can reach 120°C under 900MPa pressure combined with 80°C initial temperature. Thus, high-pressure high-temperature sterilization of low acid foods would be carried out at 120C or up with short processing time. On the other hand, higher temperature rise causes faster temperature drop and serious temperature un-uniform due to the heat loss. It is the main problem for determining inactivation kinetics of resistant bacterial spores. In order

to measure accurate destruction kinetic parameter (D values) under high pressure at elevated temperature, food compression heating behavior needs to be studied and kinetic research conditions need to be optimized to control temperature at constant conditions. Figure 2.2 gives a example to prove unstable temperature effect on spore survival curve.

2.3 High pressure inactivation of bacterial spores

2.3.1 High pressure inactivation of *Clostridium spp.*:

Most published data have dealt with the HP destruction of the thermophilic spores of *Geobacillus stearothermophilus*. *Clostridium* spores have been assumed to behave in a similar manner to *Geobacillus* spores under HP. However, recent studies (Table 2.2) on *Clostridium* spores have not confirmed this assumption.

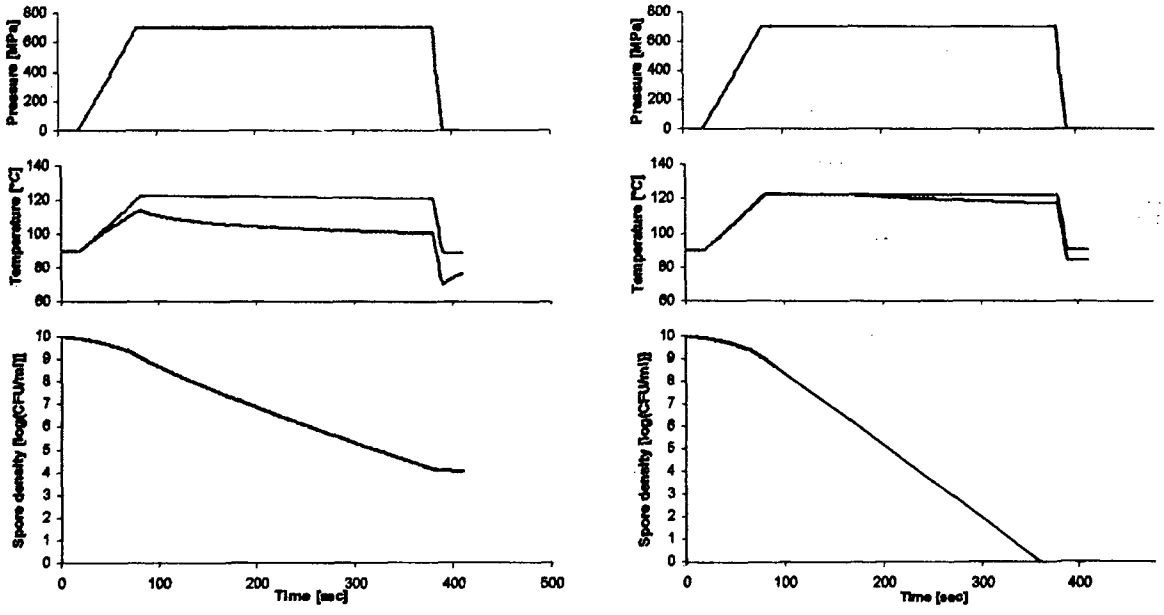


Figure 2.2 Simulation of high-pressure inactivation of spores of *B. stearothermophilus* ATCC 7953 (de Heij et al., 2003)

Table 2.2 HP inactivation of *Clostridium spp.*

Matrix	Process	Bacterial reduction	Reference
Phosphate buffer	90 °C + 200 MPa+30 min	7-log PA3679 HP+HT	Okazaki et al. (1996)
Different model food systems	54 °C+1,400 MPa + 5min 75°C +800MPa+5min	5-log PA3679	Rovere et al. (1996a)
Meat broth	30°C +900 MPa +10 min	<2.9-log, PA3679	Gola et al. (1996)
	20°C +600MPa+30min 60°C +400 MPa +30min	No effect More effective	Mills et al. (1998)
	20°C +1500MPa+5 min	No effect	Maggi et al. (1996)
Meat broth	108°C +800MPa	D value 0.695 min	Rovere et al. (1996b)
Sucrose laurate	25-70°C+404MPa; pH6-7 +nisin	<0.5-5-log	Stewart et al. (2000)
	105°C+690MPa	6-log *sporogenes*3679	Meyer et al. (2000)
phosphate buffer 0.067M	50°C+825MPa+5min 50°C+758MPa+10min 75°C +800 MPa +20min	5-log *botulinum* E 5-log *botulinum* E 2-log *botulinum s* 62-A	Reddy et al. (1999) Reddy et al. (2003)
buffer pH 7.0	75°C +800 MPa +20min	2-log *botulinum* BS-A	
crab meat	75°C+827MPa+20-30min	>6 log *botulinum* B	Reddy et al. (2006)
Mashed carrots	80-116°C+600-800MPa	*0-5.5-log botulinum*	Margosch et al. (2004)
Tris-His buffer	90-110°C+800-1400MPa+2min	0-4.4log *botulinum*TMW2.357	Margosch et al. (2006)
Phosphate buffer0.067M	T=60,65,70,75°C P=517,620,723,827MPa	D_{80C}=0.75min *botulinum* type E	Rodriguez et al. (2004)

Table 2.2 HP inactivation of *Clostridium spp. (continued)*

Matrix	Process	Bacterial reduction	Reference
buffer	T=91, 100, 108°C isothermal P=600-800MPa	*sporogenes* 3679 Kinetics	Koutchma et al. (2005)
PBS buffer	55-75°C+550-650MPa+pH4.75-6.5	*perfringens* type A *0.4-5.8* log 7955,3584	Paredes-Sabja et al. (2007)
Distilled water	121°C+700MPa+1min	7-8-log *sporogenes*7955 0.2-6.1 log *C. tyroutylicum*	Ahn et al. (2007)

In a study involving spore suspensions of *C. sporogenes* PA 3679 in meat broth, the pressure acted as a complementary synergistic process to allow reduction of the thermal processing parameters necessary to eliminate problematic spore-formers in foods. They concluded that it was important to combine HP with HT during pressure processing. Knowledge of the HP inactivation kinetics of *C. sporogenes* is essential to design a safe sterilization process.

Heredia et al. (1997) demonstrated that not only spores of *C. perfringens* will show increased heat resistance by a sublethal heat shock of 55 °C/30 min, but the vegetative cells will become more heat-resistant as well (at least two to three-fold). Spores of *C. botulinum* held in calcium acetate solutions (0.1 to 0.5 M) for 140 h at 50 °C raise heat resistance five to ten times, while heat resistance can be lowered by holding the spores in 0.1 N HCl at 25 °C for 16 h (Alderton et al., 1976). Rodriguez et al. (2004) reported *C. botulinum* type E spore had activation energy (Ea) 1.14×10^7 J/(kmol K) and $D_{80°C}$=0.75 min.

Koutchma et al. (2005) showed inactivation kinetics of *sporogenes* ATCC7955 spores in buffer under 600-800 MPa pressure combined with 91, 100, 108 °C constant temperatures. D values were 5.96, 4.9, 4.5 min and 1.13, 0.97, 0.82 min for 600, 700, 800 MPa at 91, 108 °C respectively. Thermal resistance at constant pressure was 23.7 °C and

pressure resistance at constant temperature was 1500.7 MPa. These data illustrated higher thermal resistance and pressure resistance. Finally, these spores were relatively more sensitive to temperature than to pressure.

Such observation has been indicated by exposure of spores to the natural acid conditions of some foods. The extent of variability of clostridial spores to pressure is not well known as a function of food composition. This is important, not only because of the pathogenic nature of these two species of *Clostridium*, but because strains of *C. botulinum* can produce very pressure-resistant spores. As stated earlier, spore suspensions of *C. botulinum* 17B and Cap 9B have shown little, if any reduction in viability after exposure to 827 MPa at 75 °C (FDA, 2000).

2.3.2 High pressure inactivation of *G. stearothermophilus spp.:*

High pressure processing has been proven to be an efficient method for the inactivation of vegetative bacteria, viruses, and yeasts; however, bacterial spores are not inactivated by pressure treatment at ambient temperature (Sale et al., 1970). *Bacillus stearothermophilus* (subsequently renamed by ATCC as *Geobacillus stearothermophilus)* is one of the critical species of nonpathogenic thermophilic organisms that is traditionally used in establishing thermal process specification due to its extremely high heat resistance.

Rodriguez et al. (2004) reported *G. stearothermophilus* 10149 had activation energy (Ea) 7.746×10^7 J/(kmol K) with z_T (34.5 °C) and z_P (370MPa). Patazca et al. (2006) reported a study of inactivation kinetics of *G. stearothermophilus* 10149 spores in distilled water under 500-700 MPa pressure combined with 92, 100, 111°C constant temperatures. D values were 1.8, 1.1, 0.5 min and 0.86, 0.23, 0.10 min for 600, 700, 800MPa at 91, 111 °C respectively. Thermal resistance at constant pressure was 58.8,

27.2, 27.4 °C at 500, 600, 700 MPa and pressure resistance at constant temperature was 352, 313, 216 MPa. These data illustrated higher thermal resistance and pressure resistance. Generally, these spores were relatively more sensitive to pressure than to temperature.

Several studies have been carried out on the high-pressure inactivation of *G. stearothermophilus* at different pressures, temperatures, and pH, using different inoculation media (Table 2.3). However, no detailed studies on quantifying the kinetic parameters have been reported. They found that spores could be inactivated at higher-pressures only when applied at elevated temperatures; inactivation was enhanced with an increase in treatment intensity.

These results suggested that both temperature and pressure play critical role in the HP inactivation of spores. While a steady pressure during holding time had been adequately maintained in most studies, there have always been problems with maintaining a steady temperature. Since the heat of compression of the test samples during high pressure processing is expected to increase the temperature of the sample, it is necessary to control and monitor its initial temperature before pressurization and under pressure treatment. Maintaining constant process temperature is particularly important due to heat loss through walls and closures of the pressure vessel that often results in large temperature drops during long holding time. In addition, the effects of pressurization come-up time and depressurization come-down time on process lethality have not been taken into consideration.

Table 2.3 HP inactivation of *Geobacillus stearothermophilus* articles

Matrix	Process	Bacterial reduction	Reference
Buffer (pH 7.0)	90 °C + 200 MPa+30 min	6-log	Seyderhelm et al. (1992)
Buffer (pH 7.0)	80 °C + 350 MPa +30 min	6-log	
Buffer (pH 7.0)	60°C+250MPa+5min	1.5-5 log	Ludwig et al. (1992)
	20-70°C+200-900MPa+1-10min	4-log *B. licheniformis; B. coagulans*	Fornari et al. (1995)
Buffer (pH 7.0)	90 °C + 150 MPa + 30min	5-log	Kakugawa et al. (1996)
Phosphate buffer	70 °C + 800 MPa + 3 min	5-log	Gola et al. (1996)
Phosphate buffer	70 °C + 700 MPa + 5 min	5-log	
Phosphate buffer	20°C +1500 MPa +5 min	No reduction	Miglioli et al. (1997)
Phosphate buffer	60-90°C +800-1000 MPa +5min	>5.3 -log	
Phosphate buffer	60 °C + 800 MPa + 5 min	3.5-log	
Phosphate buffer	70 °C + 700 MPa + 5 min	5-log	Rovere et al. (1998)
Phosphate buffer	20°C + 800 MPa+60min	No reduction	Hayakawa et al. (1998)
Phosphate buffer	60°C + 800 MPa+60min	4- log	
Meat batter	20-80°C+50-400MPa +1-60min	RSM	Moerman et al. (2001)
Mashed broccoli and cocoa mass	50 to 600 MPa+ 60 to 120 °C	Inactivation@ higher-pressures +elevated temperatures	Ananta et al. (2001)
Phosphate buffer0.067M	92-110°C+ 517,620,723,827MPa	Ea=7.746x10^7J/(kmolK)	Rodriguez et al. (2004)
Egg patty	105C+690MPa+2-5min	6 log verification	Koutchma et al. (2005)
Milk buffer	63-97°C+432-768MPa	RSM	Gao et al. (2006)
Distilled water	92-111°C+500-700MPa	kinetics	Patazca et al. (2006)
Egg patty	105°C+700MPa+5min	4-log reduction, D_{105C} = 0.41 min	Rajan et al. (2006)

2.3.3 High pressure inactivation of *Bacillus spp.:*

Margosch et al. (2004a) reported that spores of *B. liccheniformis* TMW 2.492 were more resistant than *B.sutilis* TMW 2.485 and were completely inactivated for 600-800MPa at 80C 16min. *B. amyloliquefaciens* spores were more pressure resistant than those of *B. liccheniformis* TMW 2.492. Table 2.4 summarized the reported data on HP destruction kinetics of *Bacillius spp.* Spores.

2.4 High pressure inactivation kinetic modeling

Scientists have always attempted to understand the mechanism of cell death beyond the quantitative description of inactivation. However, because only data on survival counts are available, the interpretation of inactivation kinetics has been a subject of speculative discussion since the beginning of the 20^{th} century.

The inactivation of microorganisms by heat and other processing methods has been traditionally assumed to follow first-order kinetics. It assumes that all the cells or spores in a population have equal resistance to lethal treatments, which results in a linear relationship between the logarithm of the number of survivors and treatment time; however, significant deviations from linearity have been reported frequently in the literature (Cerf, 1977; Peleg and Cole, 1998; Xiong et al., 1999a; van Boekel, 2002). Three kinds of deviations have been observed: curves with a shoulder, curves with a tailing, and sigmoid curves. A number of (pseudo) non-linear mechanistic and empirical models have been proposed to describe these nonlinear survival curves (Cerf, 1977; Xiong et al., 1999a; Bhaduri et al., 1991; Cole et al., 1993; Baranyi et al., 1994; Geeraerd et al., 2000).

Since the shapes of survival curves are similar to those of growth curves, models previously used for describing bacterial growth, such as the logistic, Gompertz, and Baranyi models, have also been used in studies of inactivation kinetics. The modified Gompertz and log-logistic models have been successfully used in inactivation kinetics under various experimental conditions (Bhaduri et al., 1991; Cole et al., 1993).

Table 2.4 HP inactivation of *Bacillus spp.* articles

Matrix	Process	Bacterial reduction	Reference
Buffer pH8.0	25-65°C+400MPa+30min	<1-7 log *B. coagulans*	Sale et al. (1970)
Milk	25-60°C+200MPa+5min	*B. subtilis*	Nishi et al. (1994)
Buffer	25-110°C+ ~400MPa	*B. subtilis*; *B. coagulans*	Okazaki et al. (1996)
Buffer	20-70°C+200-900MPa	*B. cereus*; *B.licheniformis*	Gola et al. (1996)
Buffer pH7.0	40°C+400-500MP+15min	2.5-3.5 log *B. cereus*	Raso et al. (1998)
Distilled water	20-75°C+280-500MPa+ 10-600min	D=0-4min *B. anthracis*	Clery-Barraud et al. (2004)
Mashed carrots	80-116°C+200-800MPa	0-6 log	Margosch et al. (2004a)
Tri-HCl buffer	60-80°C+200-800MPa +16min	*B. subtilis*, *B. amyloliquefaciens*	Margosch et al. (2004b)
Tri-HCl buffer	70-120C+0.1-1400MPa	*B. amyloliquefaciens*	Margosch et al. (2006)
Distilled water	Ti=72°C+600MPa+1min	3.6-5.6 log *B. cereus* 4.6-5.2 log *B. cirulans* 0-2.8 log *B. coagulans* 3.8-4.6 log *B. licheniformis* 3.8 log *B. mycoides* 1.7-3.2 log *B. pumilis* 1.8-3.8 log *b. subtilis*	Scurrah et al. (2006)
Distilled water	105-121°C+ 0-700MPa+1min	0.2-6.1 log *C. tyroutylicum* 0.2-4.5log *B. amyloliquefaciens* 0.1-7.5 log *B. sphaericus*	Ahn et al. (2007)

2.4.1 First-order kinetics

This model assumes that all the cells or spores in a population have identical resistance to lethal treatments and their inactivation is governed by first-order kinetics (Schaffner and Labuza, 1997).

$$\log\frac{N}{N_0} = -\frac{t}{D} \tag{2.1}$$

where N_0 = the initial number of cells (CFU/ml); N = the number of survivals after an exposure time t (CFU/ml); D (decimal reduction time) = the time required for one log reduction in the number of cells(min); t = time (min).

Published results on thermal destruction of microorganisms generally show that they follow a first order reaction, indicating a logarithmic order of death. In the semi-log plot, the logarithmic survivors under a given heat treatment at a particular temperature plotted against heating time gives a straight line. The microbial destruction rate is generally defined in terms of a decimal reduction time (D value), which is the heating time in minutes at a given temperature required to result in one decimal reduction in the surviving microbial population. D value represents a heating time that results in 90% destruction of existing microbial population (Ramaswamy, 2006).

The D value depends strongly on the temperature, with higher temperatures resulting in smaller D values. The temperature sensitivity of D values at various temperatures is normally expressed as a thermal resistance curve with log D values plotted against temperature. The temperature sensitivity indicator is defined as z, a value that represents a temperature range, which results in a 10-fold change in D values, or, on a semi-log plot, it represents the temperature range between which the D value curve passes through one logarithmic cycle. Using linear regression, z value can be obtained as the negative reciprocal slope of the thermal resistance curve.

$$z = \frac{(T_2 - T_1)}{[\log(D_1) - \log(D_2)]} \tag{2.2}$$

where D1 and D2 are D values at temperature T_1 and T_2, respectively.

In thermal processing, heat pasteurization and sterilization concept and theory was well established and well known based on the first order linear kinetic modeling.

For the inactivation of bacterial spores at constant pressure and constant temperature conditions, there should be two z values (z_P at constant pressure and z_T at constant temperature) because there are two effects (pressure and temperature). Similar to z value in thermal processing, the temperature sensitivity indicator is defined as z_P, a value that represents a temperature range, which results in a 10-fold change in D values, or, on a semi-log plot, it represents the temperature range between which the D value curve passes through one logarithmic cycle. Using linear regression, z_P value can be obtained as the negative reciprocal slope of the thermal resistance curve.

$$z_P = \frac{(T_2 - T_1)}{[\log(D_1) - \log(D_2)]} \tag{2.3}$$

where D_1 and D_2 (min) are D values at temperature T_1 and T_2 (°C), respectively.

Similar to z_P value in TDT plot, the pressure sensitivity indicator is defined as z_T, a value that represents a pressure range, which results in a 10-fold change in D values, or, on a semi-log plot, it represents the pressure range between which the D value curve passes through one logarithmic cycle. Using linear regression, z_T value can be obtained as the negative reciprocal slope of the pressure resistance curve.

$$z_T = \frac{(P_2 - P_1)}{[\log(D_1) - \log(D_2)]} \tag{2.4}$$

where D_1 and D_2 (min) are D values at pressures P_1 and P_2 (MPa), respectively. The definition of the z_T is analogous to treatment of thermal death time (TDT) data of microorganisms widely used in thermo-bacteriology and thermal process calculations.

2.4.2 Weibull model

This model assumes that cells and spores in a population have different resistances and a survival curve is just the cumulative form of a distribution of lethal agents. Assuming that inactivation times t_c follows a Weibull distribution, the cumulative form of which will yield:

$$\log\frac{N}{N_0} = -bt^n \tag{2.5}$$

where b and n are the scale and shape factors (Peleg and Cole, 1998). The Weibull distribution corresponds to a concave upward survival curve if $n < 1$, concave downward if $n>1$, and linear if $n=1$.

Although several distributions are possible, the Weibull distribution has drawn much attention due to its simplicity and flexibility. It can describe downward concave, upward concave and linear survival curves. It has been successfully used to model thermal inactivation of *Bacillus cereus*, *Bacillus pumilus*, and *C. botulinum* (Fernandez et al., 1999, 2002; Mafart et al., 2002), and pressure inactivation of *L. monocytogenes*.

Recently, high pressure inactivation kinetics of pathogenic, spoilage vegetative cells or spores needs modeling to describe non-linear survival curves mainly due to the engineering limitations of temperature control.

2.4.3 Log-logistic equation

The following equation was proposed by Cole et al. (1993) to describe the inactivation kinetics of microorganisms:

$$\log N = \alpha + \frac{\omega - \alpha}{1 + e^{4\sigma(\tau - \log t)/(\omega - \alpha)}} \tag{2.6}$$

where α = 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).

For multiple experiments, the initial numbers (N_0) are usually different. To avoid the direct use of different initial numbers, log N_0 was calculated from Eq. (2.6) and substituted back into Eq. (2.6). Since log t at t =0 is not defined, a small t ($t=10^{-6}$) was used to approximate t=0. At $t=10^{-6}$, $\log N_0 = \alpha + \frac{\omega-\alpha}{1+e^{4\sigma(\tau+\sigma)/(\omega-\alpha)}}$. The above equation can be rearranged to give:

$$\log\frac{N}{N_0} = \log N - \log N_0 = \frac{\omega-\alpha}{1+e^{4\sigma(\tau-\log t)/(\omega-\alpha)}} - \frac{\omega-\alpha}{1+e^{4\sigma(\tau+\sigma)/(\omega-\alpha)}} \qquad (2.7)$$

Let $A=\omega-\alpha$, we can reduce the parameters of the above equation from Eqs. (2.7) to (2.6):

$$\log\frac{N}{N_0} = \frac{A}{1+e^{4\sigma(\tau-\log t)/A}} - \frac{A}{1+e^{4\sigma(\tau+\sigma)/A}} \qquad (2.8)$$

Assuming that bacterial cells in a population do not have identical heat resistances and that these differences are permanent, Cole et al. (1993) proposed a log-logistic model to describe the nonlinear survival curves for *Listeria monocytogenes*. This model has been applied to describe the effect of heating rate on the inactivation of *L. monocytogenes* (Stephens et al., 1994); the survival of *Yersinia enterocolitica* at suboptimal pH and temperature (Little et al., 1994); thermal inactivation of *Salmonella enterica serovar Typhimurium* (Ellison et al., 1994); thermal inactivation of *Clostridium botulinum* (Anderson et al., 1996); thermal inactivation of *S. enterica serovar Enteritidis* and *Escherichia coli* O157:H7 with pH and NaCl as controlling factors (Blackburn et al., 1997); and more recently, pulsed electric field inactivation of S. enterica serovar Senftenberg (Raso et al., 2000).

2.4.4 Modified Gompertz equation

The modified Gompertz equation was originally proposed by Gibson et al. (1988) to model growth curves and later was used to model inactivation kinetics (Linton et al., 1995; Xiong et al., 1999b). It is also used to model the asymmetrical sigmoidal shape of microbial growth curves.

$$\log\frac{N}{N_0} = Ce^{-e^{BM}} - Ce^{-e^{-B(t-M)}} \qquad (2.9)$$

where 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.

Bhaduri et al. (1991) later demonstrated that this model could also describe the thermal inactivation of *L. monocytogenes* in liver sausage slurry. More recently, Linton et al. (1995, 1996) used the model to describe the nonlinear survival curves of *L. monocytogenes* and predicted the effects of temperature, pH, and NaCl on the inactivation of *L. monocytogenes* in infant formula. Another approach to microbial inactivation, which considers lethal events as probabilities and survival curves as the cumulative form for a distribution of lethal events, has been recently proposed (Peleg and Cole, 1998, 2000; Peleg, 1999, 2000; Peleg and Penchina, 2000; van Boekel, 2002).

2.4.5 Model simplicity

Models should be as simple as possible. A simple model is not only easy to use, but also easy to fit. Although a model with more parameters can be expected to show a better fit to data, Baranyi et al. (1996) pointed out the dangers of over-parameterization because it can result in equations that describe not only the underlying response, but the errors specific to observed data as well. Therefore, the smallest possible numbers of parameters for adequate representation of the data should be used.

2.5 High pressure sterilization and food quality

Quality change in food products during processing is always a major concern for industry and consumers. Several quality evaluation studies have been carried out on HPHT processing for improving low-acid foods such as meat, milk, and vegetable products treated at sterilization conditions were found to provide more desirable texture,

color, and flavor and aroma retention compared with traditional retorted products, and in some cases to frozen products (Hoogland et al., 2001; Krebbers et al., 2002a, 2003; Matser et al., 2004).

Matser et al. (2004) reported main flavor components (methyl chavicol, linalool) in fresh basil accounted for approximately 90% of total essential oil content (after two pulses, 85°C, 700MPa HP sterilization) and only 25% retention in conventional sterilization (Krebbers et al., 2002a). In the two pulses, at 75°C, 1000 MPa, they studied the effect of HP sterilization treatment on the retention of ascorbic acid in green beans, spinach and apple juice. They concluded that the effect of temperature and pressure on ascorbic acid is matrix dependent. In general, the high pressure sterilized samples had a significantly higher (80%) retention of ascorbic acid than the conventionally processed samples. For ascorbic acid added to apple juice, both conventional sterilization and high pressure sterilization showed only a small decrease of ascorbic acid compared to the fresh sample. This is possibly due to protective effects of the matrix. High pressure treatment also resulted in significantly better (60%) retention of the firmness of green beans compared to freezing, drying or conventional heat sterilization.

The effect of high pressure sterilization on color is strongly product dependent. In general, high pressure sterilization results in a good retention of the color which is contrary to conventional processing. Some chlorophyll containing green products are affected in a similar way as with retort e.g. green beans, while other products show less change in color e.g. spinach or a good retention of the color e.g. carrots. After two pulses, 95°C, 700 MPa HP treatment, the color of HP treated spinach and tomato puree is significantly better than that of heat sterilized (>50%), while there is no significant difference between the color of heat and pressure sterilized green beans. Color of tomato puree and spinach was retained at least 50%. All these quality retention values were higher than those of conventional sterilization (Krebbers et al., 2002b).

Meyer et al. (2000) reported that high pressure sterilized main meal entrees like macaroni and cheese, chicken ala king, salmon fettuccine, ravioli, and beef stroganoff all have a freshly prepared flavor, texture, and color. HP treated vegetables have a crispy

texture. Soups, sauces, and stews no longer have scorched flavors nor soft-textured vegetables and pasta, as seen with retort-processed products and frozen foods.

HPHT treatment offers a promising alternative for the development of shelf-stable precooked egg patties because color and appearance of selected scrambled egg patty formulations can be maintained after 700 MPa and a process temperature of 98 °C for 5 min (Juliano et al., 2004). However, negative effects on the texture and water-retention capacity were detected as the major problem in product acceptability due to the formation of a compact rubbery structure. It has yet to be proven if addition of selected ingredients can contribute to maintain water-holding capacity in scrambled eggbased foods after HPHT treatment. For example, xanthan gum can associate with proteins and improve water-holding capacity, thus reducing syneresis (Cotterill, 1995; Montero et al., 2001). Juliano et al. (2006a, 2006b) reported that the advantage of HPHT processing with respect to conventional thermal sterilization methods is the decreased process time due to "instant" compression heating, which retains appearance and flavor of scrambled egg patties and prevents the formation of green compounds attained with conventional thermal processing. This feasibility study demonstrated reformulation with pasteurized process cheese and xanthan gum significantly improved texture and water retention in HPHT-treated scrambled egg patties compared with untreated egg patties.

Riboflavin (Vitamin B2) is known to be heat-resistant, and demonstrated to be also pressure-resistant (without loss) under elevated temperatures (Butz et al., 2007). After 15 min of pressure treatment of minced pork at 600 MPa and 100 °C, Thiamin (Vitamin B1) loss of about 10% is to be expected. In contrast, conventional heat sterilization (20 min at 121 °C) may cause losses up to 45%.

Zamari et al. (2006) reported texture change of HP treated chicken breast muscle under 0.1 to 800 MPa at 20 to 70 °C. Texture profile analysis (TPA) of the treated chicken breast muscle samples was performed to determine changes in muscle hardness. At treatment temperatures up to and including 50°C, heat and pressure acted synergistically to increase muscle hardness. However, at 60 and 70°C, hardness decreased following treatments in excess of 200 MPa. TPA was performed on extracted myofibrillar protein gels that after treatment under similar conditions revealed similar effects of heat

and pressure. Differential scanning calorimetry analysis of whole muscle samples revealed that at ambient pressure the unfolding of myosin was completed at 60°C, unlike actin, which completely denatured only above 70°C. With simultaneous pressure treatment at >200 MPa, myosin and actin unfolded at 20 °C. Unfolding of myosin and actin could be induced in extracted myofibrillar protein with simultaneous treatment at 200 MPa and 40 °C. Electrophoretic analysis indicated high pressure/temperature regimens induced disulfide bonding between myosin chains.

Ma et al. (2004) reported that the effects of high pressure (to 800 MPa) applied at different temperatures (20–70°C) for 20 min on beef post-rigor longissimus dorsi texture were studied. Texture profile analysis showed that when heated at ambient pressure there was the expected increase in hardness with increasing temperature and when pressure was applied at room temperature there was again the expected increase in hardness with increasing pressure. Similar results to those found at ambient temperature were found when pressure was applied at 40 °C. However, at higher temperatures, 60 and 70°C it was found that pressures of 200 MPa caused large and significant decreases in hardness. The results found for hardness were mirrored by those for gumminess and chewiness.

In general, a number of publications proved high pressure with elevated temperatures can reserve high quality retentions in meats and vegetables depended on food matrix, compared to conventional sterilization, freezing, drying.

Preface to Chapter 3

Adiabatic temperature rise in any liquid by HP processing is an unavoidable phenomenon by compression heating and subsequent temperature drop is the result of heat loss to chamber. The strong synergistic effects between temperature and high pressure are a big concern often making uncertain the experimental results from HP microbial inactivation. In general, the temperature instability limits the meaningful evaluation of inactivation kinetic parameters of bacterial spores under high temperatures high pressure processing conditions. For this reason, kinetic data of HP inactivation of bacterial spores under these conditions are still limited. In order to solve this problem, an insulated chamber is proposed to be used to determine accurately HP resistance and kinetic parameters of resistant bacterial spores. In this chapter, the temperature rise in milk in the insulated chamber was measured under various pressures and initial temperatures, and modeled as a function of pressure, product and medium initial temperatures. Temperature verification and bacterial spore inactivation experiments prove that it is a reliable practice and suitable for HP destruction kinetic study. This work would partially fulfill the first objective of this thesis, and be helpful for works in later chapters.

Part of this research has been presented in Agricultural and Biosystems Engineering tech. Conference 2005 and has been accepted for publication in a scientific journal. The experimental work and data analysis were carried out by the candidate under the supervision of Dr. H.S. Ramaswamy. Dr. Songming Zhu assisted in the design and evaluation of the insulator chamber.

Chapter 3

Compression heating and temperature control for high pressure destruction of bacterial spores: an experimental method for kinetics evaluation

Abstract

High pressure (HP) processing is considered as an alternative technique for thermal sterilization of high quality foods. Adiabatic compression during pressurization allows for quick increase in temperature of food products which is reversed when the pressure is released, thereby providing rapid heating and cooling conditions and hence short process times. However during the pressure hold time, the product experiences a temperature drop as a result of heat loss to the vessel. The temperature variation during the process and the synergistic effect of temperature and pressure makes it difficult to get the required accurate data on microbial spore destruction kinetics. In this study, a polyoxymethylene (POM) insulated chamber was evaluated for temperature control in the test sample during pressure treatment. Temperature variations in the HP system were measured in milk test samples inside the POM insulator and pressure medium in the HP vessel under various conditions of pressures (500-900 MPa) and initial temperatures (20-80°C). Results demonstrated that the POM chamber had good thermal-insulation characteristics under pressure and was able to maintain stable operating conditions for microbial spore destruction kinetics. Based on the measured adiabatic temperature change, the required initial temperatures for the test sample and pressure medium were generated as a quadratic function of pressure and temperature. The set-up was then verified for pressure inactivation of *Clostridium sporogenes* (PA 3679) spores in UHT milk. The better temperature stability of test samples during treatment provided a means to gather accurate data on HP destruction kinetics of the microbial spores.

3. 1 Introduction

High pressure (HP) is a novel method of food processing to achieve microbial inactivation or to alter the food attributes to get desired qualities. The unique physical and sensory properties of HP-processed foods offer many potential opportunities for food processing. HP processing has been reported to be effective in reducing or eliminating microbial pathogens for refrigerated and high-acid foods (Patterson, 2005). However, bacterial spores are highly resistant to pressure destruction (Sale et al., 1970; Mills et al., 1998). HP sterilization of low-acid foods is more difficult and complex, but may be achieved by combining HP with elevated temperatures (Furukawa et al., 2003; Reddy et al., 2006; Scurrah et al., 2006).

During an HP treatment, pressurization has two physical consequences which affect the microbial spore destruction kinetics: (1) pressure increase and (2) adiabatic compression heating resulting in a temperature increase, both having potential to increase the spore kill rate. The HP treatment can thus start with a relatively lower initial temperature and use compression heating for a quick temperature increase (3-9°C/100 MPa, depending on the product temperature and composition) (Rasanayagam et al., 2003; Balasubramanian and Balasubramaniam, 2003; Balasubramaniam et al., 2004; Patazca et al., 2007). This thermodynamically generated compression heating during pressure rise can be reversed at the end of the treatment. Therefore the HP process provides the opportunity for rapid bulk heating and subsequent cooling of the product which is not possible with other conventional techniques. This is a significant step in the HP sterilization process even if pressure failed to accelerate the destruction process. Therefore, the treatment can be conducted in a short time based on quick heating/cooling. The overall treatment conditions are less severe than with conventional sterilization, resulting in shelf-stable products which are of higher quality (Matser et al., 2004). A popular technique in HP processing is to combine compression heating with conventional heating for food sterilization (Furukawa et al., 2003; Koutchma et al., 2005).

There are strong synergistic effects between pressure and temperature on the inactivation of microorganisms and bio-chemical reactions (Mills et al., 1998; Clery-Barraud et al., 2004). Compression heating is an unavoidable phenomenon during

HP processing, which makes it difficult to distinguish pressure effects on bacterial spores from temperature effects. Pressure can be instantaneously and uniformly distributed at all points of foods in an HP vessel, which is not true with temperature because the compression heating temperature rise is subsequently moderated by heat loss during holding (Denys et al., 2000; Ting et al., 2002; Balasubramanian and Balasubramaniam, 2003; Reddy et al., 2006). Many factors can affect temperature variation and distribution in a test product during HP treatment. Temperature stability during treatment is thus a big concern often making uncertain the experimental results from HP microbial inactivation. In order to assess HP effects on food systems, it is necessary to understand pressure-temperature behavior of foods during HP processing (Ting et al., 2002).

Koutchma et al. (2005) conducted HP sterilization experiments using an HP system with an internal heater to maintain stable high temperatures. However, HP systems frequently are not equipped with an internal heater in the pressure vessel. Ahn et al. (2007) controlled temperature by immersing the HP chamber (54 ml) in a bath. This may be not convenient for a system with a large HP chamber. In general, the temperature instability limits the evaluation of inactivation kinetic parameters of bacterial spores under high temperatures high pressure processing conditions. It is for this reason, kinetic data of HP inactivation of bacterial spores under these conditions are still limited.

The objectives of this study were (1) to evaluate the temperature stability during pressure holding time in the experimental set-up as a function of test conditions, and (2) to verify the use of the set-up for evaluating HP inactivation kinetics of *Clostridium sporogenes* spores.

3.2 Materials and Methods

3.2.1 High pressure unit

High-pressure (HP) treatments were carried out with an experimental set-up shown in Figure 3.1. The HP equipment (ACIP-9000/1.7/8.5VB, ACB Pressure Systems Corp., Nantes, France) had a maximum working pressure of 900 MPa and a 3-L cylindrical pressure chamber, 85 mm in diameter and 300 mm in height. The HP set-up also

equipped with a refrigerating circulator (FP45, Julabo Labortechnik GMBH, Germany) to control the vessel temperature, and a data logger (34970A, Agilent Technologies Canada Inc., Mississauga, ON) for temperature and pressure data gathering. The medium used for pressure transmission in the system was glycerin (G33-20, Fisher Scientific Corp., Whitby, ON, Canada).

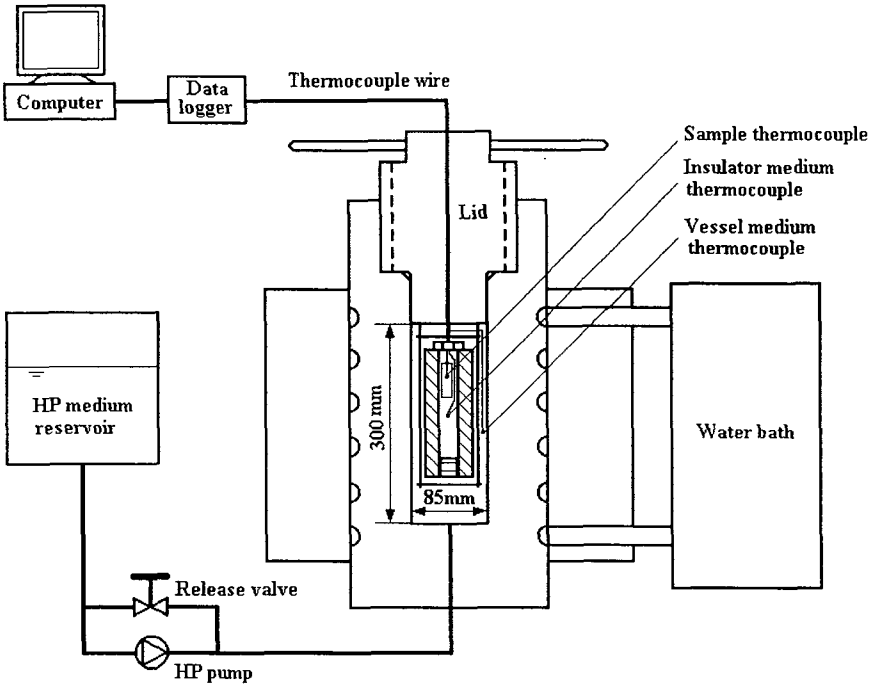


Figure 3.1 Schematic diagram of the high pressure experimental system.

3.2.2 Sample insulator

To maintain a stable temperature during HP treatment, a thick-walled cylindrical insulator was fabricated as a sample holder (Figure 3.2). The insulator (19 mm inside diameter, 60 mm outside diameter and 200 mm height) was made with polyoxymethylene plastic material (POM) (Johnston Industrial Plastic Ltd., Montreal, Canada). POM was selected as the insulator material because preliminary experiments demonstrated it to have good thermal insulation properties and a compression heating behavior similar to the common pressure media like glycerin or water (Figure 3.3). During the experiment, small flexible plastic bottles (Wheaton science, Milville, NJ) containing test sample were placed inside the POM insulated chamber. The insulator was equipped with a movable

plastic piston on the bottom side (to move in as the fluid inside chamber is compressed due to pressurization) and a threaded plug system on the top through with the thermocouples were inserted. The insulator was placed in a basket and positioned at the center of the HP vessel during HP treatment (Figure 3.1).

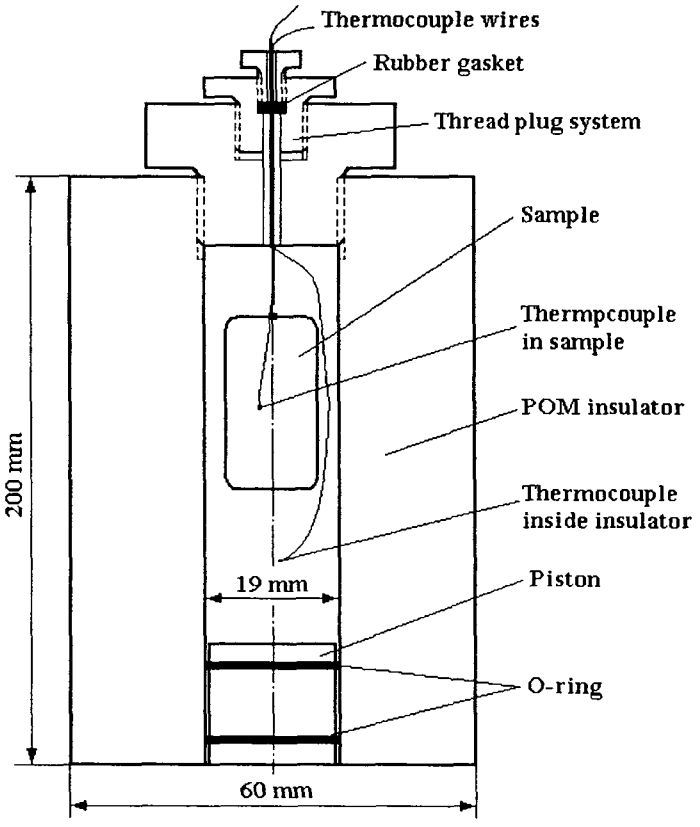


Figure 3.2 Schematic diagram of the thermal-insulator for test sample.

3.2.3 Thermal behavior measurement

The three thermocouples (K-type, OMEGA Eng. Stamford, CT) were used to monitor temperatures of the test sample (inside sample bottle) (Sample temperature), medium (water) in the insulator (outside sample bottle) (POM temperature) (Figure 3.2) and pressure medium (glycerin) (Medium temperature) in the HP chamber (outside the insulator). The thermocouple wires were installed through the top lid of the vessel with miniature connectors fixed on the inner face of the lid. UHT milk (2 % fat) was used as the test sample.

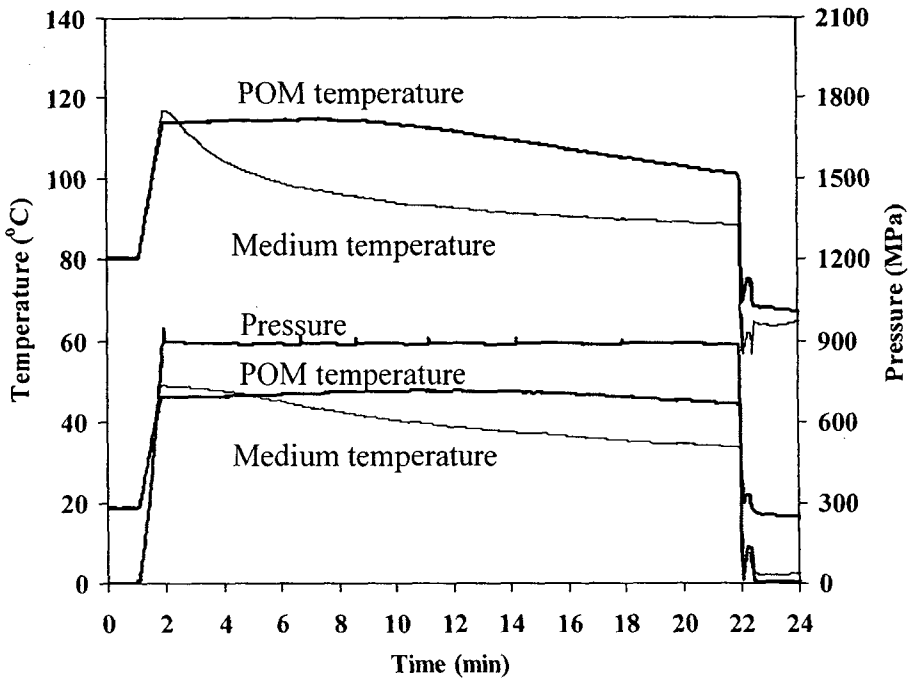


Figure 3.3 Change in temperature at the center of polyoxymethylene (POM) rod (40mm diameter) and pressure medium (glycerin) during high-pressure treatment of 900 MPa (20min) with initial temperatures at 20 and 80°C.

T_P is sample (milk) temperature when pressure reaching 900 MPa and ΔT_P is temperature change of adiabatic compression. Five pressures (500, 600, 700, 800, 900 MPa) with 20 min holding time were used for the calibration tests. Before each test, the pressure medium temperature in the HP vessel was regulated at one of the selected initial levels (20, 35, 50, 65, 80 °C). The vessel pressure was then increased to one of the five set levels. The rate of pressurization and depressurization were 15 and 45 MPa s^{-1}, respectively. Pressure and temperatures were recorded every 2 s using the data logger. Each treatment was conducted in duplicate.

3.2.4 Temperature control test

Based on the measured thermal behavior, models were developed to determine the operating conditions for any given test run. Further experiments were conducted for evaluating the temperature stability during HP treatment. The major goal in this section was to evaluate the achievement of the target processing conditions and their stability by

employing the predetermined values of initial temperature of test sample and pressure medium. Pressure and temperatures were monitored during each test.

3.2.5 Spore inactivation verification

To verify the usefulness of the temperature control HP set-up, bacterial spore inactivation experiments were carried out with *Clostridium sporogenes* (PA 3679) (ATCC, American Type Culture Collection, Rockville, MD). This strain is recognized for its high thermal resistance (greater than that of spores of *Clostridium botulinum*) and possibly a good candidate as surrogate for HP work. Commercially sterile milk (UHT 2 % fat) (Agrinove, Saint-Claire, Quebec, Canada) was inoculated with the spore suspension at a concentration of about 10^7 CFU/ml, and filled in to 1-mL sterile polypropylene bottles (Wheaton, Millville, NJ). The packed samples were kept at 4°C until HP treatment. Duplicated inoculated samples together with one for the temperature test (not inoculated) were installed in the POM insulator and subjected to HP treatments 80, 90 and 100°C at a pressure level of 900 MPa for various holding times (0-14 min). For comparison purposes, similar HP experiments were carried out without using the POM insulator. In this case sample temperature reached to the target temperature of 100°C right after pressurization, but decreased rapidly during the pressure holding period.

HP treated samples were aseptically opened and serial dilutions were made with 0.1% peptone water. For survivor enumeration, appropriately diluted samples were enumerated using a pour-plate technique on plates of MPA3679A agar (Ocio et al., 1994). The MPA3679A basal medium was prepared by adding 10g tryptone (Oxoid, Basingstoke, England, UK), 2g soluble starch (Sigma, St. Louis, MO), 2g K_2HPO_4 (Sigma, St. Louis, MO), 2g yeast extract (Sigma, St. Louis, MO) and 15g bacto agar (Fisher Scientific, Fair Lawn, NJ) into 1000ml distilled water and autoclaving for 15 min at 121°C. These plates were incubated under anaerobic conditions at 37°C for 5 days before counting. Logarithmic survivors were plotted against HP holding time. The inactivation kinetics parameter, D-value, was calculated as the negative reciprocal slope of the regression line.

3.3 Results and discussion

3.3.1 Adiabatic temperature rise

Figure 3.4 shows an example of temperature change in milk during HP treatment at 900 MPa. The initial temperature was about 80°C for both sample (milk) and pressure medium (glycerin). There was a sharp temperature increase of ΔT_P = 39.0°C in the sample as a result of pressurization. The increase the pressure medium temperature was 35.3°C, slightly less than in the sample. This could easily be explained by the heat loss from the glycerin pressure medium during pressurization (about 1 min) and/or the difference in adiabatic compression heating between milk and glycerin. However, as further experiments confirmed, the adiabatic compression temperature change in glycerin was similar to that of water or milk, therefore it is more likely caused by heat loss. During the pressure holding period, the temperature drop was 18.6, 23.0 and 26°C for the pressure medium when held for 5, 10 and 20 min, respectively. On the other hand, the sample inside the insulator had a temperature drop of 2.8, 4.9 and 12.5°C at end of 5, 10 and 20 min, respectively. Although the heat loss is not completely eliminated, the reduced temperature drop demonstrated a good thermal insulation behavior for the POM material.

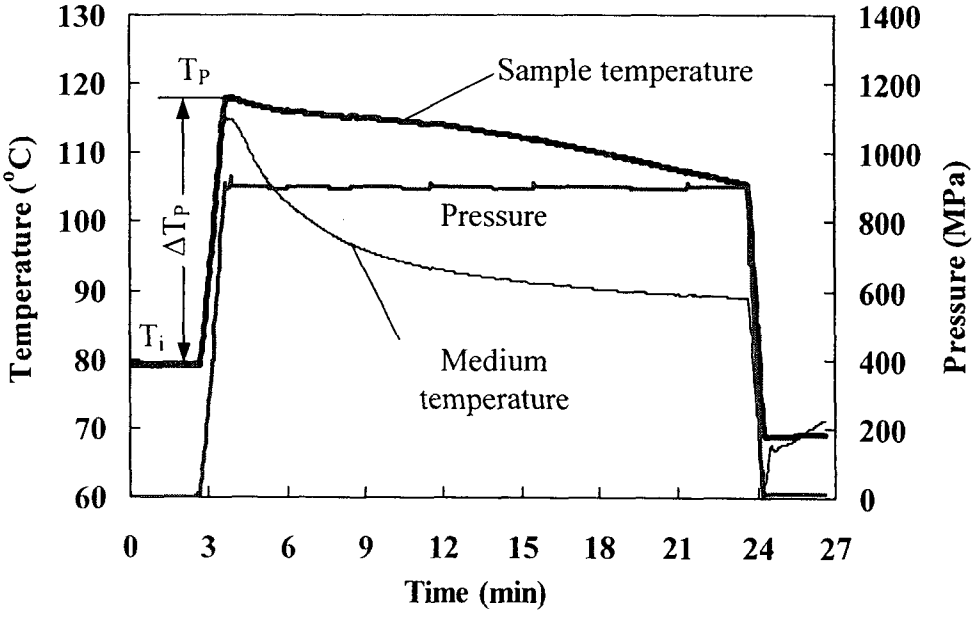


Figure 3.4 Temperature change during high-pressure treatment at 900 MPa with initial temperature T_i = 80°C.

Similar to one shown in Figure 3.4, temperature profiles were obtained from all test conditions and data were pooled to develop a multiple regression equation of sample temperature change during pressurization as a function of initial temperature and set pressure (Figure 3.5). It could be expressed as a quadratic function of pressure and initial temperature (T_i) of test sample:

$$\Delta T_P = -3.06 + 0.0224T_i + 0.0423P + 4.49\times10^{-4}T_i^2 + 1.31\times10^{-4}T_iP - 1.24\times10^{-5}P^2 \quad (3.1)$$

(R^2 =0.999, n = 50, SE = 0.20°C, P<0.05 for all items)

or a function of pressure and the desired HP processing treatment temperature (T_p):

$$\Delta T_P = -2.21 + 0.0328T_p + 0.0381P + 2.82\times10^{-4}T_P^2 + 8.64\times10^{-5}T_PP - 1.25\times10^{-5}P^2 \quad (3.2)$$

(R^2 =0.999, n = 50, SE = 0.18°C, P-value <0.05 for all items)

where T_i = initial sample temperature ($20°C \le T_i \le 80°C$), T_P = desired sample temperature (process temperature) at the operating pressure (°C), ΔT_P = adiabatic temperature rise (°C), P = pressure (500 MPa $\le P \le$ 900 MPa), R^2 = regression correlation coefficient, n = number of data, SE = standard error, P-value = statistic parameter of significance.

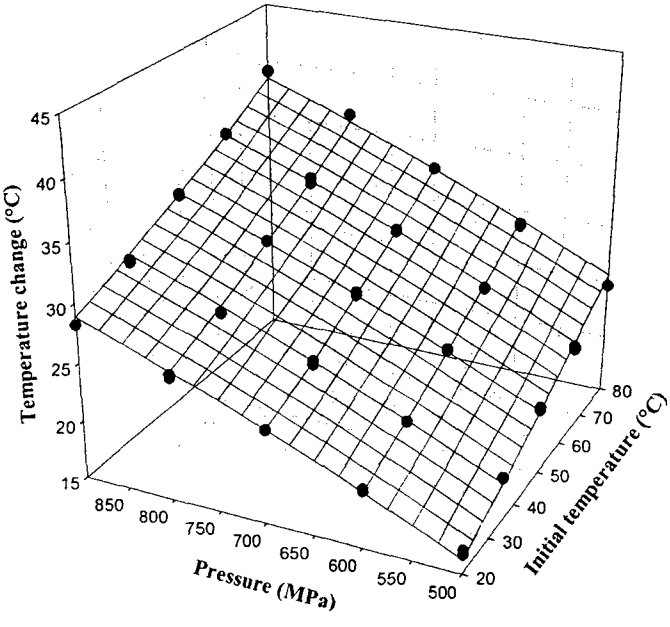


Figure 3.5 Measured temperature change of adiabatic compression and its regressed surface as a function of pressure and initial temperature.

3.3.2 Control of HP treatment temperature

In HP sterilization processes, it is important to estimate a desired initial temperature of the test sample or the pressure medium in the vessel which would give the appropriate HP treatment condition for the test. With Equation (3.2), the initial sample temperature can be easily determined as follows (Table 3.1):

$$T_i = T_P - \Delta T_P$$
$$= 2.21 + 0.9672 T_P - 0.0381 P - 2.82 \times 10^{-4} T_P^{\,2} - 8.64 \times 10^{-5} T_P P + 1.25 \times 10^{-5} P^2 \quad (3.3)$$

Because of the good thermal insulation of POM, the sample temperature was not very sensitive to the temperature changes in the pressure medium during HP treatment. However, in order to make sample temperature more stable for longer pressure holding times, the initial temperature of the vessel medium could be elevated to a slightly higher level than the initial sample temperature. This would further reduce the temperature drop in test samples during the hold period. Therefore, the following empirical equation was suggested from this study:

$$T_{Vi} = T_i + \frac{V_{In}}{V_V} \Delta T_P = T_P - \frac{V_m}{V_V} \Delta T_P \quad (3.4)$$

where T_{Vi} = initial temperature of pressure medium in the vessel (°C), V_{In} = outer volume of the POM insulator (m^3), V_V = volume of HP vessel (m^3), and V_m = volume of pressure medium inside high pressure vessel (m^3). Equation (3.4) means that the value of T_{Vi} can be set at a level between T_i and T_P, depending on the volume of both insulator and HP vessel. If the volume of the insulator is close to the vessel volume, the initial vessel temperature should be set at the same level as the desired HP treatment temperature. When the HP vessel volume is much larger than the insulator, the initial vessel temperature could be the same as initial sample temperature. This is an experimental and empirical equation but was widely tested during test runs carried out at different conditions. It provided a useful means of temperature control for the various kinetic studies using the set-up.

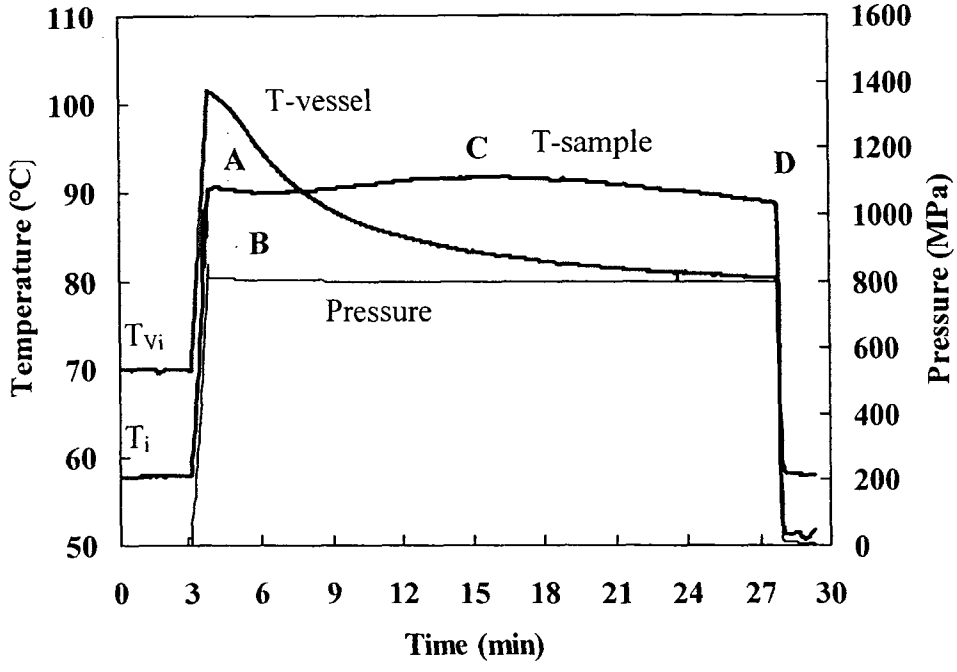
Table 3.1 Initial temperature required for desired HPP conditions

Tp (°C)	Pressure (MPa)			
	600	700	800	900
70	46.5	43.8	41.2	38.9
80	55.3	52.4	49.8	47.4
90	64.0	61.0	58.3	55.8
100	72.6	69.5	66.7	64.2
110	81.1	78.0	75.1	72.5

Tp processing temperature

Figure 3.6 shows an example of temperature profiles established by using the method as described above. The ratio of V_{In}/V_V was about 0.33 in this study, the initial temperature of the pressure medium (T_{Vi}) was set at T_i plus $0.33\Delta T_P$. After pressurization, the sample temperature reached the desired level (A in Figure 3.6a) followed by a small decrease (B in Figure 3.6). This is because the POM insulator had a slightly lower temperature increase than the sample during pressurization (Figure 3.6b), resulting in a heat transfer from sample to insulator. However, since pressure medium temperature was higher than the insulator temperature right after pressurization, and hence the sample temperature was slightly elevated (C in Figure 3.6a), and subsequently dropped more gradually until the pressure release (D in Figure 3.6a). In Figure 3.6b, the same ratio of V_{In}/V_V was used because the same insulator was used. The pressure medium temperature (T_{Vi}) was set at different value (T_i plus $0.33\Delta T_P$) because the test shown in 3.6b was carried out at a different pressure, processing temperature and holding time. The sample temperature in Figure 3.6b was even more stable than in Figure 3.6a, which confirms the insulating effectiveness of the system.

(a)



(b)

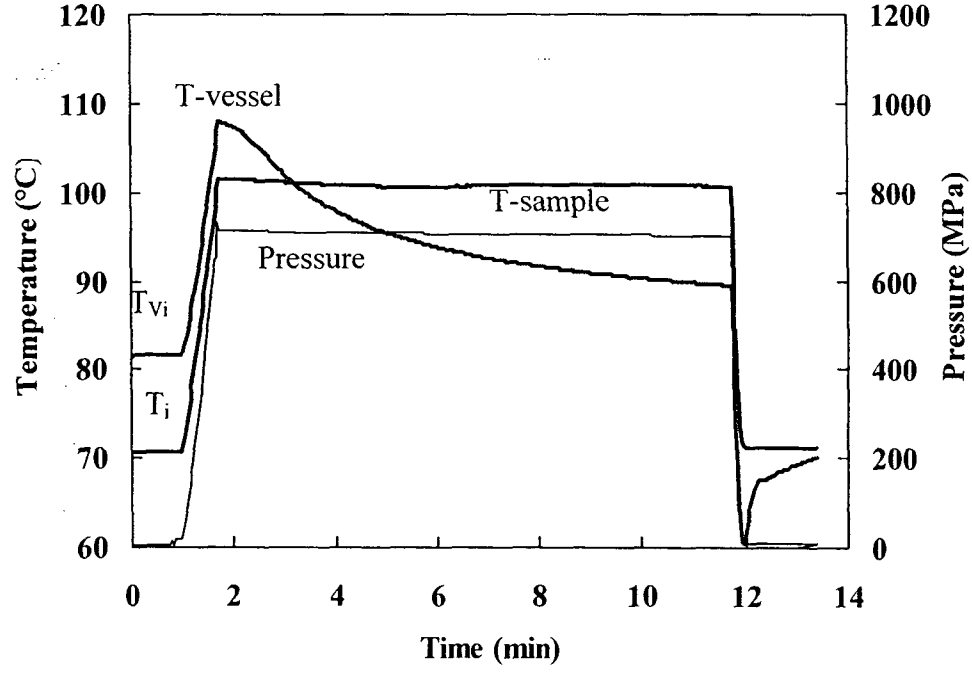


Figure 3.6 Control of sample temperature during high pressure treatment: (a) at 800 MPa and 90°C for 24 min, (b) 700 MPa and 100°C for 10 min. T_i and T_{Vi} are the initial temperature of sample and vessel medium, respectively.

Basically, temperature difference is the driving force for heat loss from sample and insulator to surrounding environment. A higher pressure and higher temperature of HP treatment usually implies a larger temperature difference for this kind of heat loss, resulting in a more rapid temperature drop during pressure holding time. Fortunately, a higher pressure/temperature requires shorter time of HP treatment for microbial inactivation kinetics analysis. By using this method, sample temperature can be maintained relatively stable at the desired level for a period long enough for a kinetics study.

3.3.3 Spore inactivation kinetics

Figure 3.7 shows the logarithmic survival reduction of the *C. sporogenes* spores in milk samples subjected to HP treatment at 900 MPa for samples in both the POM insulated set-up and without insulation. The data at zero pressure holding time means that a sample was only subjected to a pressure pulse (pressurization and depressurization without pressure holding). The logarithmic survivors in the thermal-insulated samples showed a rapid decline and followed a good linear correlation with pressure holding time indicating a first order rate of destruction. This was mainly because of the stable sample temperature maintained with the POM insulator as shown in Figure 3.8. Regression analysis using the first-order model resulted in a high value of determination coefficient (R^2 =0.99, n=16). The decimal reduction time (D value) was 1.2 min. Similarly, for the 900 MPa treatments at 80 and 90 °C (not shown in figure), data analysis resulted in D = 13.7 min (R^2 =0.98, n=20) and D=7.0 min (R^2 =0.92, n=20), respectively. Thus the thermal resistance of the spores at 900 MPa was obtained, Z_P = 18.9 °C (R^2 = 0.92), which was calculated as the negative inverse of the slope of the linear plots of logarithmic D value versus temperature.

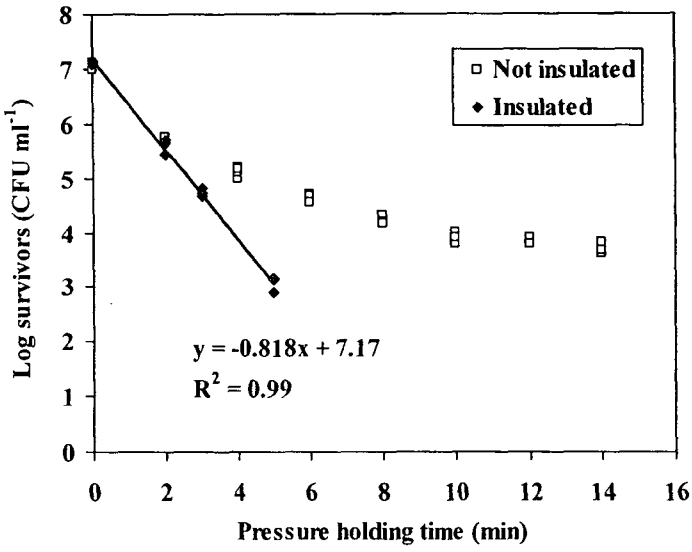


Figure 3.7 Logarithmic survivors of *C. sporogenes* (PA 3679) spores in milk samples thermally insulated during HP treatment at 900 MPa (100 °C), as compared to those not insulated (see Figure 3.8).

The sample which was not thermally insulated in the pressure vessel showed a gradual decline in the survival counts (Figure 3.7). This sample on the other hand exhibited a rapid temperature drop during pressure holding (Figure 3.8). Although immediately after pressurization to 900 MPa, the temperature reached the target level of 100 °C, it decreased to 83.5 °C during 14 min holding. As a result of the temperature drop, these samples received lower lethal treatment and hence survival counts of the HP treated spores remained relatively higher deviating from the linear trend characterized by the insulated sample (Figure 3.7). Similar reduction trends of spore survivors were reported when temperature obviously dropped during pressure holding (Ananta et al., 2001; Rajan et al., 2006). The logarithmic reduction of the spore was 3.99 ± 0.084 after 14 min (nominal D value would be 3.5 min) in non-insulated test cell at 900 MPa (100.2-83.5 °C), and even less than what was achieved for 5 min treatment in the insulated chamber (log reduction 4.48 ± 0.145; and temperature 99.7 ± 0.7 °C) (Figure 3.7). Obviously, serious experimental errors would result if the microbial destruction counts from an HP treatment with large temperature variations assigned to represent

set-point process temperature. It may be possible to make temperature corrections using an equivalent time, $t_{eq}(T)$, estimated using the concepts in thermal process calculations (Patashnik, 1953). Mathematically $t_{eq}(T)$ can be defined as:

$$t_{eq}(T) = \sum \Delta t_m 10^{\frac{T_m - T}{Z_P}} \tag{3.5}$$

where T is the target (set-point) process temperature (°C), T_m is the actual temperature of the test sample during HP treatments (°C), Δt_m is the time interval between two measured data, and z_P is the thermal resistance of the spores under high pressure (°C). Figure 3.9 shows the logarithmic survivors of the spores against the computed equivalent pressure holding times using Eq.(3.5) along with measured sample temperatures and the Z_P value obtained from insulated samples (18.9 °C at 900 MPa). Clearly there is a steeper decline this time since they now represent destruction at the constant process temperature of 100C, and is linear like in Figure 3.7 for the insulated test samples. Based on the equivalent time, a D value was determined, D = 1.7 min at 900 MPa and 100 °C (nominal temperature), which is much closer to the D value obtained using the POM insulator.

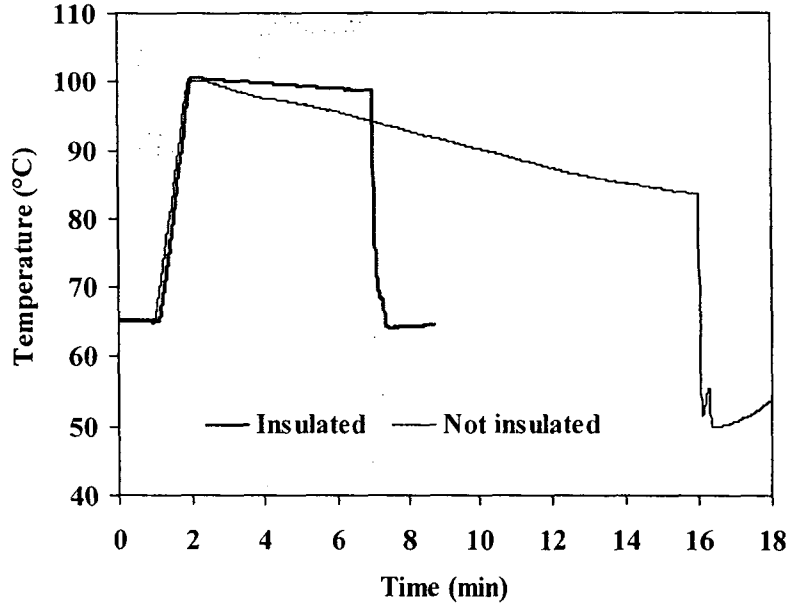


Figure 3.8 Temperature change in milk sample thermally insulated during HP treatment at 900 MPa (5 min at 100 °C), as compared to that not insulated (14 min).

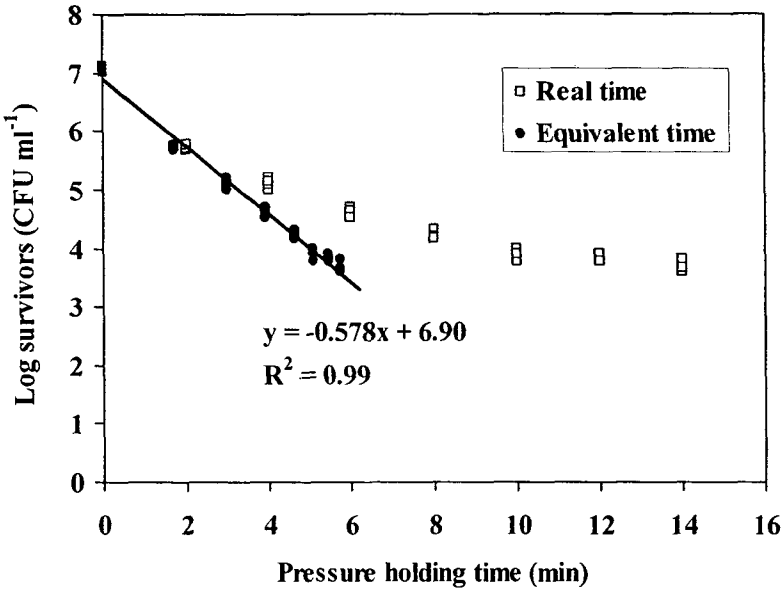


Figure 3.9 Logarithmic survivors versus equivalent pressure holding time as compared to those versus real time for *C. sporogenes* (PA 3679) spores in milk treated at 900 MPa without using POM insulator.

3.4 Conclusions

Temperature elevated by compression heating can be maintained at a desired level with good stability during an HP treatment using a thermal insulation set-up which can transmit the pressure to test samples as proposed in this study. POM is a good material for thermal insulation under HP processing conditions. More accurate results on bacterial spore destruction kinetics can be obtained from POM-insulated samples during HP treatment. Kinetic results from temperature controlled HP treatments using the POM insulator can be used to get the thermal sensitivity kinetic parameter, z value, which is needed for computing equivalent times when temperature fluctuations exist. This study suggests a useful method to the improve kinetics evaluation of HP destruction of bacterial spores at elevated temperatures.

Preface to Chapter 4

In the previous chapter, a plastic POM insulator was used to study the compression heating behavior of milk under various pressures with elevated initial temperatures. Results demonstrated that the POM chamber had good thermal-insulation characteristics under pressure and was able to maintain stable operating conditions for microbial spore destruction kinetics. The sample and equipment chamber initial temperature adjustment provided better temperature stability for temperature control and facilitated more accurate HP destruction kinetics data. By using the thick plastic POM insulator and temperature control method, the high pressure and thermal inactivation kinetics of *C. sporogenes* spores in milk at elevated temperatures were carried out to evaluate the spores resistance and this forms the subject of this chapter. This work was the first kinetic study with standardized temperature control set-up developed for the work and would partially fulfill the second objective of this thesis. The results obtained in this study are important for the further research on destruction kinetic evaluation of HP resistant bacterial spores in low acid foods detailed in later chapters.

Part of this study has been presented at the IFT conference 1996 and a manuscript is under preparation for publication. This research work was completed by the candidate under the supervision of Dr. H.S. Ramaswamy and with equipment assistance provided by Dr. Songming Zhu during experiments.

Chapter 4

High-pressure destruction kinetics of *Clostridium sporogenes* ATCC11437 spores in milk at elevated quasi-isothermal conditions

Abstract

The high-pressure sterilization establishment requires data on isobaric and isothermal destruction kinetics of target pathogenic, spoilage bacterial resistant spores. In this study, milk (2% fat) was inoculated with 1% *Clostridium sporogenes* 11437 spores and subjected to different pressure, temperature and time (P, T, t) combination treatments (700-900 MPa; 80-100 °C; 0-32 min). A previously standardized POM insulated chamber was used to enclose the test samples during the high-pressure for maintaining stable process temperatures. Destruction kinetic parameters, D values, pressure and temperature sensitivity parameters, z_T and z_P were evaluated using a 3 pressures X 3 temperatures full factorial experimental design. HP treatments generally demonstrated a pressure pulse effect (PE) (no holding time) followed by a pressure hold effect which was well described by the first order model ($R^2 > 0.90$). Higher pressures and higher temperatures resulted in a higher destruction rate and a greater microbial count reduction. At 900 MPa, the temperature corrected D values were 9.1, 3.8, 0.73 min at 80, 90, 100 °C respectively. The thermal treatments at 0.1 MPa resulted in D values 833, 65.8, 26.3, 6.0 min at 80, 90, 95, 100 °C respectively. By the comparison, HP resulted in a strong enhancement of spore destruction. Temperature corrected z_P values were 16.5, 16.9, 18.2 °C at 700, 800, 900 MPa respectively which were higher than thermal z value 9.6°C. Hence, the spores had lower temperature sensitivity at elevated pressures. Similarly, corrected z_T values were 720, 590, 1250 MPa at 80, 90, 100 °C, respectively, which illustrated lower pressure sensitivity at higher temperatures. By general comparison it could be concluded that the spores were relatively more sensitive to temperature than to pressure.

4.1 Introduction

High pressure processing at high temperatures has recognized to be a novel and potential alternative to thermal sterilization for low acid foods (Meyer et al., 2000). Since bacterial endo-spores would not be eliminated by high pressure alone (<1000 MPa) at ambient temperatures (Sale et al., 1970), high pressure treatment at low or moderate temperatures has been mostly carried out as a pasteurization process to inactivate bacterial vegetative cells and extend shelf life of high acid foods which subsequently need to be kept at refrigeration conditions (Patterson, 2005). Recent studies demonstrate that high pressure combined with elevated temperature can inactivate many pressure resistant spores and enzymes (Scurrah et al., 2006). Adiabatic compression results in quick heating with reverse achieved even quicker during decompression. Hence HP could be effectively used to provide rapid heating and cooling conditions. Processed foods could thus benefit high pressure sterilization (de Heij et al., 2003). Meyer et al. (2000) reported that the quality of HP treated foods for achieving commercial can be equal or better than that of frozen foods.

Sterile low acid food products are self stable at room temperature and currently mostly produced by thermal processing under conditions designed to destroy the most heat resistant pathogenic and spoilage microbial spores. In the canning industry, commercial sterilization is achieved by a minimum 12-log reduction of *Clostridium botulinum* spores (Pflug et al., 1978). To achieve a commercially sterile product that can be stored at ambient temperatures, HP sterilization should result in the same or better inactivation of spores than what is achieved by thermal processing. Prior to becoming a practical process, the pressure resistant pathogen (and their surrogates) and spoilage bacterial spores need to be identified and their pressure resistance and survival behavior under pressure need to be determined (Margosch et al., 2006). Moreover, the high pressure process need to be verified by a biological validation to assure the desired log cycle reductions of resistant non-pathogenic surrogate spores (Sizer et al., 2002; Koutchma et al., 2005).

There have been some studies dealing with high-pressure inactivation of *Bacillus* spp.; *Geobacillus* spp. and *Clostridium* spp. spores at elevated temperatures, detailed data

on destruction kinetics gathered at near isobaric and isothermal conditions is still limited because the samples and chamber medium temperature dynamically change (compression heating and heat loss) and affects the inactivation behavior of spores during the HP treatment (de Heij et al., 2003). Recently, Koutchma et al. (2005) carried out inactivation kinetics of *C. sporogenes* PA3679 spores in buffer with engineering improvement by using a internal heater to maintain pressure chamber temperatures. The study detailed in Chapter 3 with a thick wall insulator showed good insulation is another practical method to carry out kinetic study at high temperatures.

Nakayama et al. (1996) reported that bacterial spore pressure resistance did not correlate with their heat resistance. Thus, the species and strains whose spores were used in thermal processing as resistant targets need to be re-identified by pressure processing. *C. sporogenes* is gram positive, anaerobic, spore forming and motile bacteria. It is widely distributed in nature and also in the intestines of animals. In food sterilization studies, non-pathogenic *C. sporogenes* spores which have a heat resistance similar to target *Clostridium botulinum* spores are often used (Koutchma et al., 2005). *Clostridium sporogenes* ATCC 11437 spores have been widely used as standard reference for sterility determinations in the pharmaceutical industry. *Clostridium sporogenes* ATCC 11437 spores are commercially available and are also easily obtained coated on strips at high concentration levels. There are no published reports on high pressure destruction kinetics of *Clostridium sporogenes* ATCC 11437 spores in milk. The objectives of this study were (1) to evaluate the HP destruction kinetics of *C. sporogenes* 11437 spores in milk under isobaric and isothermal processing conditions (2) to investigate the pressure and temperature sensitivity of these bacterial spores.

4.2 Materials and Methods

4.2.1 *Clostridium sporogenes* culture preparation

Freeze-dried cultures of *C. sporogenes* (ATCC-11437) was obtained from the American Type Culture Collection (ATCC, Rockville, MD) and stored at –40 °C. This culture was hydrated in 10 ml Reinforced Clostridial Medium (RCM) broth (Oxoid, Basingstoke, Hampshire, UK) at 37°C for 24 h in anaerobic condition, and then stored at

4°C. To prepare the inoculation culture, 0.1ml of the cultured broth was transferred into 50 ml of fresh RCM broth and incubated at 37°C for 24 h in anaerobic condition. Two such transfers were made in succession to obtain the culture with viable counts of 10^8 CFU ml^{-1} approximately.

4.2.2 Preparation of *C. sporogenes* spores

C. sporogenes culture (0.2 ml) was transferred and spread on Campdem Sporulating Agar plate (CSA) and incubated at 37°C for 7 days in anaerobic condition for growth and forming bacterial spores. The CSA medium formula was 5g tryptone (Oxoid, Basingstoke, Hampshire UK), 5g bacterial peptone (BD, Co., Spark, MD), 1 g Lab Lemco meat extract (Oxoid, Basingstoke, Hampshire UK), 2g yeast extract (BD, Co., Spark, MD), 0.056 g calcium chloride (BDH, Inc., Toronto, ON), 0.062 g $MnSO_4.H_2O$ (Fisher Scientific, Fair Lawn, NJ), 1 g glucose (Fisher scientific, Fair Lawn, NJ) and 15 g bacterial agar (Fisher scientific, Fair Lawn, NJ) in 1 liter distilled water. Spores were collected by flooding the agar surface with 10 ml sterile distilled water and scraping the colonies with a sterile glass spreader. After harvest, the spores were washed three times by the centrifugation at 4000 x g for 15 min each at 4 °C and suspended in sterile distilled water to give approximately 10^9 CFU/ml and stored at 4 °C until use.

4.2.3 Sample preparation

Spore suspension (1%) inoculated in commercially sterile milk (UHT 2% fat, 3.2% protein, 6% carbohydrate, Agrinove, Saint-Claire, Quebec, Canada) to give a final concentration of about 10^7 CFU/ml and was filled up to the brim in 1mL sterile polypropylene bottles (Wheaton, Millville, NJ, USA) and closed with sterile screw caps. The sample tubes were kept in 4°C until use.

4. 2.4 Thermal treatment

A glycerin bath (SL26, Julabo, Labortechnik GMBH, Germany) was used to treat samples at 90, 95 and 100 ± 0.1 °C. Glycerin (G33-20, Fisher scientific, whiteby, ON) was fluid media. A temperature reference milk tube (with a K-type thermocouple

tip at sample tube center) was placed in constant temperature glycerin bath fluid with sample tubes simultaneously. A datalogger (Agilent 34970A, formerly HP, Agilent Technologies Canada Inc., Mississauga, ON) monitored and recorded sample temperatures. When the reference milk tube temperature reached the desired temperature, the timer started to measure the holding time. The come-up time was 5 to 6 min, which depended on process temperature, and was taken in to account for computing the effective time. Treated samples were taken out at different points of time for microbiological enumeration and kept in ice water bath.

4.2.5 High pressure equipment and high pressure treatment

HP treatments were carried out in the isostatic press (Model ACIP9000/1.7/8.5VB, ACB Corp., Nantes cedex 3 France) with a cylindrical pressure vessel (8.5 cm diameter and 30 cm high). This was described in detail in Chapter 3 Section 3.2.1. A data-logger (Agilent 34970A, formerly HP, Agilent Technologies Canada Inc., Mississauga, ON) connected K-type thermocouple (OMEGA Eng. Stamford, CT) which went though high pressure chamber cover, was used to collect online pressure and temperatures data every 1 s. The pressurization medium was pure glycerin G33-20 (Fisher Scientific Corp., Whiteby, ON, Canada) to meet the requirement of its pump.

A thick wall POM (Polyoxymethylene, Johnston Industrial Plastic Ltd., Montreal, Canada) plastic cylinder (1.9 cm inside diameter, 6.0 cm outside diameter and 20 cm height) was used as insulator for temperature control. The description of this insulating chamber was also given in Chapter 3, Section 3.2.2. Three tubes (1 temperature reference and 2 samples) were kept inside and this insulator was assembled in pressure vessel for delaying heating transfer from sample tubes to pressure vessel during high pressure processing. This design allowed pressure equivalence between inside and outside of insulator.

Before HP treatment, all sample and reference tubes and insulator were preheated 10 min in a water bath (SL26, Julabo, Labortechnik GMBH, Germany) for increasing temperature to sample initial temperature. Two K-type thermocouples (OMEGA Eng.

Stamford, CT) attached to a data-logger were installed through the top plug: one installed in a tube center for recording experimental reference temperature and the other for monitoring insulator inside fluid temperature. The insulator inside was filled with distilled water. The 3rd thermal couple was installed outside insulator for monitoring pressure vessel temperature.

A treatment in the pressure holding mode consisted of pressurization, pressure holding and depressurization. The equipment was operated at a maximum speed 15.4±0.1 MPa/s and up to a maximum pressure of 900 MPa. Pressurization come-up time ranged from 45 to 60 s (depending on the target pressure level), and depressurization time was <15 s. The pulse mode was similar except that there was no holding time. Three pressure levels (700, 800, 900 MPa) and process temperatures (80, 90, 100°C) full factorial combinations were selected for kinetic study. Data logger was used to record the treatment temperature profiles.

After pressure treatment, the treated samples were kept in an ice water bath prior to bacterial enumeration. Each experiment was conducted in duplicate and the results were averaged for each sample. Two 80°C 10 min thermal treated samples were used as control to compare with treated ones.

4.2.6 Sample temperature control during HP treatments

Based on our previous milk compression heating study (detailed in Chapter 3), the plastic POM provided a good material to work at high temperature and high pressure environment for insulation and regression equation (1) was established to compute Ti (initial sample temperature ($20°C \le T_i \le 80°C$), (°C)) as a function of pressure processing temperature (Tp) and pressure level (MPa) during pressurization (Table 4.1).

$$T_i = T_P - \Delta T_P$$
$$= 2.21 + 0.9672 T_P - 0.0381 P - 2.82 \times 10^{-4} T_P^{\,2} - 8.64 \times 10^{-5} T_P P + 1.25 \times 10^{-5} P^2 \qquad (4.1)$$

(R^2=0.99, n=50, SE=0.22°C, p-value<0.05 for all items)

where T_i = initial sample temperature ($20°C \le T_i \le 80°C$), T_P = sample temperature when pressure reaching target level (°C), ΔT_P = compression adiabatic temperature change (°C),

P = pressure (500 MPa $\leq P \leq$ 900 MPa), R^2 = regression correlation coefficient, n = number of data, SE = standard error, P-value = statistic parameter of significance.

Before high-pressure treatment, sample tubes and insulator were preheated in a water bath (10 min, distilled water as medium) to increase their temperature to process initial temperature (T_i). And then sample tubes were placed in the insulator and the set up was closed by a piston and a plug without air inside. The insulator was quickly assembled in HP chamber and started to run.

Table 4.1 High pressure high temperature processing experimental setup: initial temperature (°C), pressure (MPa) and HP process temperature (°C)

Process temperature (°C)	Initial temperature(°C)		
	Pressure 700(MPa)	Pressure 800(MPa)	Pressure 900(MPa)
80	52.4	49.8	47.4
90	61.0	58.3	55.8
100	69.5	66.7	64.2

Due to the good thermal insulation of the insulator, sample temperature was not sensitive to the variation in the temperature of the vessel medium during HP treatment. However, in order to provide a better stability for longer pressure holding times, the initial temperature of the vessel medium was elevated to a level higher than the initial sample temperature (details in Chapter 3). Therefore, the following empirical equation was used for setting up pressure vessel temperature:

$$T_{Vi} = T_i + \frac{V_{In}}{V_V}\Delta T_P = T_P - \frac{V_m}{V_V}\Delta T_P \qquad (4.2)$$

where T_{Vi} = initial temperature of vessel medium (°C), V_{In} = outer volume of the POM insulator (m^3), V_V = volume of HP vessel (m^3), and V_m = volume of pressure medium inside high pressure vessel (m^3).

4.2.7 Enumeration of survival spores

After thermal and pressure treatments, two treated samples were kept in a refrigerator (4°C) within 10 min for bacterial enumeration. Each experiment was conducted in duplicate and the results were averaged for each sample. Two 80°C 10min thermal treated samples were used as control to compare with treated ones.

For the enumeration, the sample tubes were aseptically opened and serial dilutions were made with 0.1% peptone water. Spores of *C. sporogenes* were enumerated in Yeast Dextrose Tryptone Agar with enriched starch (YDTAS) plates by using a pour-plate technique. The medium has 5 g peptone (Oxiod, Basingstoke, England, UK), 2.5 g tryptone (Oxiod, Basingstoke, England, UK); 1 g beef extract ("Lab lamco" Oxiod, Basingstoke, England, UK), 2 g yeast extract (BD, Co. Spark, MD, USA); 1 g glucose (Fisher Scientific, Fair Lawn, NJ), 1 g soluble starch (Sigma, St. Louis, MO); and 15 g bacterial agar (Fisher Scientific, Fair Lawn, NJ) in 1 liter distilled water. Duplicate plates were used for each dilution. These YDTAS plates were incubated at anaerobic 37°C condition for 5days before counting.

4.2.8 Kinetic data analysis

Since tests were carried out over several days, small variations in the initial concentrations were unavoidable and therefore the residual counts needed to be nominal. In this study, all results were nominal by multiplying the survival fraction with their nominal initial counts (10^7 CFU ml^{-1}).

The pressure inactivation kinetics of microorganisms at constant temperature during pressure holding time was analyzed using a first-order kinetic model:

$$\log(N/N_0) = -t/D \qquad (4.3)$$

where N is the number of survival microorganisms (CFU ml^{-1}) after a pressure treatment at time t (min); N_0 is the initial number of microorganisms (CFU ml^{-1}); D is referred as the decimal reduction time or D value and is the treatment time at any given pressure that will result in destruction of 90% of the existing microbial population (i.e., resulting in one decimal reduction in the surviving population). The D value were obtained from the

linear regression slope of log (N/N_o) vs. t (or time taken to traverse one logarithmic cycle):

$$D = -1/\text{slope} \quad (4.4)$$

The pressure resistance was analyzed by pressure death time (PDT) model. In PDT model, the pressure resistance at a constant temperature was determined by plotting the decimal logarithm of decimal reduction time (log D value) against the pressure. From the regression of log (D) vs. pressure, the pressure resistance z-value (Z_T) was determined as the negative reciprocal of the slope as shown below:

$$Z_T = (P_2 - P_1) / \log (D_1 / D_2) = -1/\text{slope} \quad (4.5)$$

where D_1 and D_2 (min) are D values at pressures P_1 and P_2 (MPa), respectively.

Based on food thermal processing theory, the temperature inactivation kinetics of microorganisms at different constant pressure during pressure holding time was analyzed as a first-order kinetic model (TDT model). In this model, the thermal resistance of the D values at a constant pressure was determined by plotting the decimal logarithm of decimal reduction time (log D value) against the temperature. From the regression of log (D) vs. temperature, the thermal resistance z-value (z_P) was determined as the negative reciprocal of the slope as shown below:

$$Z_P = (T_2 - T_1) / \log (D_1 / D_2) = -1/\text{slope} \quad (4.6)$$

where D_1 and D_2 (min) are D values at temperature T_1 and T_2 ($^{\circ}C$), respectively. The definition of the $z_{(p=0.1\ MPa)}$ is analogous to treatment of thermal death time (TDT) data of microorganisms widely used in thermo-bacteriology and thermal process calculations.

4.2.9 Kinetic data temperature calibration

The plastic insulator was used to delay heat transfer and keep sample temperature relatively constant in pressure holding period, but the chance was always there that temperature imbalance between sample and pressure vessel would cause sample temperature fluctuation during pressure holding period. Therefore, it was necessary to use temperature calibration in kinetic data computations to eliminate sample temperature

minor fluctuations (Figure 4.2) (Patazca et al., 2004). The calibration was based on the first order kinetic analysis theory and the concepts in thermal process calculations (Patashnik, 1953). An effective time t_{eT} equation was established as the accumulation of temperature and time integration:

$$t_{eT} = \int 10^{\left(\frac{T-Tp}{z}\right)} dt = \sum 10^{\left(\frac{T-Tp}{z}\right)} * \Delta t \qquad (4.7)$$

where t_{eT} = temperature time integration or accumulated effective time, T = real temperature (°C) (data logger recorded), T_p = process temperature (°C), z = temperature sensitivity (°C), Δt = real time interval (min).

Pressure and temperature profiles were obtained from each HP holding time experiment. Time-temperature profiles and residual count data were initially used by taking treatment time as the real time, and initial values characteristic D and associated z values were computed from Log(N/No) values to $t_{e(T)}$ and Log(D) vs T relationships. The computed D and z values were used to calculate effective time $t_{e(T)}$ at the nominal temperature (Eq.4.7). A linear regression was then used to relate the Log(N/No) values to $t_{e(T)}$ for each condition in order to get the new values of D and z (which are more accurate than before). For z value calculation, at least three experiments needed to be set at different temperatures. The calibration cycle was run continually until z value difference was between successive computation was less than 0.5%. In this study, three pressures (700, 800, 900 MPa) and three temperatures (80, 90, 100°C) with full factorial experiments were used. These calculations were preformed using Microsoft Excel 2000. Figure 4.1 shows the calculation algorithm for calibration as detailed in Patazca et al. (2004). In this calibration, the effects of come-up time and come-down time were also considered and corrected by this method. Similar corrections were also applied to the thermal destruction kinetics.

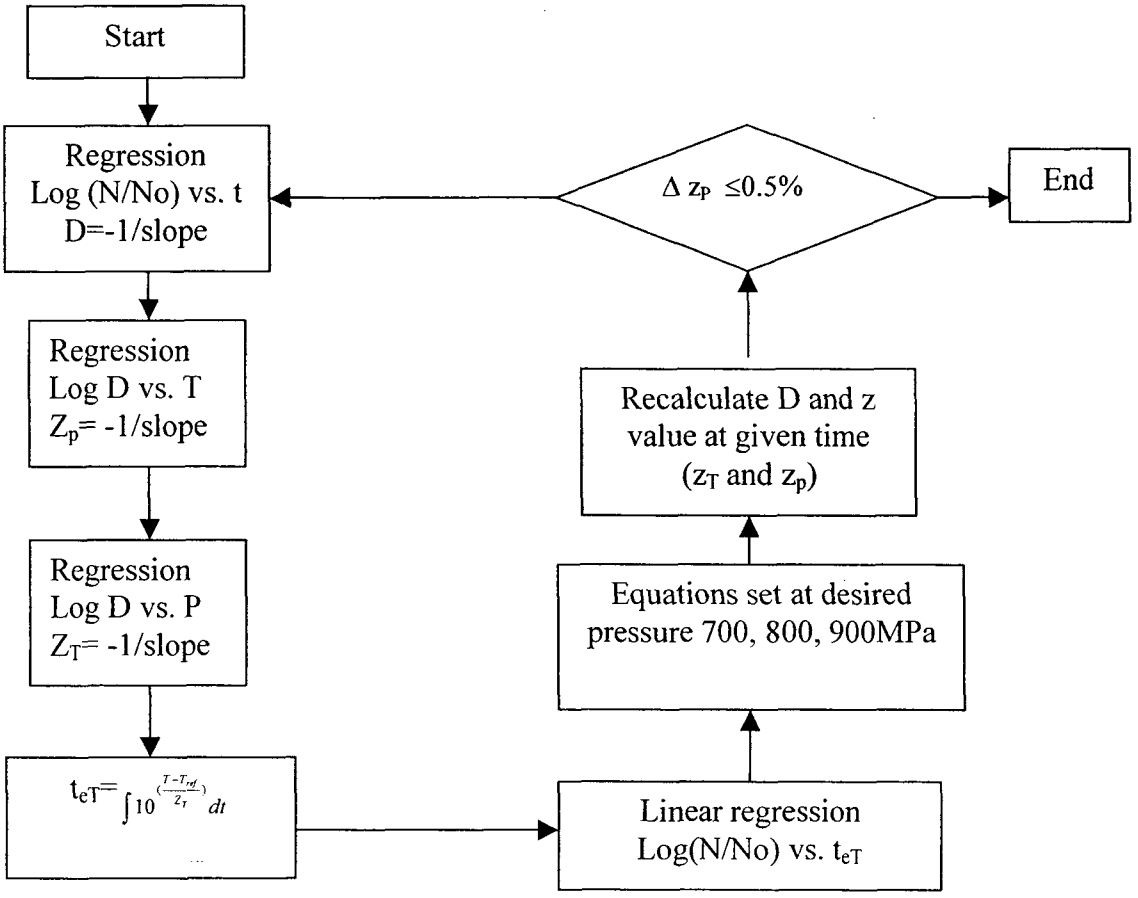


Figure 4.1 Diagram for computing kinetic parameter calibration

The method that used temperature profile to calibrate inactivation kinetics data has been widely used in microwave processing and thermal processing research (Tajchakavit et al., 1998; Awuah et al., 1993). To our knowledge, Patazca et al. (2004) first used this method to calibrate temperature and pressure minor fluctuation in high-pressure high temperature kinetic study. In this study, pressure calibration was not considered due to the automatic pressure compensation during pressure holding.

4.3 Results and discussion

4.3.1 Thermal destruction kinetic of *C. sporogenes* spores

Figure 4.2 show the survivor curves for *C. sporogenes* 11437 spores under thermal processing conditions at 80, 90, 95, 100 °C and Table 4.2 summarizes the associated D and z values of thermal destruction kinetics. Figure 4.2 indicated that higher temperatures resulted in higher rates of microbial destruction and resulted in steeper slopes giving lower D values. The D values were 833, 65.8, 26.3, 6.0 min at 80, 90, 95, 100 °C, respectively. The survivors of 80 °C decreased a little and the reduction was quite small. From microbiological experiment point of view, D value 833 min at 80 °C was very large and count differences during a normal test run could be considered negligible. Hence, only data at 90, 95, 100 °C have employed to calculate z value. The z value was 9.6 °C. Stumbo (1973) reported that *C. sporogenes* 7955 spore has 0.1-1.5 min $D_{121.1\,°C}$ and 7.8-10.0°C z value. Compared with these heat resistant bacterial spores used for commercial sterilization reference, the spores *C. sporogenes* 11437 were not as resistant. However, this strain has been widely used in pharmaceutical applications for sterility tests.

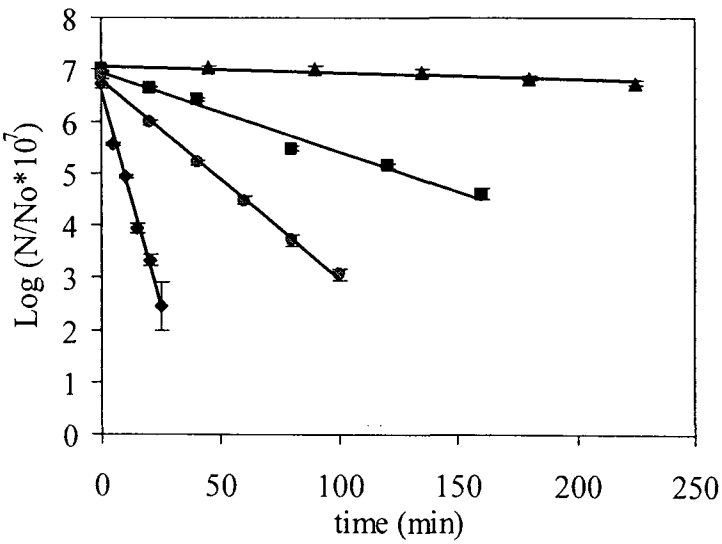


Figure 4.2 Nominal survivors of *C. sporogenes* 11437 spores in thermally treated milk (1 atm) at temperature (▲)80, (■) 90, (●) 95, (♦) 100 °C

Table 4.2 Decimal reduction time (D values) of *C. sporogenes* 11437 spores in milk associated with HP and thermal treatment

Pressure (MPa)	Temperature (°C)	Uncorrected		Corrected	
		D value (min)	R^2	D value (min)	R^2
700	80	14.5	0.95	17.0	0.97
	90	7.4	0.96	8.4	0.95
	100	1.0	0.98	1.0	0.97
800	80	10.6	0.96	12.3	0.95
	90	6.0	0.93	5.7	0.92
	100	0.76	0.95	0.8	0.98
900	80	6.6	0.96	9.11	0.99
	90	3.7	0.91	3.83	0.91
	100	0.63	0.93	0.73	0.96
0.1	80	833	0.80		
	90	65.8	0.98		
	95	26.2	0.99		
	100	6.0	0.99		

4.3.2 High pressure destruction kinetics

Figure 4.3 shows the temperature changes in the test sample and pressure chamber during a typical test run. Before treatment, the milk initial temperature and chamber medium temperature were set to 54.0 and 62.9°C. During pressurization, milk and medium temperature increased quickly due to the adiabatic heat. When the pressure reached desired process pressure, 700 MPa, milk reached predicted temperature 81.0 °C and chamber medium at 91.3 °C. This gave an adiabatic temperature rise of 27 °C in milk and 28 °C in the pressure medium, and kept the medium temperature at a slightly elevated level to prevent the temperature drop in the test sample during the test run. During 32 min pressure holding time, chamber medium temperature dropped steadily to 71.9 °C because of heat loss to the metal chamber wall. If the milk sample tubes were placed directly in equipment chamber, sample temperature would have decreased by the same 20 °C margin as observed for the chamber medium. Compression heating and heat loss were unavoidable natural phenomenon during HP process and care must be taken to interpret data obtained from situations involving excessive drop in sample temperature. Several

studies show that temperature and pressure have synergetic effect on biological materials. The simulation study of Heij et al. (2003) on high pressure inactivation of spores of *B. stearothermophilus* ATCC 7953 showed that spores survival curves had a significant tail at the end of pressure holding period when sample temperature was allowed to drop. These results indicate that high-pressure destruction kinetics of microorganisms must be carried out at isobaric and isothermal conditions. Ting et al. (2002) suggested that all of the pressure, temperature and time points should be reported to indicate conditions of HPP test. The temperature curve of milk with the insulated chamber illustrates only some minor temperature drop around desired temperature because of chamber medium heating and cooling effects on the insulator.

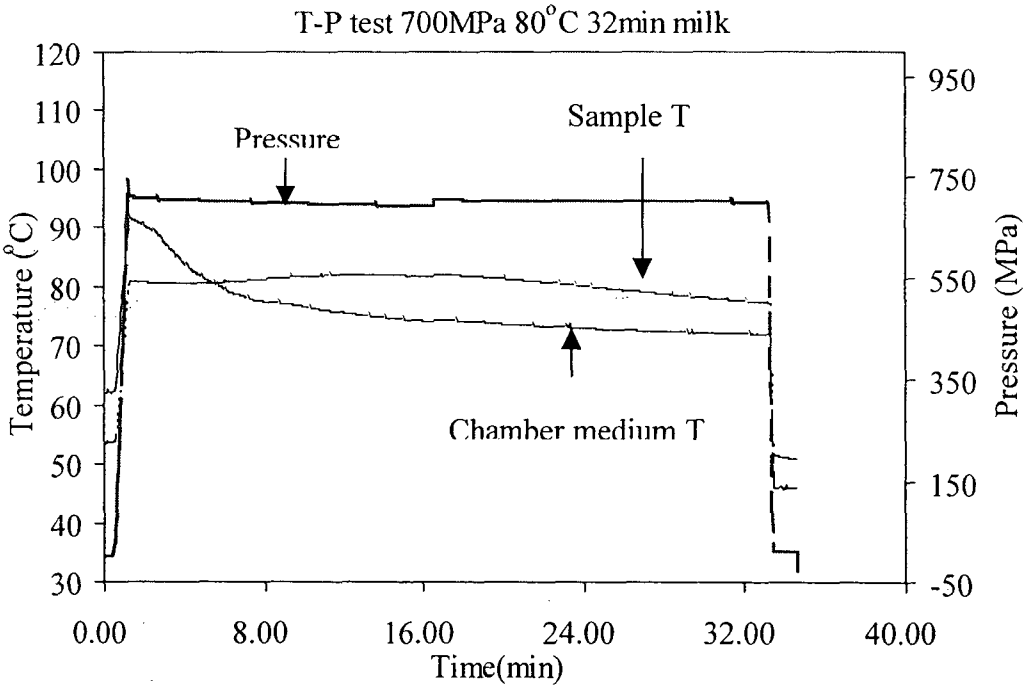
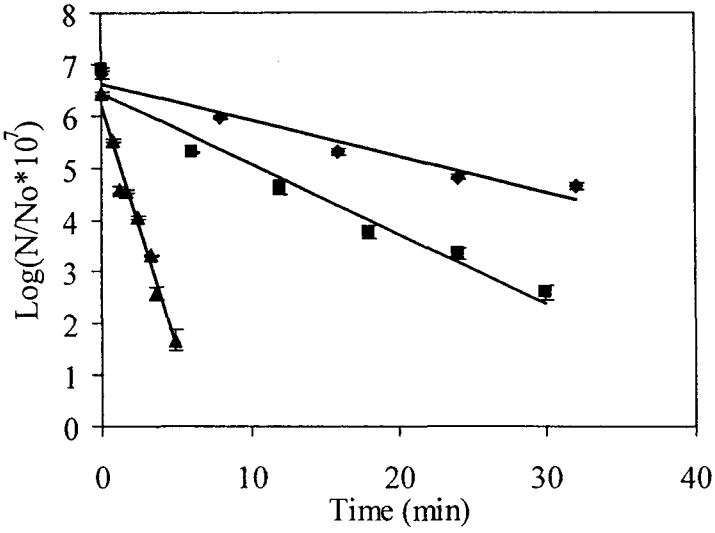


Figure 4.3 The changes in milk temperature in the insulated chamber and pressure chamber medium temperature outside the chamber during a pressure treatment at 700 MPa, 80 °C for 32min.

Figures 4.4(a), 4.5(a), 4.6(a) show *C. sporogenes* spores survival curves at 700, 800 and 900 MPa, respectively, at 80, 90 and 100°C as a function of pressure holding time.

(a)



(b)

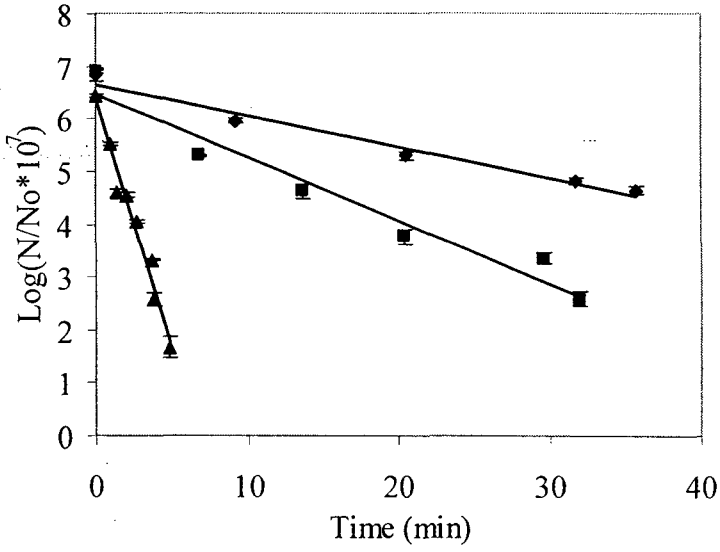
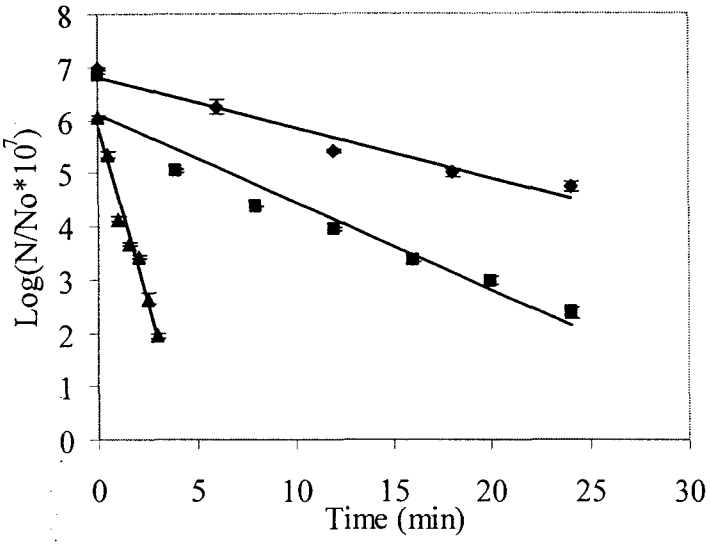


Figure 4.4 Uncorrected (a) and temperature corrected (b) nominal survivors of *C. sporogenes* 11437 spores in high pressure treated milk at 700 MPa and different temperatures (♦) 80, (■)90, (▲) 100 °C

(a)



(b)

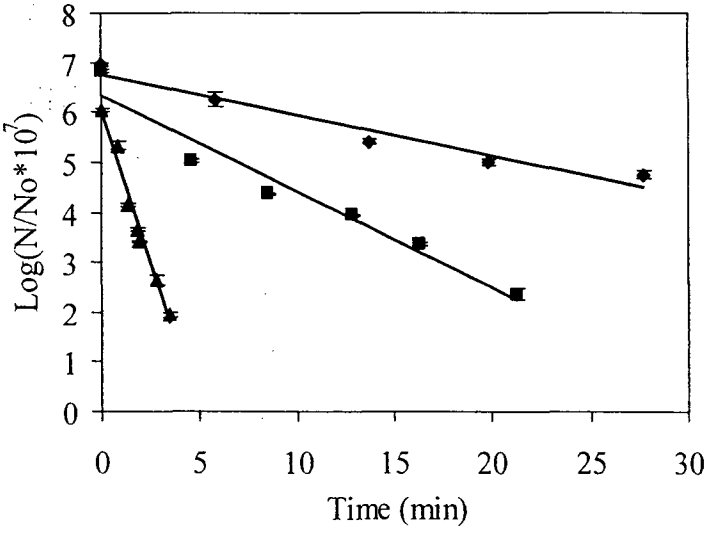
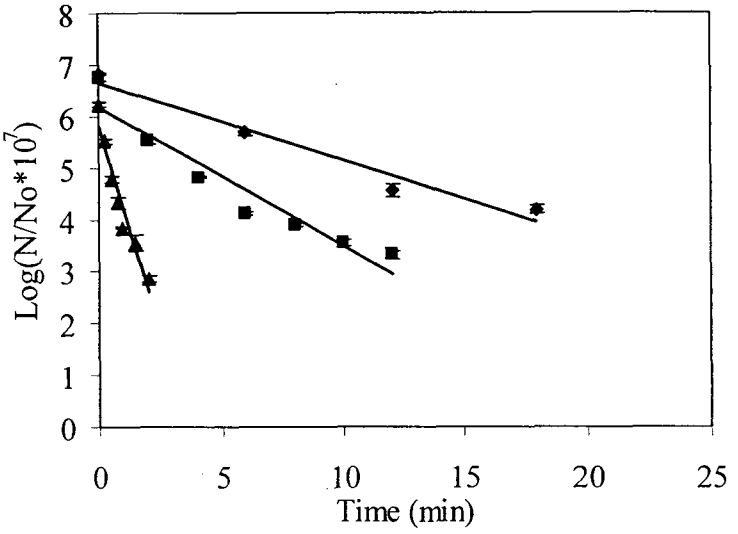


Figure 4.5 Uncorrected (a) and temperature corrected (b) nominal survivors of *C. sporogenes* 11437 spores in high pressure treated milk at 800 MPa and different temperatures (♦) 80, (■)90, (▲) 100 °C

(a)



(b)

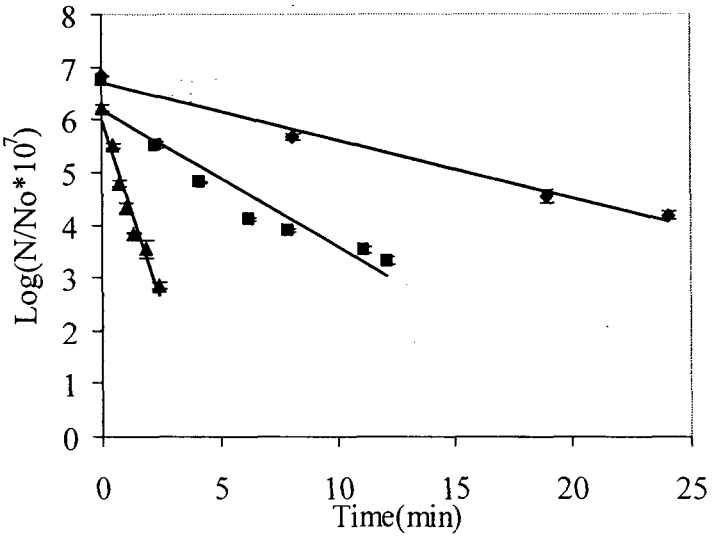


Figure 4.6 Uncorrected (a) and temperature corrected (b) nominal survivors of *C. sporogenes* 11437 spores in high pressure treated milk at 900 MPa and different temperatures (♦) 80, (■)90, (▲) 100 °C

The pressure destruction of the bacterial spore was found to follow a dual effect behavior as reported by Basak et al. (1996): a rapid destruction just due to pressurization and depressurization (without holding) (pressure pulse effect) followed by a first order rate destruction during the pressure holding. Consistently, there was a pulse effect that depended on the pressure level (difference between the original nominal microbial population (10^7 CFU g^{-1} on log scale 7.0) and the survivors at "zero" time). All survival curves confirmed the dual effect pressure destruction to be associated with HP treatments, with higher pressure and higher temperature treatments resulted in higher pulse effect and a faster destruction rate.

Table 4.3 shows the effect of high pressure pulse (come-up time and come-down time) on *C. sporogenes* 11437 spore nominal reductions. It can be realized that high pressure (700-900 MPa) combined with 100°C can result in 0.60-1.0 log-reduction compared to 0.05-0.18 log-cycle at 80 °C. The margin is relatively small compared in other studies with vegetative bacterial cells (Ramaswamy et al., 2003), nevertheless appropriate to recognize.

Table 4.3 Pressure pulse effect on *C. sporogenes* 11437 spores in milk

Process temperature (°C)	Spores reductions Log (N/No*10^7)		
	Pressure 700 (MPa)	Pressure 800 (MPa)	Pressure 900 (MPa)
80	0.18±0.10	0.05±0.035	0.18±0.017
90	0.10±0.036	0.18±0.041	0.27±0.062
100	0.60±0.031	1.0±0.033	0.82±0.056

During pressure holding, the destruction trend could be well fitted to the first order model ($R^2 > 0.90$). Computed D values from the regression analysis are also summarized in Table 4.2. An increase in pressure resulted in a decrease in D value meaning acceleration in destruction of bacterial spores. For example, the D values at 700, 800, 900 MPa were 14.5, 10.6, 6.6 min, and 1.0, 0.76, 0.63 min for HP treatment at 80 and 100 °C, respectively. These D values were lower than those from Rovere et al. (1996).

After kinetic calibration (Figures 4.5-4.7, b captions), D values 700, 800, 900 MPa changed to 17.0, 12.3, 9.11 min and 1.0, 0.8, 0.73 min at 80 and 100 °C, respectively. According to the concept of thermal process calculation, corrected D value depend on the accumulation of real temperature and holding time. When the real temperature was higher than the desired temperature, based on Eq. (7), the expected effective time becomes longer than the experimental holding time, and the temperature corrected D value becomes larger than the uncorrected one. On the contrary, lower real temperature for the same level of survivor reduction have a lower effective time, and the resulting corrected D would be lower. With the new linear regression of nominal survivors against effective time, D values will revert back to their true values.

The D values of *C. sporogenes* 7955 spore in buffer (Koutchma et al., 2005) and in milk were 2.83, 2.3 min and 2.7, 1.8 min for 700, 800 MPa at 100°C, respectively. Compared with *C. sporogenes* 7955 spores in buffer and in milk, 11437 spore D values were lower, which indicates the high-pressure resistance of *C. sporogenes* 7955 strain was higher than that of *C. sporogenes* 11437 strain.

Comparing HPHT with thermal treatments at the same temperature conditions, it can be observed that pressure treatment was efficient and resulted in D values to decrease significantly ($P<0.05$). At 900 MPa and 100°C, the associated D value was one tenth of D values for thermal treatment at 100 °C. This indicated that high pressure combined with mild heat can inactive bacterial spores more efficiently and shorten the processing time.

4.3.3 Pressure resistance and thermal resistance

According to the TDT and PDT theories, Z_T and Z_P were analyzed from the three pressures and three temperatures full factorial experiment design. Thermal resistance or thermal sensitivity (at constant pressure) of D value variation against temperature was well described by the Z_p value. Through linear regression analysis, the thermal resistance (z_p) value was determined using Eq. (5) at each constant pressure (Figure 4.8). The Z_P were 17.2, 17.4, 18.2 °C for 700, 800, 900 MPa respectively (Table 4.4). After kinetic D value corrections, Z_P values changed slightly to 16.5, 16.9, 18.2 °C, respectively and which illustrated thermal resistance increased by 10.3% with pressure increasing from

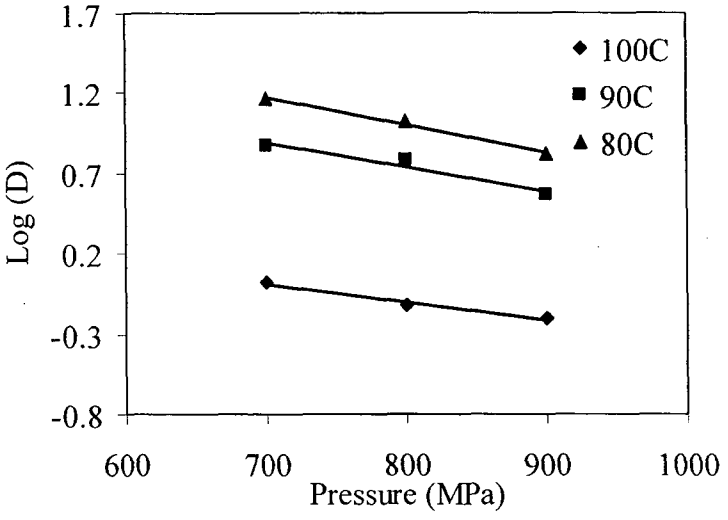
700 to 900 MPa. Koutchma et al. (2005) reported Z_P value of *C. sporogenes* 7955 spores in buffer was 23.7 °C, higher than found in these studies. Perhaps the difference could be the milk medium. Higher Z_P value means lower temperature sensitivity at higher pressuress. Compared with 9.6 °C thermal treatment z value, one can find that these bacterial spores had higher temperature resistance under pressure, even though they had relatively lower D values.

Similarly, the pressure resistance (Z_T) value was obtained by Eq. (6) using the linear relationship at each constant temperature (Figure 4.9). Uncorrected and corrected 11437 Z_T values were 590, 660, 910 MPa and 720, 590, 1250 MPa at 80, 90, 100 °C, respectively, which indicated pressure resistance increased (by 73.6 %) with temperature increasing from 80 to 100 °C) (Table 4.5). This meant that at higher temperature, these bacterial spores were not sensitive to the pressure increasing.

Koutchma et al. (2005) showed Z_T value of *C. sporogenes* 7955 spore in buffer was 1500 MPa. Rovere et al. (1996) reported that Z_T values of *C. sporogenes* 7955 were 725 (90 °C), 962 (100 °C), 752 (108 °C) MPa and Reddy et al. (1999) pointed out that Z_T value of *C. botulinum* Type A was 1524 MPa (75 °C). Compared with literature values Z_T for *C. sporogenes* 7955 (no data available for *C. sporogenes* 11437), the values of Z_T found in this study were higher, again perhaps because of the milk medium. Ramaswamy et al. (2003) reported that the z_T values of *E. Coli* (29055) in apple juice (25 °C) were 126 MPa (total vegetative survivors) and 140 MPa (healthy vegetative cells). By comparison of bacterial vegetative cells and spores, spores had much higher Z_T value, which indicated that the inactivation mechanism of spores was different.

As we know, D value calibration could change Z_P and Z_T values. The method can compensate for minor variations in temperature during the test and improve computed D values. From z value regression coefficient variation, we could find z value calibration also improved their regression coefficient R^2 (Tables 4.4; 4.5).

(a)



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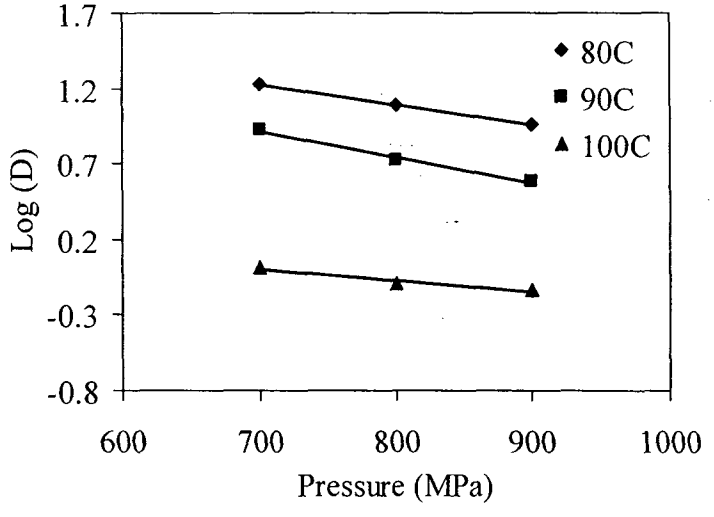


Figure 4.7 Uncorrected (a) and corrected (b) D value curves of *C. sporogenes* 11437 spores in milk subjected to HP and thermal treatments at different temperatures (♦) 80, (■) 90, (▲)100 °C as a function of pressure

Table 4.4 High pressure Zp value (°C) of *C. sporogenes* 11437 spores in milk subjected to HP and thermal treatments

Pressure (MPa)	Uncorrected		Corrected	
	Z_p value	R^2	Z_p value	R^2
700	17.2	0.92	16.5	0.92
800	17.4	0.90	16.9	0.96
900	19.6	0.92	18.2	0.97
0.1	9.6	0.98		

Table 4.5 High pressure Z_T value (MPa) of *C. sporogenes* 11437 spores in milk subjected to HP treatment

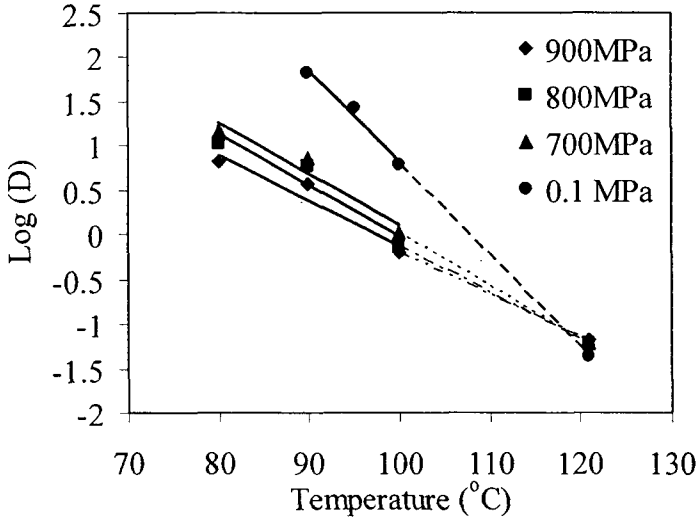
Temperature(°C)	Uncorrected		Corrected	
	Z_T value	R^2	Z_T value	R^2
80	590	0.98	714	0.99
90	660	0.95	588	0.99
100	910	0.98	1250	0.95

4.3.4 Predicted D values at higher temperature

Using the Zp and Z_T, D values were computed for an extrapolated temperature of 121C to compare the thermal and pressure D values at commercial processing temperatures. Figure 4.9 also illustrates the D value curves against temperature and Table 4.6 shows the computed D values at specific temperatures (105, 110, 115, 120 and 121 °C). It can be found that extrapolated HP D values of *C. sporogenes* 11437 were in fact slightly higher than thermal D values at around 121°C. Because of the larger Z_P values associated with pressure, their D values changed relatively slowly with temperature. On the other hand, the thermal D values which were several fold higher at lower temperatures converge rapidly because of their lower z values. It meant that at the temperature close to 121°C, high pressure treatment may not accelerate clostridial spore destruction, in fact it can even offer protection to thermal destruction with an associated D value higher than the thermal D. Margosch et al. (2006) observed that a pressure-mediated protection happened on *B. amyloliquefaciens* TMW 2.479 spores at 120 °C and 800 to 1200 MPa. Ahn et al. (2007) reported that at 700 MPa at 121°C, *B.*

amyloliquefaciens ATCC 49763 spores hand a lower spore count reduction than in a thermal treatment at 121°C.

(a)



(b)

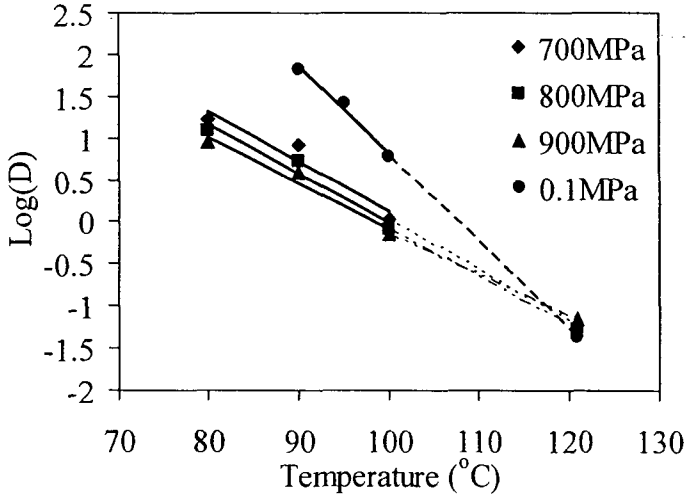


Figure 4.8 Uncorrected (a) and corrected (b) D value curves of *C. sporogenes* 11437 spores in HP treated and thermally treated milk at different pressures (♦) 700, (■) 800, (▲) 900 MPa, (●) 0.1 MPa as a function of temperature

Table 4.6 Predicted decimal reduction times of *C. sporogenes* 11437 spores in milk under HP and thermal processing conditions

Pressure (MPa)	Predicted D value (min) at higher temperature (°C)				
	105	110	115	120	121
700	0.65	0.33	0.16	0.081	0.070
800	0.48	0.25	0.12	0.063	0.055
900	0.44	0.23	0.12	0.066	0.058
0.2	2.0	0.61	0.18	0.056	0.044

4.4 Conclusions

Overall, the study indicated that compression heating and heat loss were unavoidable and can significantly affect sample temperature during HP treatment. To obtain appropriate experimental data, a thermally insulated chamber that permits pressure application is necessary. The developed set-up provided good temperature control during the kinetic studies. Temperature calibration results showed that it is necessary and able to correct the effective time and kinetic parameters based on real temperature profiles. Kinetic data showed higher pressures and higher temperatures always resulted in a faster microbial reduction (shorter D value). High pressure combined with elevated temperature was able to destroy the pressure resistant bacterial spore. However, at higher pressures, the thermal resistance increased with temperature and at higher temperatures, the pressure resistance increased with pressure. Comparison of kinetic data with literature values of *C. sporogenes* 3679 indicated a lower resistance for *C. sporogenes* 11437. At 121°C, high pressure lost its inactivation acceleration, and only high temperature played an important role in spore destruction. However, high-pressure as a powerful quick heating and cooling assisted method has an advantage for low acid sterilization. Recently, researchers who are looking for a method to evaluate pressure treatment effects at higher temperatures. The potential use this spore trips as a temperature and time indicator (TTI) under a specific high-pressure should be possible.

Preface to Chapter 5

In the previous chapter, the high pressure and thermal destruction kinetics of *C. sporogenes* 11437 spores suspended in milk were evaluated at elevated temperatures. This strain was selected because of its wide use in pharmaceutical application as a sterility indicator. However, the study showed that the high pressure resistance of *C. sporogenes* 11437 spores were not very high relative to those reported in literature for the other frequently used *C. sporogenes* strain, namely 7955, which is commonly referred to as PA3679, although most of the data reported in literature were for spores suspended in a buffer. Although the different media used could explain some of the differences observed between the kinetic data found in Chapter 4 and those reported in literature, real differences could only be realized by carrying out a detailed destruction kinetics for the *C. sporogenes* 7955 in milk as was done for the other strain. Hence the purpose of this chapter was evaluation of HP destruction kinetic study on *C. sporogenes* 7955 spores suspended in milk at elevated temperatures. This study was necessary also to make meaningful comparison with literature data to confirm *C. sporogenes* 7955 is a more pressure resistant strain. Results of this chapter would partially fulfill the second objective of this thesis.

This study was submitted for the IFTPS 2006 student paper competition and won the 4th place finish amongst 14 other papers. A manuscript is in preparation for publication. This research work was completed by the candidate under the supervision of Dr. H.S. Ramaswamy.

Chapter 5

High pressure destruction kinetics of *Clostridium sporogenes* ATCC7955 spores in milk elevated temperatures

Abstract

C. sporogenes 7955 spores inoculated in milk (2% fat) were subjected to high pressure (HP) treatments at 700-900 MPa and at elevated temperatures 80-100 °C for selected times up to 32min. Samples in 1 mL plastic vials were placed in a specially constructed insulated chamber to minimize temperature drop during the treatment. As in previous studies, the HP treatments demonstrated a biphasic inactivation reduction, first a pulse effect which is the result of pressurization and depressurization and a pressure hold time effect that was well fitted by the first order model ($R^2 > 0.90$). Destruction kinetics and pressure sensitivity parameters were evaluated by the first order reaction rate calculation. Higher pressures and higher temperatures resulted in a faster rate of spore destruction. Temperature corrected D values ranged from 38.2 to 2.4 min at 700 MPa and 13.7 to 1.2 min at 900 MPa with process temperatures set between 80 and 100 °C. Thermal treatments gave D values 156, 57.5, 12.1 min at 90, 95, 100 °C, respectively. Thus, HP treatment resulted in significant reduction in D values in the temperature range studied. HP Z_P values were 16.5, 20.3, 18.9 °C at 700, 800, 900 MPa, respectively and therefore were higher than the thermal z value 9.0 °C, indicating that spore's thermal resistance increased with an increasing pressure. Likewise the Z_T values were 450, 690, 680 MPa at 80, 90, 100 °C, respectively, demonstrating the spore's pressure resistance to also increase with an increasing temperature. Overall, *C. sporogenes* 7955 spore was relatively more sensitive to temperature than pressure. This strain was also more resistant than *C. sporogenes* 11437 and thus a better candidate for a surrogate. The predicted D values at 121 °C under HP were higher than under thermal treatment indicating that the conventional commercial sterility approach cannot be overlooked.

5.1 Introduction

High pressure processing has been recognized as novel and innovative food preservation alternative method to high temperature sterilization of low acid foods because high pressure combined with elevated temperature can inactivate highly resistant bacterial spores (Sale et al., 1970; Seyderhelm et al., 1992; Mills et al., 1998). Adiabatic compression temperature rise caused by pressurization results in quick heating to process temperatures or the subsequent decompression results in rapid cooling. Thus HP can be used as a special technique for rapid bulk heating and cooling of foods which should provide quality advantage for the process. Some studies confirm that HP sterilized food quality is equal to or better than that of frozen foods (Krebbers et al., 2002; de Heij et al., 2003; Matser et al., 2004). The process being novel, it requires a large amount of data on microbial destruction kinetics to create a valid knowledge base for making wise regulatory decisions for commercial approval of the process.

As note in the previous chapter, the currently, sterile low acid food (LCAF) products are produced by heat processing, and generally a minimum process to result in 12-log reduction of spores of *Clostridium botulinum* (Stumbo, 1973), an anaerobic, mesophilic, spore forming pathogen that poses health risk in low acid foods. Most commercial processes for LACF are based on a process lethality of at least 5 min (which would statistically achieve over 20D of the target *Clostridium botulinum* spore). Since *C. botulinum* is a pathogen capable of producing a serious toxic neurotoxin, it is rarely used in process development studies. Instead surrogates of comparable resistance are always looked for. *Clostridium sporogenes*, a typical thermal resistant, mesophilic, spore-former, is traditionally used in confirmation of thermal processing specifications, when microbiological techniques are warranted. In the previous chapter, *C. sporogenes* 11437 were studied for HP resistance as a potential surrogate as used in pharmaceutical sterility tests. However, its HP resistance appeared to be lower than some published reports for PA3679. Hence this study focuses on evaluating the HP resistance of *C. sporogenes* ATCC7955 (PA3679).

Rovere et al. (1996) determined kinetic D values of *C. sporogenes* 7955 spores were 3.5, 3.2 min for 600, 700 MPa at 100°C respectively. Reddy et al. (1999, 2000, 2003 and 2006) studied the effect of HP in combination with elevated temperatures (i.e.,

35 to 75°C) on spores of *C. botulinum* type A, B and E and found it had greater inactivation when the temperature was increased to 75°C at 827 MPa. Type A spores were more resistant and only 3 log unit reduction achieved at 827 MPa and 75°C combination. Margosch et al. (2004, 2006) reported that proteolytic TMW2.357 (*C. botulinum* types B) and TMW 2.479(*B. amyloliquefaciens*) exhibited a greater resistance to pressure than bacterial spores (*Bacillus* spp. and *C. botulinum* spp.). Recently, Kouchma et al. (2005) conducted a destruction kinetic study of *C. sporogenes* PA3679 spore in phosphate buffer with constant temperature control, which showed higher resistance D values with Z_P value (23.7°C) and Z_T value (1500 MPa). Milk is a complete food, rich in nutrients, but low acid food nature and is highly perishable. To our knowledge, there has been no high pressure destruction kinetic study on *C. sporogenes* 7955 spore in milk.

The objectives of this study were to evaluate the HP destruction kinetics of *C. sporogenes* 7955 spores suspended in milk and compare it with other pressure resistant spores under similar treatment conditions.

5.2 Materials and Methods

5.2.1 *Clostridium sporogenes* culture preparation

Freeze-dried cultures of *C. sporogenes* (ATCC-7955) was obtained from the American Type Culture Collection (ATCC, Rockville, MD) and stored at –40°C. The original number of this species is PA3679 which is widely used in food research. The culture was hydrated in 10 ml Reinforced Clostridial Medium (RCM) broth (Oxoid, Basingstoke, Hampshire, UK) at 37°C for 24 h in anaerobic condition. Preparation details were the same as those used for *C. sporogenes* 11437 in Chapter 4.

5.2.2 Preparation of *C. sporogenes* spore

Same as detailed for *C. sporogenes* 11437 in Chapter 4.

5.2.3 Sample inoculation and packing

Same as detailed for *C. sporogenes* 11437 in Chapter 4.

5.2.4 High pressure equipment

Same as detailed in Chapter 4.

5.2.5 Plastic insulator

Same as detailed for *C. sporogenes* 11437 in Chapter 4.

5.2.6 High pressure treatment

Same as detailed for *C. sporogenes* 11437 in Chapter 4.

5.2.7 Sample temperature control during HP treatments

Same as detailed for *C. sporogenes* 11437 in Chapter 4.

5.2.8 Thermal process treatment

Same as detailed for *C. sporogenes* 11437 in Chapter 4.

5.2.9 Enumeration of survival cells

After HP treatments, the sample tubes were aseptically opened and serial dilutions were made with 0.1% peptone water. Spores of *C. sporogenes* were enumerated in Modified PA3679 agar (MP3679A) plates (Ocio et al., 1994) which the medium has 10 g tryptone (Oxiod, Basingstoke, England, UK); 2 g yeast extract (BD, Co. Spark, MD, USA); 2 g K_2HPO_4 (Acros Organics, NJ, USA); 2 g soluble starch (Sigma, St. Louis, MO); and 15 g bacterial agar (Fisher Scientific, Fair Lawn, NJ) in 1 liter distilled water, by using a pour-plate technique. These MP3679A plates were incubated at anaerobic 37°C condition for 5 days before counting.

5.2.10 Kinetic data analysis

Same as detailed for *C. sporogenes* 11437 in Chapter 4.

5.2.11 Kinetic data temperature calibration

Same as detailed for *C. sporogenes* 11437 in Chapter 4.

5.3 Results and discussion

5.3.1 Thermal destruction kinetic of *C. sporogenes* spores

Figure 5.1 shows the survivor curves for *C. sporogenes* 7955 spores suspended in milk under thermal processing conditions. The water bath was set at 90, 95 and 100 °C and holding time ranged from 50 to 180 min. Samples were treated at each temperature and two were taken out at each time interval. After treatment samples were immediately cooled and held in ice water bath until enumeration within 30min. Table 5.1 summarizes the associated D and z values of thermal destruction kinetics.

Figure 5.1 demonstrates characteristic thermal destruction curves similar to what was observed previously for *C. sporogenes* 11437 spores. Survival curves were well described by the first order log linear trends (R^2 >0.98). Higher temperatures resulted in higher rates of microbial destruction and resulted in steeper slopes giving lower D values. The D values were 156, 57.5, 12.1 min at 90, 95, 100 °C, respectively, with a corresponding z value of 9.0 °C (Table 5.1). Stumbo (1973) reported that *C. sporogenes* PA3679 spore had $D_{121.1}$ in the 0.1 to 1.5 min range with z value in the 7.8-10.0 °C range. The projected value from these studies would yield a $D_{121.1}$ around 0.1 min which indicates the strain is perhaps a fairly thermal resistant strain. Compared with our previous results with *C. sporogenes* 11437 in milk (Chapter 4), the D values of *C. sporogenes* 7955 spores were double of those of *C. sporogenes* 11437, again indicating *C. sporogenes* 7955 spores to be more resistant and possibly a better candidate than *C. sporogenes* 11437 for HPHT processing study.

5.3.2 High pressure destruction kinetics

Figures 5.2(a), 5.3(a), 5.4(a) show *C. sporogenes* 7955 spores survival curves at 700, 800 and 900 MPa, respectively, at 80, 90 and 100 °C as a function of pressure holding time. Again, the pressure destruction was found to follow a dual inactivation effect behavior detailed in Chapter 4: a rapid destruction just due to pressure pulse effect

followed by a first order rate destruction during the pressure holding. The survival curves confirmed the dual effect pressure destruction to be associated with HP treatments, with higher pressure and higher temperature treatments resulted in higher pulse effect and a faster destruction rate.

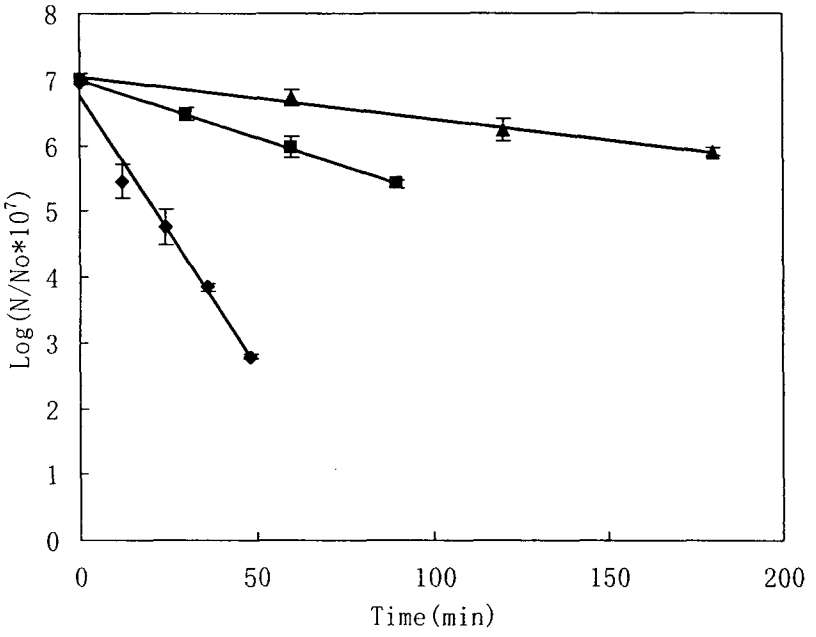


Figure 5.1 Nominal survivors of *C. sporogenes* 7955 spores in thermally treated milk (1 atm) at temperature (▲) 90, (■) 95, (♦) 100 °C

Table 5.2 summarizes the effect of pressure pulse effect on *C. sporogenes* 7955 spore nominal reductions. High pressure (700-900 MPa) combined with 100°C caused 0.29-0.53 log-reduction as compared to 0.06-0.16 log-cycle at 80 °C. Compared with previous *C. sporogenes* 11437 spore in milk results, *C. sporogenes* 7955 spore was more resistant to pulse pressure. Koutchma et al. (2005) reported the inactivation reduction of *C. sporogenes* 7955 spore in buffer as 0.39, 0.37, 0.65 log-unit for 600, 700, 800 MPa at 100°C, indicating that more reduction achieved in buffer samples resulted from longer come-up time.

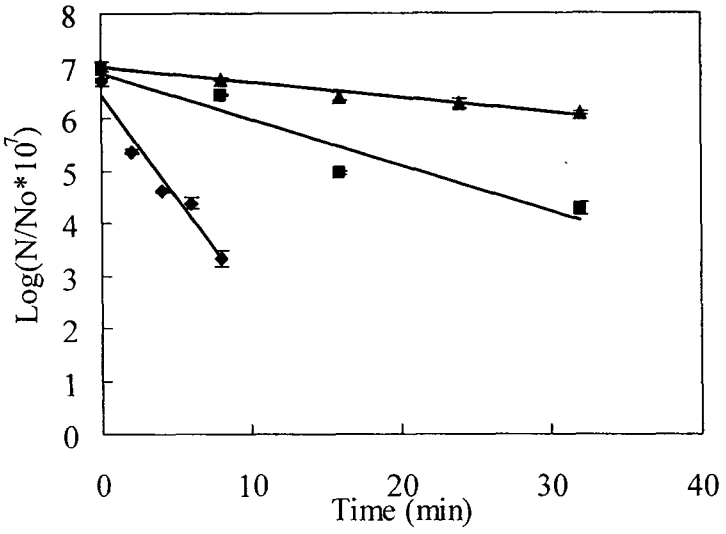
Table 5.1 Decimal reduction time (D values) of *C. sporogenes* 7955 spores in milk associated with HP and thermal treatment

Pressure (MPa)	Temperature (°C)	Uncorrected		Corrected	
		D value (min)	R^2	D value (min)	R^2
700	80	34.8	0.97	38.2	0.97
	90	15.9	0.90	13.6	0.99
	100	2.7	0.95	2.4	0.97
800	80	17.2	0.94	15.9	0.98
	90	10.7	0.96	11.4	0.96
	100	1.8	0.96	1.7	0.97
900	80	12.5	0.98	13.7	0.99
	90	7.0	0.92	7.0	0.94
	100	1.3	0.99	1.3	0.99
0.1	90	156.3	0.99		
	95	57.5	0.99		
	100	12.1	0.98		

Table 5.2 Pressure pulse effect on *C. sporogenes* 7955 spores in milk

	Spores reductions Log(N/No*10^7)		
Process temperature (°C)	Pressure 700 (MPa)	Pressure 800 (MPa)	Pressure 900 (MPa)
80	0.02±0.05	0.05±0.04	0.14±0.04
90	0.06±0.04	0.13±0.02	0.16±0.05
100	0.29±0.07	0.42±0.03	0.53±0.02

(a)



(b)

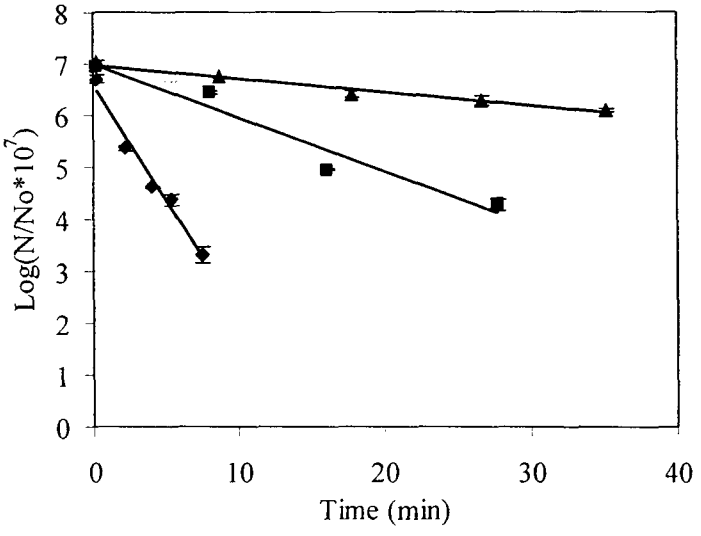
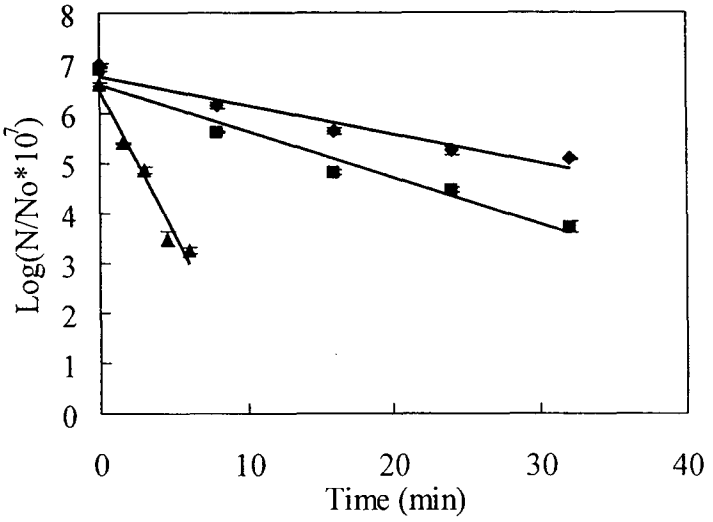


Figure 5.2 Uncorrected (a) and temperature corrected (b) nominal survivors of *C. sporogenes* 7955 spores in high pressure treated milk at 700 MPa and different temperatures (♦) 80, (■)90, (▲) 100 °C

(a)



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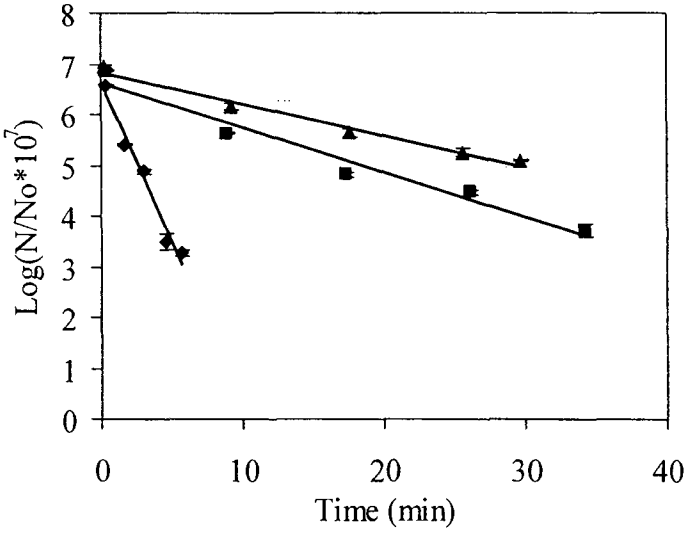
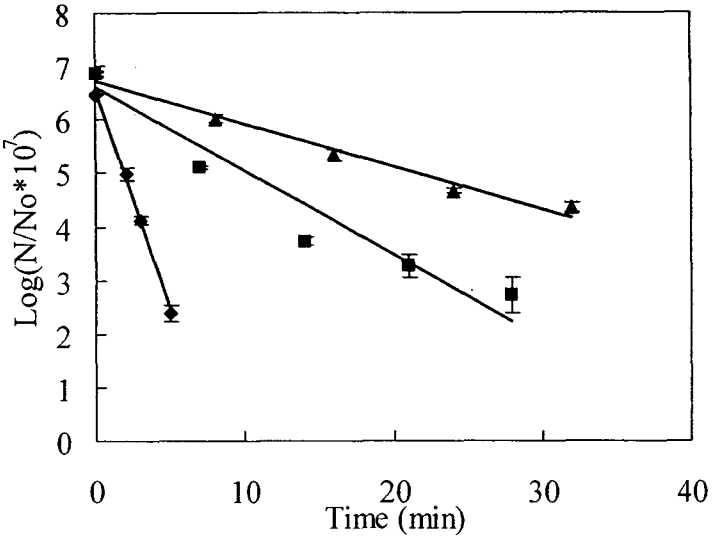


Figure 5.3 Uncorrected (a) and temperature corrected (b) nominal survivors of *C. sporogenes* 7955 spores in high pressure treated milk at 800 MPa and different temperatures (♦) 80, (■)90, (▲) 100 °C

(a)



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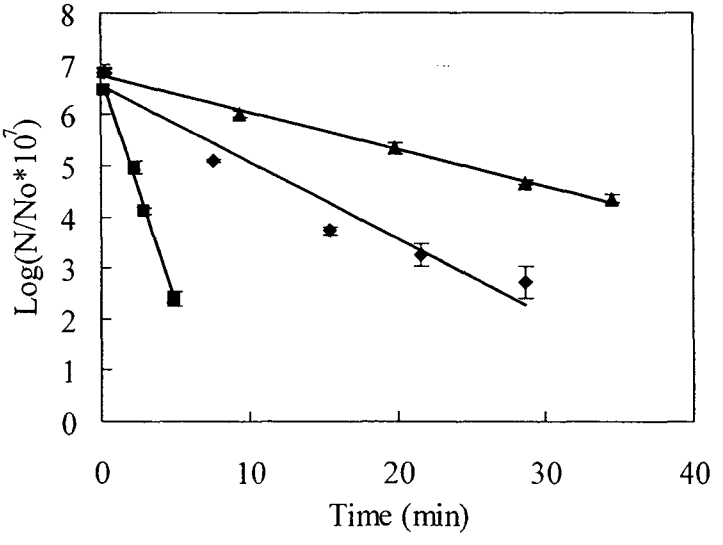


Figure 5.4 Uncorrected (a) and temperature corrected (b) nominal survivors of *C. sporogenes* 7955 spores in high pressure treated milk at 900 MPa and different temperatures (♦) 80, (■)90, (▲) 100 °C

During pressure holding, the destruction trend could be well fitted to the first order model. Computed D values from the regression analysis are summarized in Table 5.1. An increase in pressure resulted in a decrease in D value meaning acceleration in destruction of bacterial spores. For example, the D values of 700, 800, 900 MPa were 34.8, 17.2, 12.5 min and 2.7, 1.8, 1.3 min for HP treatment at 80, 100 °C respectively. These values were 14.5, 10.6, 6.6 min, and 1.0, 0.76, 0.63 min respectively, for *C. sporogenes* 11437. It can be recognized that the values for 7955 are almost twice those for 11437 under each of the testing conditions. After kinetic calibration, D values 700, 800, 900 MPa changed to 38.2, 15.9, 13.7 min and 2.4, 1.7, 1.2 min at 80, 100 °C respectively (Figure 5.2-5.4, b captions; Table 5.1).

The D values of *C. sporogenes* 7955 spore in buffer were 6.0, 4.9, 4.5 min and 3.2, 2.83, 2.3 min for 600, 700, 800 MPa at 91, 100 °C, respectively (Koutchma et al., 2005). At the comparable pressures and temperatures of 700 and 800 MPa at 90 and 100 °C, D values from this study were 13.6 and 11.4 min at 90 °C and 2.4 and 1.7 at 100 °C for 700 and 800 MPa, respectively. The values at 100 °C were relatively more closer while at 90 °C, the values from this study are considerably higher. Rovere et al. (1996) reported inactivation kinetics of *C. sporogenes* 7955 in meat broth, which were 6.8, 5.3 min for 700, 800 MPa at 93 °C and 3.5, 3.2 min for 600, 700 MPa at 100 °C respectively. These were lower than found in the present study. As noted before, the differences in the suspension medium could be a significant factor with milk used in this study compared to buffer in the previous. Baro-resistance due to milk components such as fat, lactose and casein has been recognized in other studies. Overall, compared with high pressure kinetics of *C. sporogenes* 11437 spores in milk, D values of 7955 spore in milk were certainly higher.

Comparing HPHT processing with thermal treatments at the same temperature conditions (90, 100 °C), it can be recognized that HPHT was more efficient and made D value decrease significantly ($P<0.05$) (Table 5.1). The HPHT process at 90 °C with either 700 or 800 MPa resulted in more than ten-fold decrease in conventional D values at 90 °C (the 900 MPa process achieved almost a 100 fold decrease). At 100 °C, however, the differences were narrower raging from one fifth to one tenth the conventional values. These results indicate that high pressure combined with mild heat can inactive bacterial

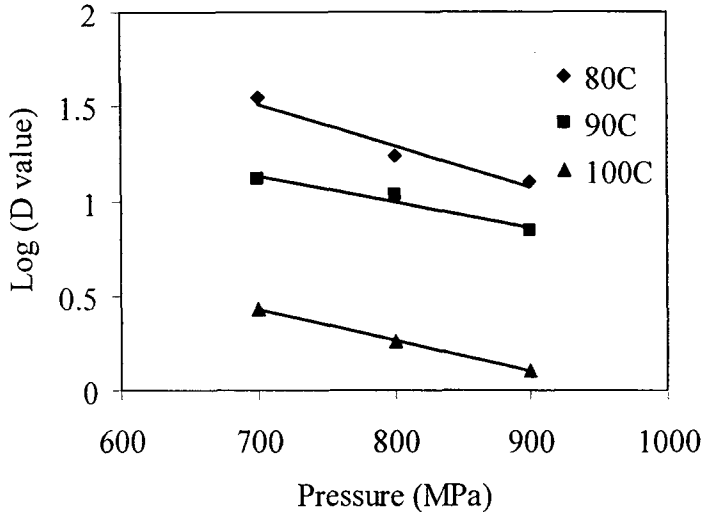
spores and effectively shorten the processing times, especially at lower processing temperatures.

5.3.3 Pressure and temperature sensitivity studies

Based on TDT and PDT models, Z_T and Z_P of *C. sporogenes* 7955 spores suspended in milk were analyzed from full factorial experimental designs (three pressures by three temperatures). Thermal resistance or thermal sensitivity (at constant pressure) of D value variation against temperature was well described by the Z_p value. Thermal resistance (at constant pressure) of D value variation with temperature was different among the different microorganisms. Through linear regression analysis, the thermal resistance (Z_p) value was determined at each pressure (Figure 5.5). The Z_P were 18.0, 20.6, 20.2 **°C** for 700, 800, 900 MPa respectively (Table 5.3). After temperature correction of the kinetic D values, Z_P values changed to 16.5, 20.3, 18.9°C, respectively. The thermal resistance increased by 14.5% with pressure increasing from 700 to 900 MPa. By comparison, Z_P values of *C. sporogenes* 7955 spores in milk were similar to those of *C. sporogenes* 11437 in milk which were 16.5, 16.9, 18.2 C for 700, 800, 900 MPa, respectively (Chapter 4). Higher Z_P value meant lower thermal sensitivity. Comparing Z_P values with 9.6 **°C** thermal treatment z value, one could find that the bacterial spore had higher thermal resistance under pressure, even though it had lower HPHT D values.

Similarly, the pressure resistance (Z_T) value was obtained from the linear relationship at each constant temperature (Figure 5.5). Uncorrected and corrected 7955 Z_T values were 450, 560, 625 MPa and 450, 690, 680 MPa at 80, 90, 100 **°C**, respectively, which indicated pressure resistance increased by 51% with process temperature increasing from 80 to 100°C (Table 5.4). This again meant that at higher temperature, bacterial spores were more resistant to increasing pressures.

(a)



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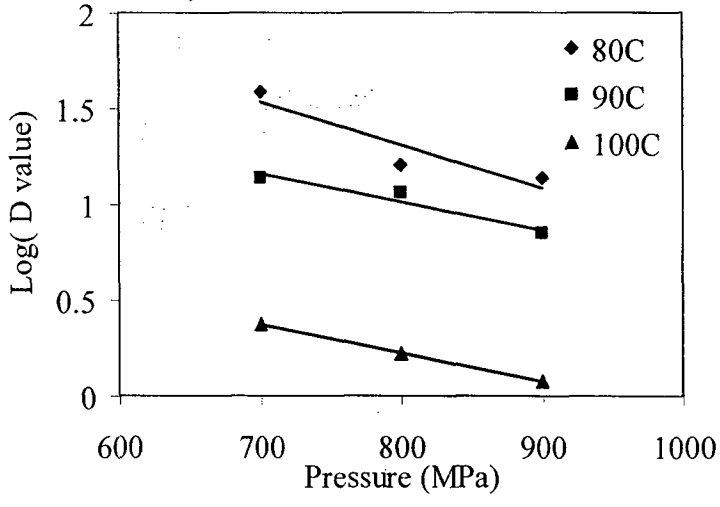


Figure 5.5 Uncorrected (a) and corrected (b) D value curves of *C. sporogenes* 7955 spores in milk subjected to HP and thermal treatments at different temperatures (♦) 80, (■) 90, (▲)100 °C as a function of pressure

Table 5.3 High pressure Zp value (°C) of *C. sporogenes* 7955 spores in milk subjected to HP and thermal treatments

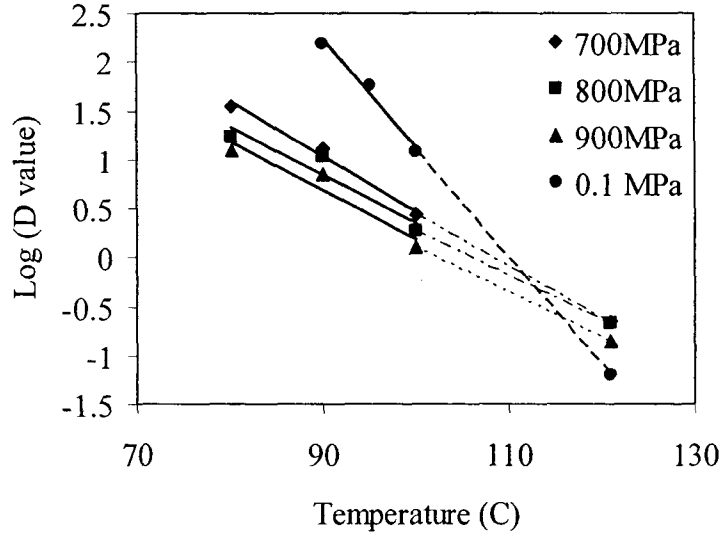
Pressure(MPa)	Uncorrected		Corrected	
	Z_p value	R^2	Z_p value	R^2
700	18.0	0.98	16.5	0.98
800	20.6	0.90	20.3	0.86
900	20.2	0.93	18.9	0.94
0.1	9.0	0.98		

Table 5.4 High pressure Z_T value (MPa) of *C. sporogenes* 7955 spores in milk subjected to HP and thermal treatments

Temperature(°C)	Uncorrected		Corrected	
	Z_T value	R^2	Z_T value	R^2
80	450	0.95	450	0.86
90	560	0.95	690	0.93
100	625	0.99	680	0.99

Koutchma et al. (2005) pointed out that Z_T values of *C. sporogenes* 7955 spore in buffer were 1500 MPa and in Chapter 4 it was shown than the z_T values of 11437 in milk were 720, 590, 1250 MPa for 80, 90, 100 °C, respectively. Rovere et al. (1996) reported that Z_T values of *C. sporogenes* were 725 MPa (90 °C), 962 MPa(100 °C), 752 MPa (108 °C) and Reddy (1999) pointed out that Z_T value of *C. botulinum* Type A was 1524 MPa (75 °C). These data confirm that *Clstridium spp.* spores had lower pressure sensitivity at higher temperatures.

(a)



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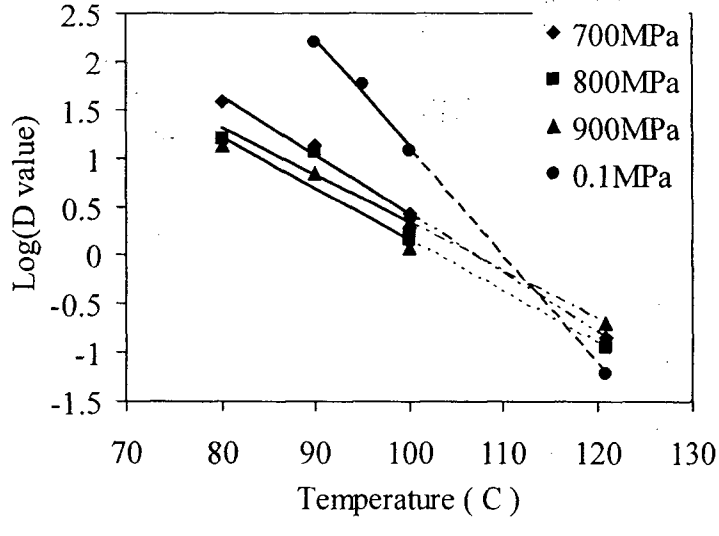


Figure 5.6 Uncorrected (a) and corrected (b) D value curves of *C. sporogenes* 7955 spores in milk subjected to HP and thermal treatments at different pressures (♦) 700, (■)800, (▲)900 MPa, (●) 0.1 MPa as a function of temperature

5.3.4 Predicted D values at higher temperature

Using the Zp and Z_T, D values were again computed for an extrapolated temperature of 121 °C to compare the thermal and pressure D values at commercial processing temperatures. Figure 5.6 also illustrates the D value curves against temperature and Table 5.5 shows the computed D values at specific temperatures (105, 110, 115, 120 and 121 °C). It can be found that extrapolated HP D values of *C. sporogenes* 7955 were higher than thermal D values at around 121°C. Again, because of the larger Z_P values associated with pressure, HPHT D values changed relatively slowly with temperature. On the other hand, the thermal D values which were several folds higher at lower temperatures converge rapidly because of their lower z values. It meant that at temperatures close to 120 °C, high pressure treatment may not accelerate clostridial spore destruction. It can, in fact, even offer protection to thermal destruction with an associated D value higher than the thermal D. Other researchers have also made some similar observations (Margosch et al., 2006; Ahn et al., 2007).

Table 5.5 Predicted decimal reduction time (D values) of *C. sporogenes* 7955 spore in HP and thermal treated milk at higher temperatures

Pressure (MPa)	Extrapolated D value (min) at Temperature(°C)				
	105	110	115	120	121
700	1.3	0.66	0.33	0.16	0.14
800	1.2	0.70	0.39	0.22	0.20
900	0.78	0.42	0.23	0.13	0.11
0.1	3.7	1.0	0.29	0.080	0.062

5.4 Conclusions

The study showed that, within the range of experimental conditions, higher pressures and high temperatures always resulted in a faster microbial destruction (smaller D value). High pressure combined with elevated temperature was able to destroy *C. sporogenes* 7955(PA3679) spores and had a greater efficiency than thermal processing. In this case their higher pressure resistance was correlated with their heat resistance. *C. sporogenes* 7955(PA3679) spores were more pressure resistant than *C. sporogenes* 11437

spores suggesting that *C. sporogenes* 7955 spores are better surrogates for *C. botulinum* surrogate and would be a better reference for establishing HP sterilization processing and processing validation. Media in which the kinetic tests are carried out plays a significant role. Comparing with literature data on destruction kinetics of *C. sporogenes* 7955 (PA3679) in meat and buffer, milk used in this study appear to offer a greater baro-protection.

Preface to Chapter 6

In the previous Chapters 4 and 5, the HP destruction kinetic study on *C. sporogenes* 11437 and 7955 spores suspended in milk were evaluated and compared at elevated process temperatures to determine their HP spores resistance for use as possible surrogates in low acid foods. *C. sporogenes* 7955 spores were found to be of higher pressure resistance than *C. sporogenes* 11437 and both had accelerated destruction kinetics when combined with pressure rather than when subjected to thermal treatment alone. It was also clear that the pressure resistance of *C. sporogenes* 7955 and 11437 was correlated with their heat resistance. It was also found by comparing with literature data, destruction kinetics of *C. sporogenes* likely depended also the nature of the media used for suspending them, with milk offering better baro-protection than meat and buffer. However, the experimental conditions used were and equipment were quite different. Seafood are a group of important and highly perishable low acid foods with typical high values. High pressure sterilization as a value added alternative processing technique, the application to seafood processing is very important and necessary. In this study, pressure resistance of *C. sporogenes* 7955 spore suspended in a salmon slurry are evaluated for comparison. The results obtained in this research is important for further research works on HP sterilization application in low acid foods, especially seafood. It would partially fulfill the second and third objectives of this thesis.

Part of this study has been presented at the IFTPS 2007 student paper competition and won the second place. It will be submitted for publication. This research work was completed by the candidate under the supervision of Dr. H. S. Ramaswamy.

Chapter 6

High pressure destruction kinetics of *Clostridium sporogenes* ATCC7955 spores in salmon meat base at elevated temperatures

Abstract

Salmon meat slurry (50% fish meat) was inoculated with *C. sporogenes* 7955 spores to give a cell concentration of 10^6/ml and subjected to high pressure (HP) treatments (700-900 MPa; 80-100 °C and 0- 24min). Samples were placed in a thick wall plastic insulator for providing temperature stability. As in previous studies, the HP treatments generally demonstrated a dual effect of bacterial spore destruction consisting of a pulse effect (step change) and a hold time effect (first order). Destruction rates (D value), pressure and temperature sensitivity parameters (Z_P and Z_T values) were evaluated by the first order reaction rate model. Higher pressures and temperatures resulted in accelerated spore destruction rate. For 800 and 900 MPa HP treatments, the D values were 14.0, 4.0, 1.0 min and 10.9, 2.9, 0.6 min at 80, 90, 100 °C, respectively. Temperature corrections gave a better first order fit for the survivor curves. Thermal treatments alone had D values 104, 19.9, 7.5 min at 90, 95 and 100 °C respectively, thereby with substantially higher D values than HPHT D values. Temperature sensitivity Z_P values (at constant pressure) were 14.5, 17.3, 15.5C at 700, 800, 900 MPa respectively, with z value increasing with pressure and almost 50% higher than the thermal treatment z value 9.0 °C. The pressure sensitivity parameter Z_T values (at constant temperature) were 440, 540, 550 MPa at 80, 90 and 100 °C, respectively, and with the z values increasing with temperature. *C. sporogenes* 7955 spores were relatively more sensitive to temperature than to pressure, and then pressure resistance were higher in milk base than in salmon base.

6.1 Introduction

High pressure processing as a novel non-thermal food processing technique has been widely applied for pasteurization of foods to extend food shelf life and preserve desirable qualities, such as natural color, flavor and nutrients. However, some resistant microorganisms and enzymes can survive the pasteurization. Therefore, high pressure treated foods should be kept at low temperature to prevent spoilage (Cheftel, 1995; Farkas et al., 2001). Seafood products are the favorite on the table delicacy for consumers due to its specific flavor, texture and rich of nutrients. They are low in acid, highly perishable and usually spoil faster than other muscle foods. Nowadays, high pressure processing is recognized as a value added pasteurization process for seafood processing. Recently, commercial HP treated seafoods such as oyster, mussel and lobster, are available on the global food markets (Hugas et al., 2002).

However, this technique was not used in low acid food sterilization because the more resistant bacterial spores were not adequately inactivated by HP applications at room or refrigerated temperatures (Knorr, 1995; Maggi et al., 1996). Although the potential for the use of HP treatment as an alternative method to thermal process is possible, commercial high pressure sterilization technology has not been realized yet because of lack of data on spore destruction kinetics by high pressure. Sterile shelf-stable seafood products are currently produced by thermal processing under conditions designed for low acid canned foods (LACF) respecting the 12 log cycle reduction approach based on the most heat resistant pathogen *Clostridium botulinum* (Stumbo, 1973). Several studies demonstrate that with high pressure processing combined with elevated temperature it is possible to achieve the desired spore inactivation (Mills et al., 1998; Patterson et al., 1995; Reddy et al., 2003, 2006). Adiabatic temperature change associated with pressure processing make them ideal for achieving rapid heating to processing temperatures and subsequent rapid cooling. These HPHT techniques would benefit the product in terms of retaining higher quality in the product. Such advantages have been demonstrated in some studies (Hoogland et al., 2001; de Heij et al., 2003; Matser et al., 2004).

In the previous Chapters 4 and 5, the HP destruction kinetic study on *C. sporogenes* 11437 and 7955 spores suspended in milk were evaluated and compared at

elevated process temperatures to determine their HP spores resistance for use as possible surrogates in low acid foods. *C. sporogenes* 7955 spores were found to be of higher pressure resistance than *C. sporogenes* 11437 and both had accelerated destruction kinetics when combined with pressure rather than when subjected to thermal treatment alone. It was also clear that the pressure resistance of *C. sporogenes* 7955 and 11437 was correlated with their heat resistance. It was also found by comparing with literature data, destruction kinetics of *C. sporogenes* likely depended on the nature of the media used for suspending them, with milk offering better baro-protection than meat or buffer. However, the experimental conditions used were and equipment were quite different. Seafoods are a group of important and highly perishable low acid foods with typical high values. High pressure sterilization as a value added alternative processing technique, the application to seafood processing is very important and necessary. The objectives of this study was to evaluate the pressure resistance of *C. sporogenes* 7955 spore suspended in a salmon slurry.

6.2 Materials and Methods

6.2.1 *Clostridium sporogenes* culture preparation

Same as detailed for *C. sporogenes* 11437 in Chapter 4.

6.2.2 Preparation of *C. sporogenes* spore

Same as detailed for *C. sporogenes* 11437 in Chapter 4.

6.2.3 Sample preparation

Fresh Atlantic salmon fish was obtained locally (La mer, Montreal, QC), removed skin and bone. The pure fish fillet (68.6% moisture and pH 6.32) was cut into small cubs (1x1x1 cm), distributed 100 g to sterile stomacher plastic bags and quickly frozen and kept in -40 °C freezer until use. The salmon meat was thawed overnight at 4°C, mixed with 100 g low concentration spore suspension and then processed 3 min in a stomacher (Bagmixer 400, St Nom, France) to get 50% meat slurry (initial spore count about 1 x 10^6CFU/g). Approximately 12 g spore slurry was distributed into each sterile plastic

pouches (Nasco, Toronto, ON). Each sample was squeezed into the shape of a thin plate (60 mm length, 40 mm width and 3 mm thickness) and heat sealed (National instrument Co., Baltimore, MD) after removing most of the trapped air. All sample bags were maintained at 4°C refrigerator until use within 12 h. All the operations above were carried out in a Class 100 Forma laminar airflow workstation (Thermo Electron Co., Mariette, Ohio).

6.2.4 High pressure equipment

Same as detailed for *C. sporogenes* 11437 in Chapter 4.

6.2.5 Insulator chamber

A thick wall POM (Polyoxymethylene, Johnston Industrial Plastic Ltd., Montreal, Canada) plastic cylinder (4.0 cm inside diameter, 8.0 cm outside diameter and 22 cm height) was used as insulator placed in pressure chamber for temperature control. Similar to the previous one described in Chapter 4, this insulator also had a plug with threads installed on the top for thermocouple insertion and a plastic moveable stopper at the bottom. The insulator inside was filled with distilled water. Two samples bags were kept inside with thermocouple attached to one of them and this insulator assembly was positioned inside the pressure vessel for delaying heat transfer from sample bags to pressure vessel during high pressure processing (Figure 6.1).

6.2.6 High pressure treatment

Even though a larger volume POM insulator was used in this experiment for the larger sample size, this insulator was made up of the same material and the design was similar to insulator used in Chapter 4. So the HP treatments were given the same way as detailed for *C. sporogenes* 11437 in milk in Chapter 4. One K-type thermocouples (OMEGA Eng. Stamford, CT) attached to a data-logger was installed through the top plug and installed in the sample pouch in the insulator for recording product temperature. The second thermal couple was installed outside insulator for monitoring pressure vessel

temperature. Before HP treatment, all sample bags and insulator were preheated 10 min in a water bath (SL26, Julabo, Labortechnik GMBH, Germany) for increasing temperature to sample initial temperature.

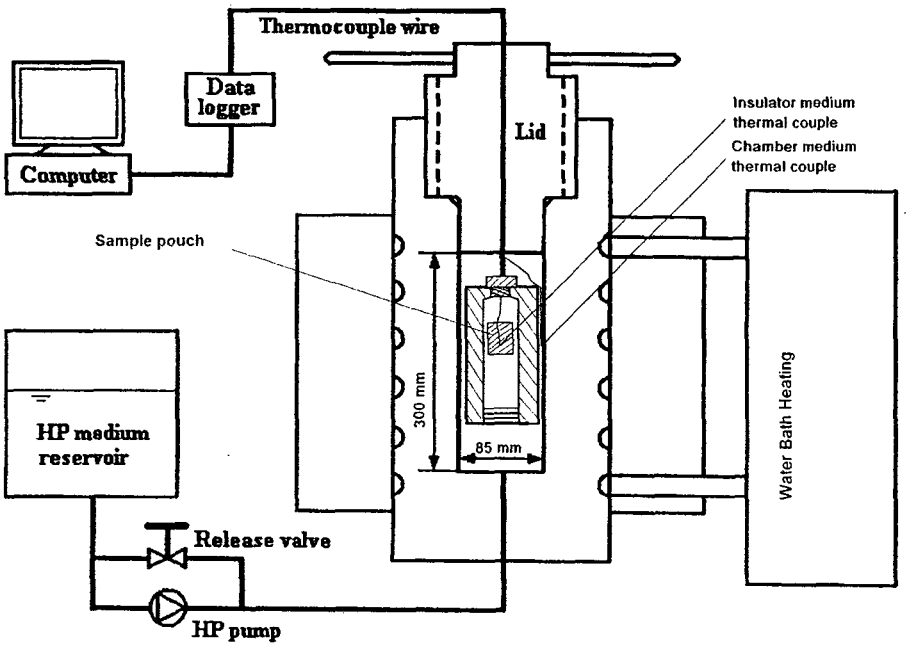


Figure 6.1 **Schematic diagram of the high pressure experimental setup**

6.2.7 Sample temperature control during HP treatments

Several high pressure experiments treatments were given at different pressure (500 –900 MPa) and initial temperature (50 – 80 °C) combinations. Experimental data showed that the temperature changes with this unit were similar to that used with milk (Chapter 4.2.6).

Figure 6.2 illustrates the temperature profile for a test run at 700 MPa at 80°C for a 24 min treatment. Before treatment, the water initial temperature and chamber medium temperature were set at 52.5°C and 61.5°C. During pressurization, water, sample and glycerine temperatures increased due to the adiabatic heating. When the pressure reached desired process pressure 700 MPa, the sample reached a temperature 79.8°C and chamber medium at 93.7°C. Due to the higher temperature of the medium in pressure chamber outside the insulator, the product internal temperature inside the insulator rose to a

maximum of 80.8°C at the sixth min and then dropped gradually to 78.6°C before depressurization. All the data of temperature verification experiments demonstrated that the large volume insulator temperature change had the same trend as those in the smaller volume insulator used in Chapter 4. Because the volume of two sample bags used in this research was small and the slice shape of them pretty much eliminated the temperature difference between sample and water medium, the temperature of sample was constant and as the same as water medium during high pressure treatments.

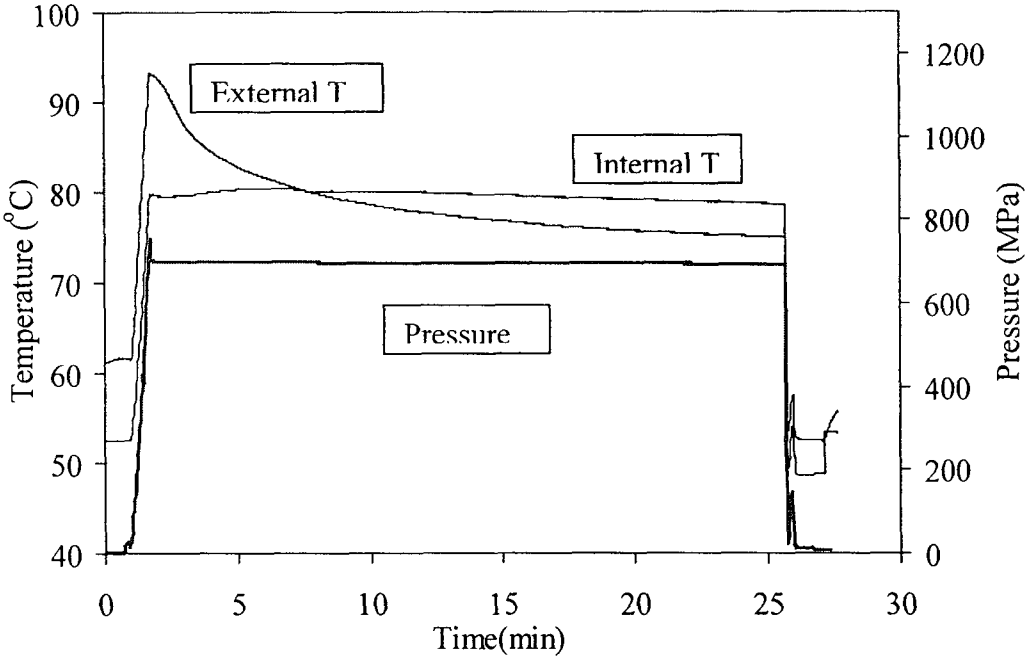


Figure 6.2 Changes in the pressure, medium temperature in the pressure vessel and product temperature inside the insulator in a test run: 700 MPa, 80°C, 24min holding time.

6.2.8 Thermal process treatment

Same as detailed for *C. sporogenes* 11437 in Chapter 4.

6.2.9 Enumeration of survival spores

After HP treatments, the sample bags were aseptically opened and 10g sample transfer to stomach bag mixed with 90g sterile distilled water. Serial dilutions were made with 0.1% peptone water. Spores of *C. sporogenes* were enumerated in Modified PA3679 agar (MP3679A) plates (Ocio, et al. 1994) which has 10 g tryptone (Oxiod, Basingstoke, England, UK); 2 g yeast extract (BD, Co. Spark, MD, USA); 2 g K_2HPO_4 (Acros Organics, NJ, USA); 2 g soluble starch (Sigma, St.Loouis, MO); and 15 g bacterial agar (Fisher Scientific, Fair Lawn, NJ) in 1 liter distilled water, by using a pour-plate technique. These MP3679A plates were incubated at anaerobic 37°C condition for 5days before counting.

6.2.10 Kinetic data analysis

Same as detailed for *C. sporogenes* 11437 in Chapter 4.

6.2.10 Kinetic data temperature calibration

Same as detailed for *C. sporogenes* 11437 in Chapter 4.

6.3 Results and discussion

6.3.1 Thermal destruction kinetic of *C. sporogenes* spores

Figure 6.3 shows the survivor curves for *C. sporogenes* 7955 spores suspended in salmon meat slurry under thermal processing conditions (90-100 °C) and selected holding times. Table 6.1 summarizes the associated D and z values of thermal destruction kinetics. The thermal destruction characteristics of *C. sporogenes* 7955 spores suspended in salmon meat slurry were similar to those observed for *C. sporogenes* 11437 and 7955 in milk. They were well described by the first order log linear trends (R^2 >0.98). Higher temperatures resulted in lower D values. The associated D values were 104, 19.9, 7.5 min at 90, 95, 100 °C respectively. These D values were lower than those found for the same strain in milk (156, 57.5, 12.1 min found at 90, 95, 100 °C), but higher than observed for *C. sporogenes* 11437 spores in milk (65.8, 26.3, 6.0 min at 90, 95, 100 °C, respectively). Hence the strain dependent and media dependent variations become obvious. The corresponding z value was 8.5 °C (Table 6.1), compared to 9.0 °C found for the same in

milk and 9.6°C for *C. sporogenes* 11437 spores. Again there were smaller differences, but qualitatively the temperature difference was somewhat more similar. Overall, for thermal destruction, *C. sporogenes* 7955 spores in milk offered the most resistance.

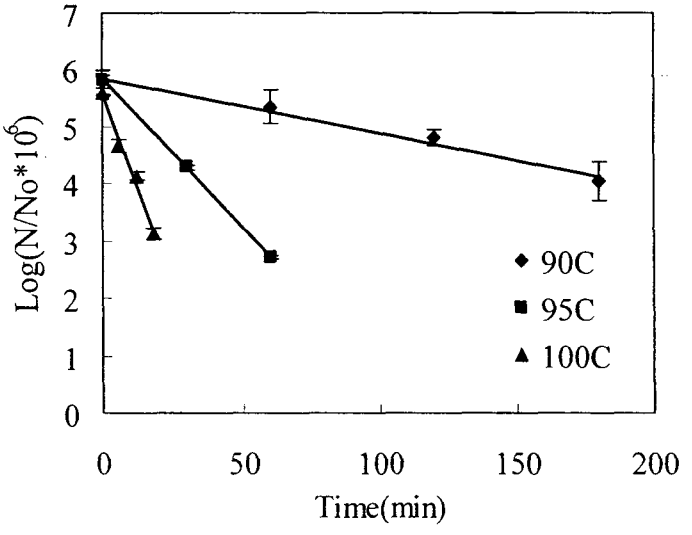


Figure 6.3 Nominal survivors of *C. sporogenes* 7955 spores in thermally treated salmon meat slurry (1 atm) at temperature (▲) 90, (■) 95, (♦) 100 °C

Table 6.1 Decimal reduction time (D values) of *C. sporogenes* 7955 spores in salmon meat slurry with HP and thermal treatment

Pressure (MPa)	Temperature (°C)	Uncorrected		Corrected	
		D value (min)	R^2	D value (min)	R^2
700	80	32.8	0.97	31.2	0.99
	90	9.0	0.90	6.8	0.99
	100	1.3	0.95	1.3	0.99
800	80	13.6	0.94	14.0	0.97
	90	5.0	0.96	4.0	0.98
	100	1.0	0.96	1.0	0.97
900	80	9.4	0.98	10.9	0.98
	90	2.8	0.92	2.9	0.99
	100	0.6	0.99	0.6	0.99
0.1	90	104.2	0.99		
	95	19.9	0.99		
	100	7.5	0.98		

6.3.2 High pressure destruction kinetics

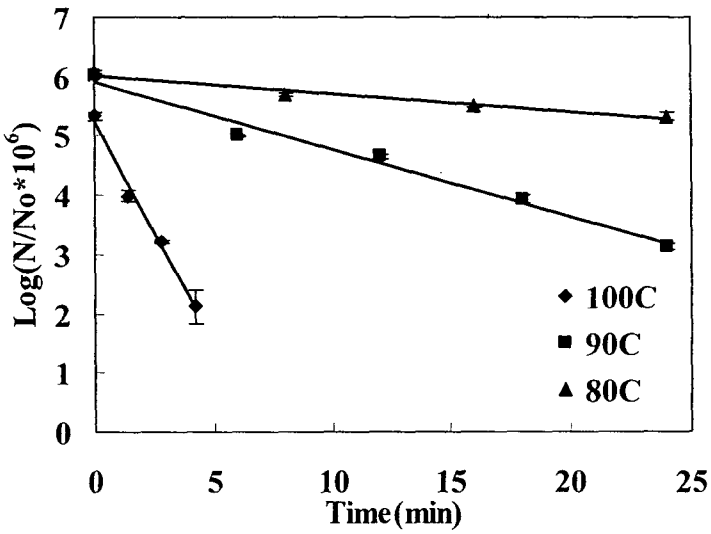
Figures 6.4-6.6 (a captions) show *C. sporogenes* 7955 spores survival curves at 700, 800 and 900 MPa, respectively, at 80, 90 and 100°C as a function of pressure holding time. Again, the pressure destruction was found to follow a dual inactivation effect behavior detailed in Chapters 4 and 5 with higher pressure and higher temperature treatments resulted in higher pulse effect and a faster destruction rate. Table 6.2 summarizes the pulse of the pressure treatment.

During pressure holding time, the destruction trend was well fitted to the first order model. Computed D values from the regression analysis are summarized in Table 6.1. Higher pressures and higher temperatures resulted in a decrease in D value meaning acceleration in destruction of bacterial spores. The D values at 700, 800, 900 MPa were 32.8, 13.6, 9.4 min and 1.3, 1.0, 0.6 min for HP treatment at 80 and 100 °C, respectively. These values were slightly lower than for the same strain in milk medium (34.8, 17.2, 12.5 min and 2.7, 1.8, 1.3 min for HP treatment at 80, 100 °C respectively), but higher than those found for *C. sporogenes* 11437 (14.5, 10.6, 6.6 min, and 1.0, 0.76, 0.63 min respectively, at 80 and 100°C). After corrections, D values at 700, 800, 900 MPa were 31.2, 14.0, 10.9 min and 1.3, 1.0, 0.6 min at 80, 100°C respectively (Figure 6.2-6.4, b captions; Table 6.1). Thus overall, the kinetic results are comparable to those observed with *C. sporogenes* 7955 suspended in milk, but milk as media definitely provided better baro-protection than salmon meat. In general there is also a general agreement that higher thermal resistance translated to higher pressure resistance within the range of experiments.

Table 6.2 Effect of pressurization come-up time (CUT) and depressurization come-down time(CDT) on *C. sporogenes* 7955 spores reduction

Process temperature (°C)	Spores reductions Log(N/No*10^6)		
	Pressure 700(MPa)	Pressure 800(MPa)	Pressure 900(MPa)
80	-0.07±0.03	0.01±0.07	0.01±0.06
90	0.01±0.03	0.16±0.03	0.24±0.03
100	0.67±0.06	0.80±0.04	1.35±0.16

(a)



(b)

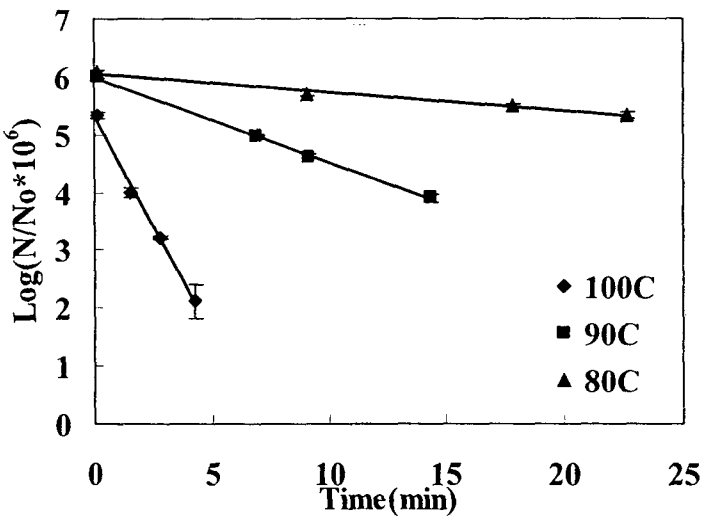
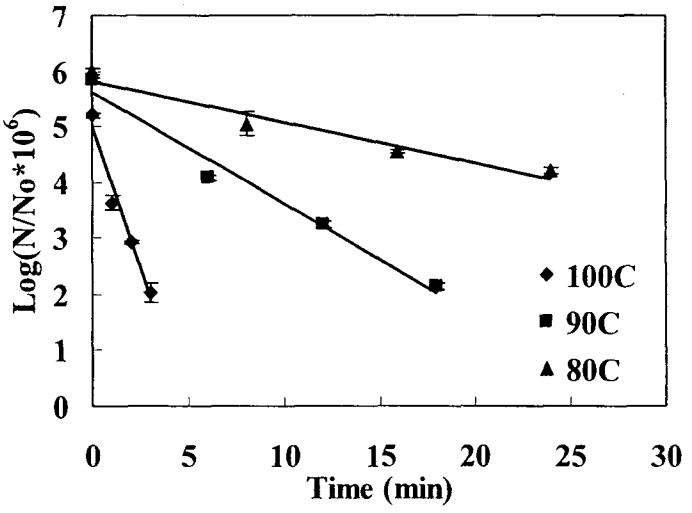


Figure 6.4 Uncorrected (a) and temperature corrected (b) nominal survivors of *C. sporogenes* 7955 spores high pressure treated salmon meat slurry at 700 MPa and different temperatures (♦) 80, (■)90, (▲) 100 °C

(a)



(b)

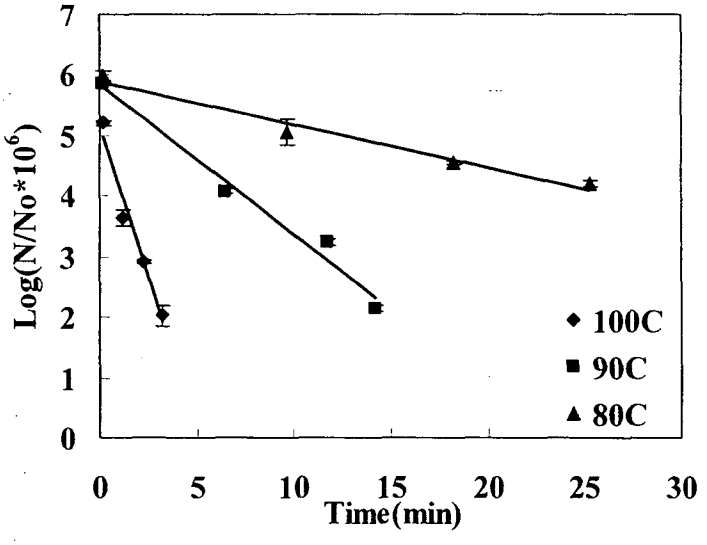
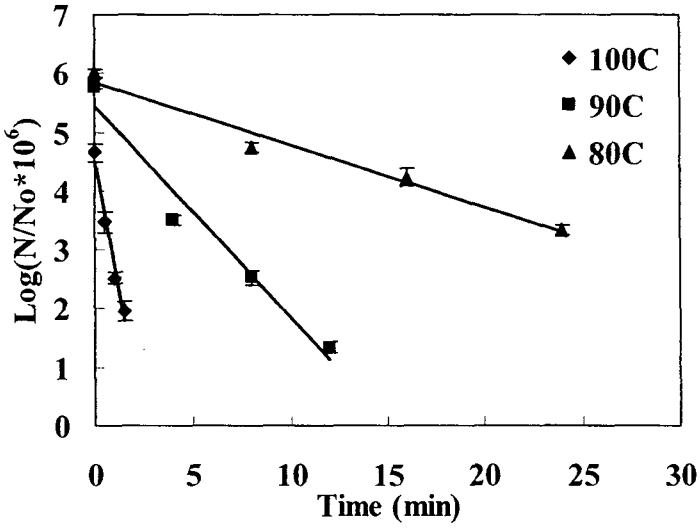


Figure 6.5 Uncorrected (a) and temperature corrected (b) nominal survivors of *C. sporogenes* 7955 spores high pressure treated salmon meat slurry at 800 MPa and different temperatures (♦) 80, (■)90, (▲) 100 °C

(a)



(b)

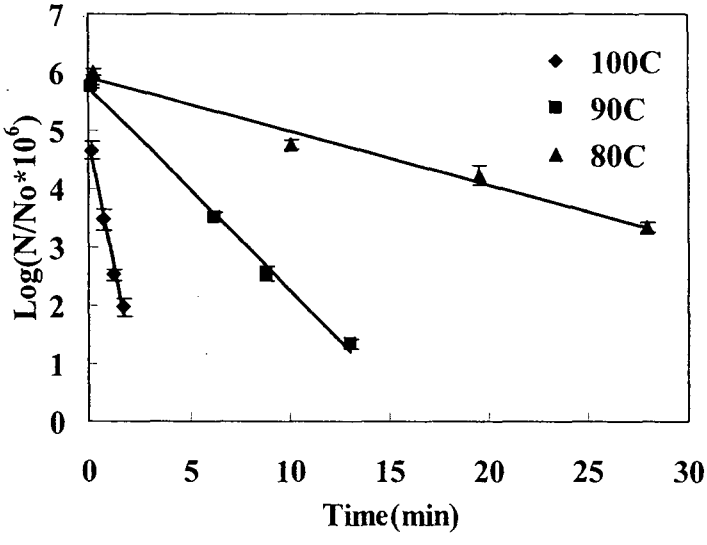


Figure 6.6 Uncorrected (a) and temperature corrected (b) nominal survivors of *C. sporogenes* 7955 spores high pressure treated salmon meat slurry at 900 MPa and different temperatures (♦) 80, (■)90, (▲) 100 °C

The D values of *C. sporogenes* 7955 spores in buffer for 700, 800 MPa were 4.9, 4.5 min and 2.83, 2.3 min at 91, 100 °C, respectively (Koutchma et al. 2005). Compared with the kinetics of *C. sporogenes* 7955 spores in buffer and in milk, D values of 7955 spores in fish meat were lower, indicating that *C. sporogenes* 7955 spores in fish meat were less resistant to high-pressure treatment.

Comparing HPHT processing with thermal treatments at the same temperature conditions (90, 100°C), again it can be recognized that HPHT was more efficient and made D values of *C. sporogenes* 7955 spores decrease significantly ($P<0.05$) (Table 6.1). The thermal D value at at 90°C was about 15 times higher than the HPHT D value at 700 MPa, 25 times at 800 MPa and 75 times at 900 MPa. Likewise at 100 °C, the relative D values were 6 times at 700 MPa, 8 times at 800 MPa and 12 times at 900 MPa. The differences were noticeably getting narrower as the process temperature increased. These results indicate that high pressure combined with mild heat can inactive bacterial spores and effectively shorten the processing times, especially at lower processing temperatures.

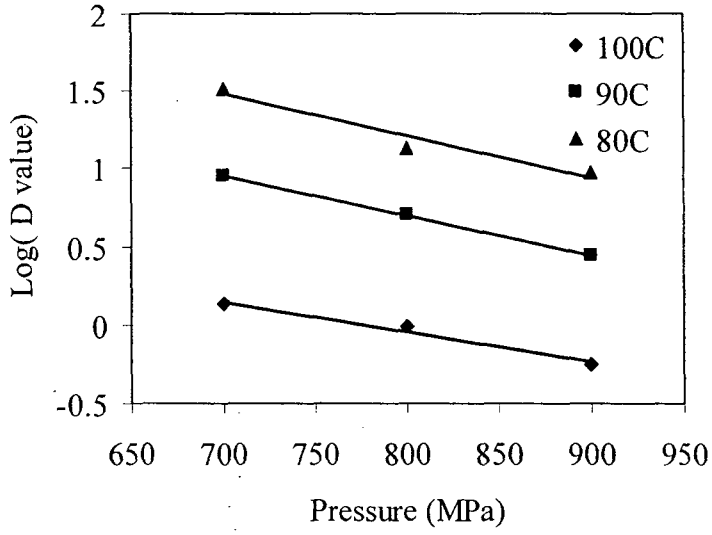
6.3.3 Pressure and thermal dependency study

Z_T and Z_P of *C. sporogenes* 7955 spores suspended in salmon meat were analyzed from the respective D value curves either as a function of pressure or temperature. Thermal sensitivity (at constant pressure) of D values was described by the Z_P value (Figure 6.8). The Z_P were 14.5, 17.3, 15.5 °C for 700, 800, 900 MPa respectively (Table 6.3), compared to 16.5, 20.3, 18.9°C, for 700, 800, 900 MPa respectively (in milk, Chapter 5) and 16.5, 16.9, 18.2 C for 700, 800, 900 MPa, respectively for *C. sporogenes* 11437 in milk (Chapter 4). By comparison, Z_P values of *C. sporogenes* 7955 and 11437 spores in milk and salmon meat were relatively similar. Higher Z_P value meant lower thermal sensitivity. Comparing these Z_P values with 8.6°C thermal treatment z value, one can find that the bacterial spores had higher thermal resistance under pressure, even though it had lower HPHT D values.

Similarly, the pressure resistance (Z_T) value was obtained from the linear relationship at each constant temperature (Figure 6.7). Corrected Z_T values *C. sporogenes*

7955 in salmon meat slurry were 440, 540, 550 MPa at 80, 90, 100 °C, respectively (Table 6.4), which indicated pressure resistance increased with process temperature. These Z_T values of *C. sporogenes* 7955 spore in fish meat were similar to those in milk.

(a)



(b)

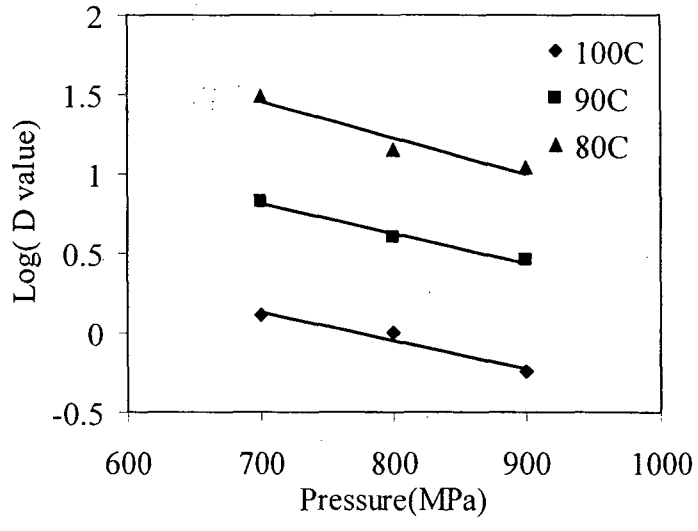
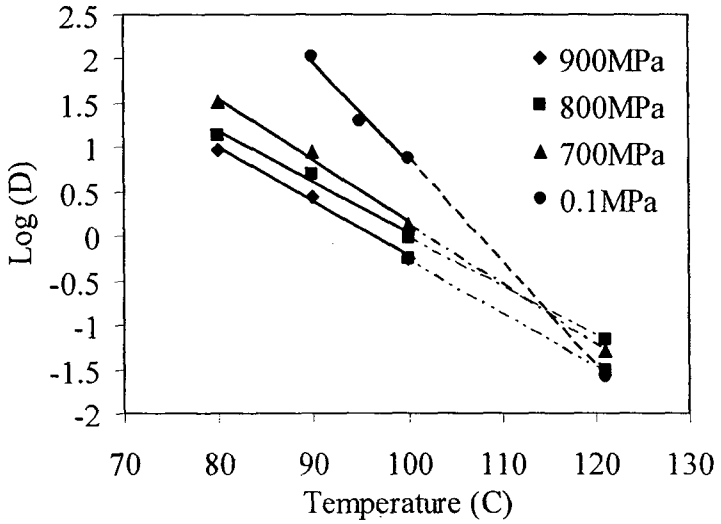


Figure 6.7 Uncorrected (a) and corrected (b) D value curves of *C. sporogenes* 7955 spores in salmon meat slurry subjected to HP and thermal treatments at different temperatures (♦) 80, (■) 90, (▲)100 °C as a function of pressure

(a)



(b)

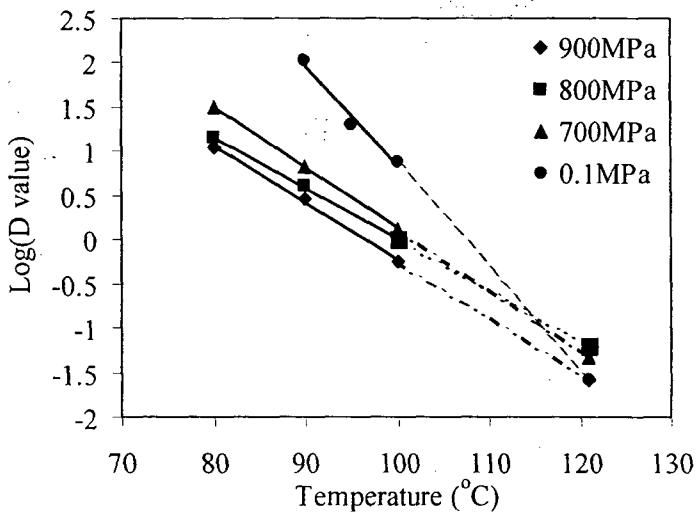


Figure 6.8 Uncorrected (a) and corrected (b) D value curves of *C. sporogenes* 7955 spores in salmon meat slurry subjected to HP and thermal treatments at different pressures (♦) 700, (■)800, (▲)900 MPa, (●) 0.1 MPa as a function of temperature

Table 6.3 High pressure Z_P value (°C) of *C. sporogenes* 7955 spores in salmon meat slurry subjected to HP and thermal treatments

Pressure (MPa)	Uncorrected		Corrected	
	Z_p value	R^2	Z_p value	R^2
700	14.4	0.98	14.5	0.98
800	17.5	0.90	17.3	0.86
900	16.4	0.93	15.5	0.94
0.1	9.0	0.98		

Table 6.4 High pressure Z_T value (MPa) of *C. sporogenes* 7955 spores in salmon meat slurry subjected to HP and thermal treatments

Temperature (°C)	Uncorrected		Corrected	
	Z_T value	R^2	Z_T value	R^2
80	370	0.95	440	0.92
90	395	0.99	535	0.98
100	535	0.98	550	0.96

6.3.4 Predicted D values at higher temperature

Using the Z_P and Z_T, D values were again computed for an extrapolated temperature of 121C to compare the thermal and pressure D values at commercial processing temperatures. Figure 6.8 also illustrates the D value curves against temperature and Table 6.5 shows the computed D values at specific temperatures (105, 110, 115, 120 and 121°C). It can be found that extrapolated HP D values of *C. sporogenes* 7955 were almost the same as the thermal D values at around 121°C. Again, because of the larger Z_P values associated with pressure, HPHT D values changed relatively slowly with temperature. On the other hand, the thermal D values which were several folds higher at lower temperatures converge rapidly because of their lower z values. It meant that at the temperature close to 121°C, high pressure treatment might not accelerate clostridial spore destruction.

Table 6.5 Predicted decimal reduction time (D values) of *C. sporogenes* 7955 spore in HP and thermal treated salmon meat at higher temperatures

Pressure (MPa)	Extrapolated D value (min) at Temperature(°C)				
	105	110	115	120	121
700	0.60	0.27	0.12	0.056	0.048
800	0.52	0.27	0.14	0.071	0.062
900	0.28	0.13	0.064	0.031	0.026
0.1	1.8	0.48	0.13	0.035	0.027

6.4 Conclusions

The study showed that, within the range of experimental conditions, higher pressures and high temperatures always resulted in a faster microbial destruction (shorter D value). High pressure combined with elevated temperature was able to destroy *C. sporogenes* 7955(PA3679) spores in salmon meat and had a greater efficiency than thermal processing. Within the range of experimental conditions, higher pressure resistance was correlated with heat resistance. *C. sporogenes* 7955(PA3679) spores in milk were more pressure resistant than *C. sporogenes* 7955(PA3679) spores in salmon meat which were more resistant than *C. sporogenes* 11437 spores suggesting that the pressure resistance depends on the strain as well as the medium. Media in which the kinetic tests are carried out plays a significant role.

Preface to Chapter 7

In the previous chapters, the HP inactivation kinetics of *C. sporogenes* 11437 in milk, and *C. sporogenes* 7955 spores in milk and salmon fish meat base were carried out to evaluate their pressure resistances at elevated temperatures. *C. sporogenes* 7955 spores had higher resistance than *C. sporogenes* 11437 in thermal treatments as well as HPHT treatments. *C. sporogenes* 7955 spores also demonstrated higher resistance in milk than in salmon meat base, in consistence with their thermal resistances therefore giving a general correspondence between thermal and high pressure resistances. The literature references indicate one of the most thermal resistant spore former to be a thermophile, *G. stearothermophilus.* However its pressure resistance studies have been limited and variable. In this paper, the thermal and high pressure inactivation kinetics of *G. stearothermophilus* 10149 spore in milk at elevated temperatures were studied so that they can be compared with those of *C. sporogenes* 7955 spores. This study would partially fulfill the second and third objectives of this thesis and would be useful and important for research work on high pressure sterilization.

Part of this study has been presented at CIFST conference 2006 and will be submitted for publication. This research work was completed by the candidate under the supervision of Dr. H. S. Ramaswamy.

Chapter 7

High pressure destruction kinetics of *Geobacillus stearothermophilus* 10149 spores in milk elevated temperatures

Abstract

G. stearothermophilus 10149 spores were inoculated in milk (2% fat) and subjected to high pressure (HP) treatments (500-900 MPa) at elevated temperatures (70-90 °C) and holding times (0-32min). Samples in 1 mL plastic vials were placed in a specially constructed insulated chamber to prevent temperature drop during the treatment. The study confirmed the dual effect HP destruction of bacterial spore with a step change pulse effect and a pressure holding time effect. As before, destruction rates (D value), pressure and temperature sensitivity parameters (Z_P and Z_T values) were evaluated by the first order reaction rate model. Higher pressures and temperatures resulted in steep acceleration of spore destruction rate. For 700 and 900 MPa HP treatments, the D values were 13.9, 3.4, 1.3 min and 8.7, 2.2, 0.6 min at 70, 80, 90°C respectively. After compensating for the small temperature variations, the D values changed to 15.9, 3.6, 1.3 min and 13.4, 2.5, 0.6 min at 70, 80, 90 °C respectively. Thermal treatments had D values 49.4, 16.1, 6.3 min at 110, 115, 120 °C respectively, indicating very high thermal resistance. By comparison, HP destruction rates were much smaller. Temperature sensitivity Z_P values (at constant pressure) were 25.3, 18.1, 15.5 °C at 500, 700, 900 MPa, respectively, with z value increasing with pressure and much higher than the thermal z value 11.2 °C. The pressure sensitivity parameter Z_T values (at constant temperature) were 2560, 770, 610 MPa at 70, 80, 90 °C, respectively, with the z values *decreasing* with temperature. This behavior was different compared to previous studies, but the overall pressure resistance of *G. stearothermophilus* 10149 spores in milk was much lower compared with that of *C. sporogenes* 7955 spores.

7.1 Introduction

The importance of high pressure processing as novel and innovative food preservation method has been highlighted in the previous chapters. High pressure high temperature (HPHT) processing is getting recognized as alternative technique to high temperature sterilization because it is able to inactivate highly resistant bacterial spores in low acid (pH 4.5) foods. Adiabatic compression results in quick heating, and has the potential to raise initial product temperatures from 90 °C to sterilizing levels that are generally possible under HTST and UHT techniques. A brief holding at this peak pressure will be sufficient to achieve the desired sterility in the product. A quick pressure release will reverse the heating effect resulting in almost instantaneous cooling to around 90 °C. Thus the pressure processing can be used to simulate HTST operation in bulk foods which are impossible under conventional heating conditions. Hence the HPHT process has potential to produce the same or better high quality products than possible with HTST and UHT techniques.

Low acid food sterilization achieved by commercial thermal processing aims to destroy the most heat resistant microbial spores that cause public health concern or spoilage. The target has always been *Clostridium botulinum* in low acid foods. Because *C. botulinum* is a pathogen, a typical nonpathogenic surrogate such as *Clostridium sporogenes* strain PA3679 (ATCC7955) which has a similar or greater heat resistance than that of *Clostridium botulinum* has been traditionally used in verification of thermal processing specification. In the previous chapters, the HP inactivation kinetics of *C. sporogenes* 11437 in milk, and *C. sporogenes* 7955 spores in milk and salmon fish meat base were carried out to evaluate their pressure resistances at elevated temperatures. *C. sporogenes* 7955 spores was shown to have higher resistance than *C. sporogenes* 11437 in thermal treatments as well as HPHT treatments. *C. sporogenes* 7955 spores also demonstrated higher resistance in milk than in salmon meat base, in consistent with their thermal resistance and therefore gave a general correspondence between thermal and high pressure resistances. The literature references indicate one of the most thermal resistant spore former to be a thermophile, *G. stearothermophilus* ($D_{121°C}$ 5.5 min). For high pressure sterilization, it was initially assumed that these *heat resistant* spores would have

similar higher resistance in HPHT processing. However, some studies show that the thermal resistance was not necessarily correlated with resistance under high pressure (Hayakawa et al., 1994; Pataszca et al., 2005).

Several publications mentioned that high pressure did not affect *G. stearothermophilus* spore at room temperature and high pressure combined with elevated temperature had great efficacy on its inactivation (Seyderhelm et al., 1992; Miglioli et al., 1997; Gola et al., 1996; Rovere et al., 1998; Ananta et al., 2001; Kakugawa et al., 1996; Estrada-giron et al., 2007). A HP destruction kinetics of *G. stearothermophilus* spore in distilled water showed lower D values with z_P values (27.2-58.8 °C) and z_T values (216-352 MPa) (Patazca et al., 2006). Rajan et al. (2005) demonstrated that a process temperature of 105 °C/700 MPa for 5 min could accelerate the inactivation of *G. stearothermophilus* spores suspended in egg patties. Thus, the pressure resistance studies related to *G. stearothermophilus* have been limited and variable.

The objectives of this study were (1) to evaluate the HP destruction kinetics of *G. stearothermophilus* 10149 spores and compare with other pressure resistant spores at the same treatment conditions (2) to investigate the pressure and temperature resistance of this bacterial spore (3) to apply temperature calibration method to improve kinetic results accurately.

7.2 Materials and Methods

7.2.1 *Geobacillus stearothermophilus* culture preparation

Freeze-dried cultures of *G. stearothermophilus* (ATCC-10149) was obtained from the American Type Culture Collection (ATCC, Rockville, MD) and stored at –40°C. The culture was rehydrated in 10 ml TYG broth at 55 °C for 24 h in aerobic shaking water bath (SW22, Julabo, Labortechnik GMBH, Germany). Two such transfers were made in succession to obtain the culture with viable counts of 10^8 CFU ml^{-1} approximately and then stored at 4 °C (Kim and Naylor, 1966). This TYG medium formula is 10 g tryptone (BD, Co., Spark, MD), 5 g yeast extract (BD, Co., Spark, MD), 2 g K_2HPO_4 (Acros Organics, NJ, USA) in 1 liter distilled water (adjust pH 7.2 before autoclave). To prepare the inoculation culture, 1ml of the cultured broth was transferred into 50 ml of fresh TYG broth and incubated at 55 °C for 24 h.

7.2.2 Preparation of *G. stearothermophilus* spores

A 0.2 ml aliquote of *G. stearothermophilus* culture was transferred and spread on sporulation agar plate and incubated at 55 °C 7 days aerobic condition for growth and forming bacterial spores (Kim and Naylor, 1966). This sporulation medium formula was 8 g nutrient broth (BD, Co., Spark, MD), 4g yeast extract (BD, Co., Spark, MD), 0.01 g $MnCl_2.4H_2O$ (Acros Organics, NJ, USA) and 20 g agar (Fisher scientific, Fair Lawn, NJ) in 1 liter distilled water (Kim and Naylor, 1966). Spores were washed with 5ml cold sterile distilled water by using a sterile glass spreader. After harvest, the spores were washed three times by the centrifugation at 4000 x g and suspended in cold sterile distilled water to approximate 10^9 CFU/ml and stored at 4 °C until use. In this preparation, we did not use lysozyme treatment to kill vegetative cells; because microscope examination showed there were few vegetative cells left.

7.2.3 Sample inoculation and packing

The prepared spore suspension was added to milk (2% fat, 3.2% protein, 6% carbohydrate, Agrinove, Saint-Claire, Quebec, Canada) to a final concentration of about 10^7 CFU/ml and was distributed in 1ml sterile polypropylene bottles (Wheaton, Millville, NJ, USA) which were made full without air inside for high pressure kinetic study. For thermal treatment study, sterile glass pipettes (No. P1005, Chase Scientific Glass, Inc.) sealed both ends by gas burner flame were used as sample container, which had 3 ml milk sample each tube. All of sample tubes were kept at 4°C until use.

7.2.4 High pressure equipment

Same as detailed for *C. sporogenes* 11437 in Chapter 4.

7.2.5 Insulated chamber

Same as detailed for *C. sporogenes* 11437 in Chapter 4.

7.2.6 High pressure treatment

Same as detailed for *C. sporogenes* 11437 in Chapter 4.

7.2.7 Sample temperature control during HP treatments

Same as detailed for *C. sporogenes* 11437 in Chapter 4.

7.2.8 Thermal process treatment

An oil bath (SL26, Julabo, Labortechnik GMBH, Germany) was used to treat samples at 110, 115 and 120 °C. The heating medium was pure glycerin G33-20 (Fisher Scientific Corp., Whiteby, ON, Canada). A temperature reference milk tube (a K-type thermocouple tip at this tube center) was also placed in constant temperature oil bath with sample tubes simultaneously. Real temperature was recorded by the data logger. When the reference milk tube temperature reached the desired temperature, the timer started to measure the holding time. The come-up time was about 2 min. Two sample tubes were treated at each temperature and taken out by the time interval. After treatment, samples were immediately cooled and held in ice water bath until enumeration within 30min.

7.2.9 Enumeration of survival spores

After HP treatments, the sample tubes were aseptically opened and serial dilutions were made with 0.1% peptone water. Spores of *G. stearothermophilus* were enumerated in tryptic soy agar (TSA) (BD, Co., Spark, MD) plates by using a pour-plate technique. Pour plate method allowed us to do one more log cycle counting. These TSA plates were incubated at aerobic 55°C condition for 5 days before counting.

7.2.10 Kinetic data analysis

Same as detailed for *C. sporogenes* 11437 in Chapter 4.

7.2.11 Kinetic data temperature calibration

Same as detailed for *C. sporogenes* 11437 in Chapter 4.

7.3 Results and discussion

7.3.1 Thermal destruction kinetic of *G. stearothermophilus* spores

Figure 7.1 illustrates nominal survivors of *G. stearothermophilus* spores in heat treated milk and Table 7.1 shows the computed D values for thermal destruction kinetics of *G. stearothermophilus* 10149 spores in milk. Figure 7.1 illustrates that as usual higher temperatures resulted in a higher rate of microbial destruction and lower D values, but the survivor curves did not have very a very good log linear trend and the regression R^2 varied from 0.82 to 0.89. The computed D values were 49.4, 16.1, 6.3 min at 110, 115, 120 °C, respectively with a z value was 11.2 °C.

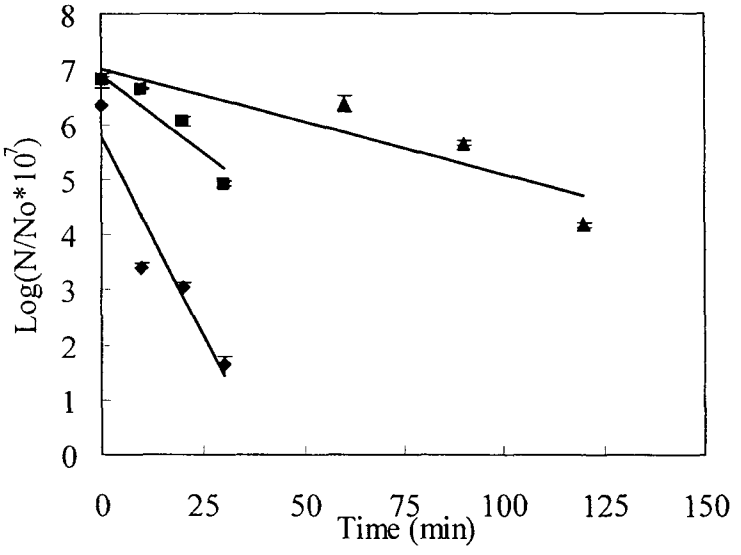


Figure 7.1 Nominal survivors of *G. stearothermophilus* spores in thermal treated milk (2 atm) at temperature (▲) 110, (■) 115, (♦) 120 °C

It has been reported that *G. stearothermophilus* spore had 4.0-5.0 min $D_{121.1°C}$ and z value 7.8-12.2 °C (Ramaswamy, 2006). Patazca et al. (2006) reported $D_{121.1°C}$ and z values of 5.5 min and 10.8 °C for *G. stearothermophilus* 10149 spores in water. Compared with these reference values, the results from this study were nearly the same

and confirmed that these spores had high thermal resistance and was suitable for HPHT kinetic study.

Table 7.1. Decimal reduction time (D values) of *G. stearothermophilus* 10149 spores in thermal treated milk

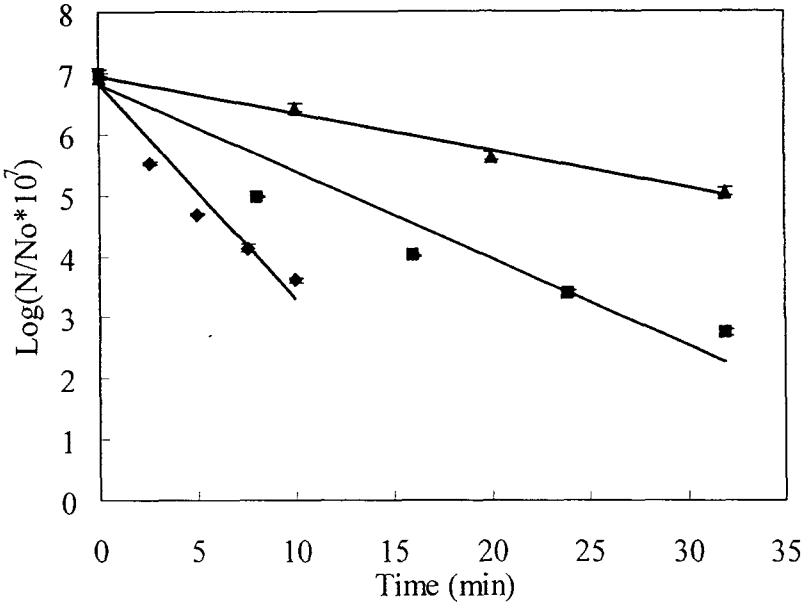
Pressure (MPa)	Temperature (°C)	D value		z value	
		D value (min)	R^2	z (°C)	R^2
0.1	110	49.4	0.82	11.2	0.99
	115	16.1	0.89		
	120	6.3	0.87		

7.3.2 High pressure destruction kinetics

Figures 7.2-7.4 (a captions) show *G. stearothermophilus* spores nominal survivor curves under various pressures at 70, 80, 90 °C during pressure holding. In "zero" min holding time experiments, the survivors were not observed to have significant reduction in counts ($p<0.05$) except 0.12±0.04, 0.16±0.05 for 700, 900 MPa at 90 °C. This indicated the pressure come-up time at temperatures below 90 °C did not have inactivation effect on this spore. Patazca et al. (2006) reported the inactivation happened at pressure over 600 MPa and temperature over 100 °C combinations. So unlike in previous chapters, there was no significant pulse effect on pressure inactivation of *G. stearothermophilus.*

During pressure holding period, the nominal survivors were well fitted with the first order model ($R^2 > 0.90$). Regression details are listed in Table 7.2. An increase in pressure resulted in a decrease in D value and acceleration in destruction of bacterial spores. For example, the uncorrected D values at 500, 700, 900 MPa were 16.6, 13.9, 8.8 min and 3.1, 1.3, 0.6 min for HP treatment at 70, 90 °C respectively (Table 7.2). After kinetic correction, the D values at 500, 700, 900 MPa changed to 19.1, 15.9, 13.4 min and 3.1, 1.3, 0.6 min at 70, 90 °C respectively (Figures 7.2- 7.4 b captions). The uncorrected and corrected D values at 90 °C were the same due to the accurate temperature control and short treatment times involved. The D values at 70, 80 °C treatments changed a little due to the small variation in temperature. The experiments at lower temperature required longer holding times. So D value correction at 70 °C was more than that at 80 °C.

(a)



(b)

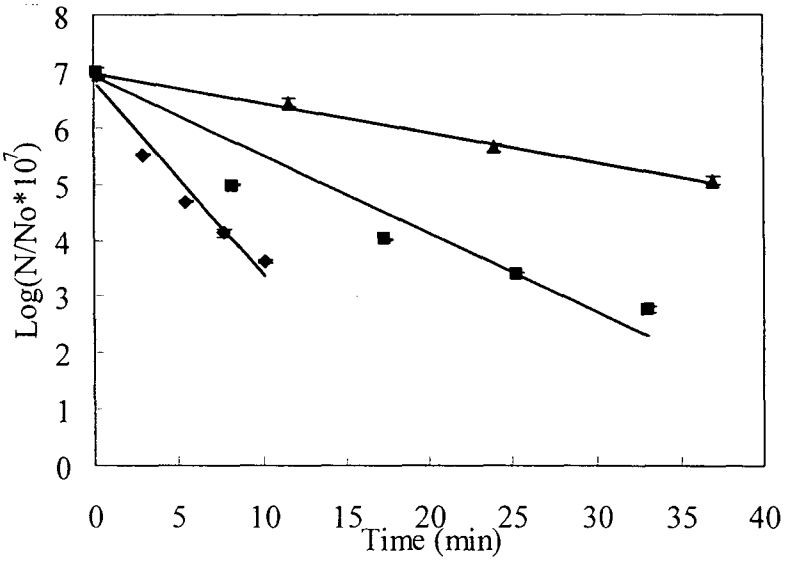
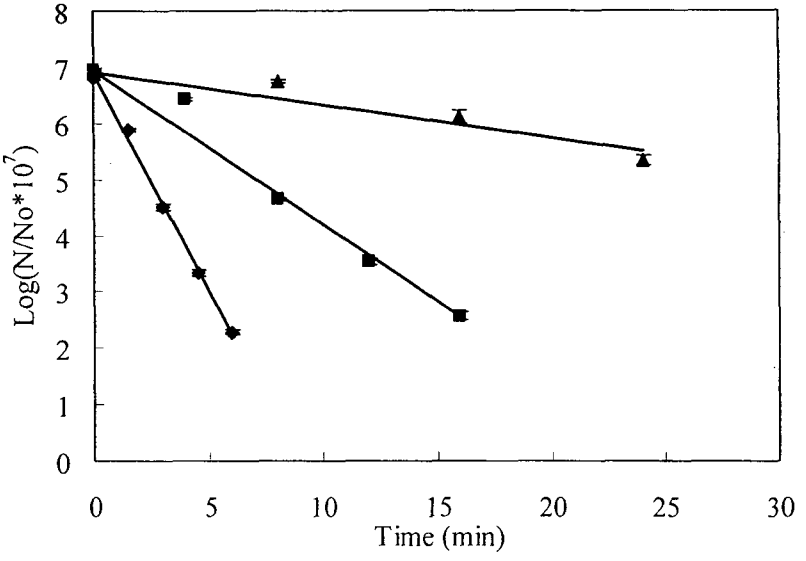


Figure 7.2 Mathematic uncorrected (a) and corrected (b) nominal survivors of *G. stearothermophilus* spores milk subjected to 500 MPa high pressure treatment at temperature (♦) 70, (■)80, (▲) 90 °C

(a)



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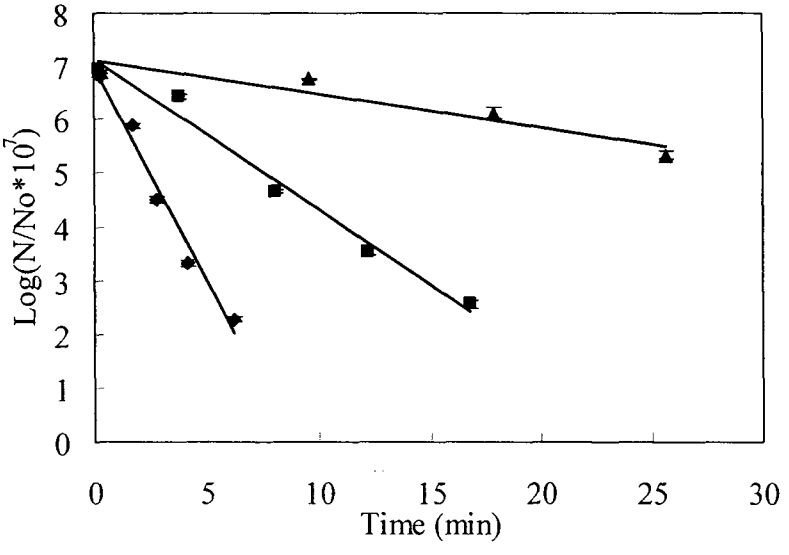
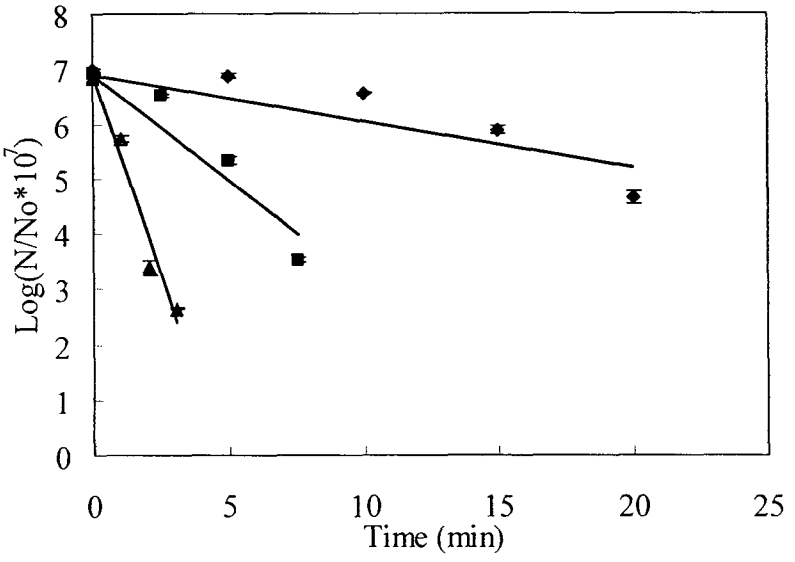


Figure 7.3 Mathematic uncorrected (a) and corrected (b) nominal survivors of *G. stearothermophilus* spores milk subjected to 700 MPa high pressure treatment at temperature (♦) 70, (■)80, (▲) 90 °C

(a)



(b)

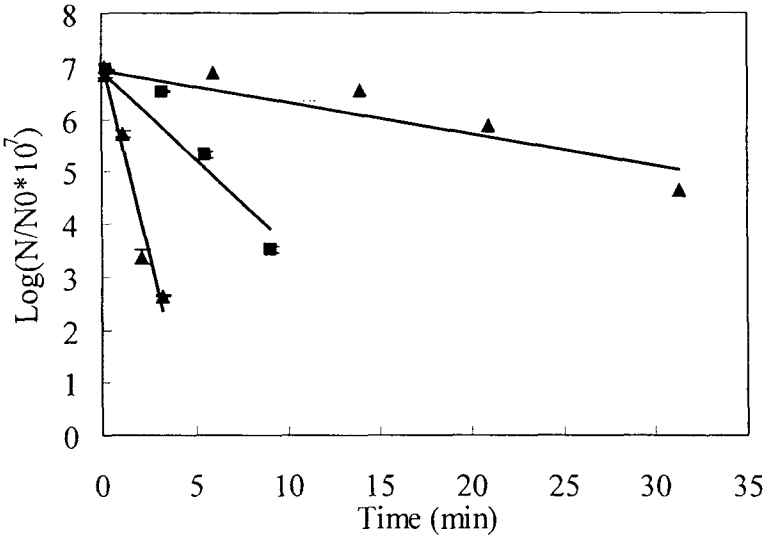


Figure 7.4 Mathematic uncorrected (a) and corrected (b) nominal survivors of *G. stearothermophilus* spores milk subjected to 900 MPa high pressure treatment at temperature (♦) 70, (■)80, (▲) 90 °C

Table 7.2. Decimal reduction time (D values) of *G. stearothermophilus* 10149 spores in HP treated milk

Pressure (MPa)	Temperature (°C)	Uncorrected		Corrected	
		D value (min)	R^2	D value (min)	R^2
500	70	16.6	0.97	19.1	0.98
	80	8.0	0.93	9.2	0.93
	90	3.1	0.95	3.1	0.96
700	70	13.9	0.94	15.9	0.91
	80	3.4	0.97	3.6	0.98
	90	1.3	0.99	1.3	0.98
900	70	8.7	0.99	13.4	0.95
	80	2.2	0.93	2.5	0.94
	90	0.6	0.96	0.6	0.95

Comparing HPHT treatments with thermal treatment, it can be easily seen that the high pressure results in significant enhancement of destruction rate. The D value even at 70 °C and 500 MPa pressure was about equal to that at 115 °C in the absence of added pressure (which is 45 °C higher in temperature). With a thermal z value of around 11 °C, this would mean at the same temperature (70 °C), the projected thermal D value would be 4 order higher in magnitude (because of 4z temperature between them). Likewise, at the higher pressure of 900 MPa, the D value at 90 °C was one order of magnitude lower than at 120 °C in the thermal mode, and again when adjusted for the same temperature these will differ by 3 orders of magnitude. Thus the pressure-temperature combination would be of significant advantage in this case.

Patazca et al. (2006) reported D values of *G. stearothermophilus* 10149 spores in distilled water were 1.8, 1.27, 0.86 min and 0.5, 0.29, 0.10 min for 500, 700 MPa at 92, 100, 111 °C respectively. Comparing with their extrapolated data at 70, 80, 90 °C for 500, 700 MPa treatments, the D values in this study were higher than for the spores suspended in water. The pressure-induced pH shift depends on pressure, partial molar volume change value and the number of charges of the anion (El'Yanov and Hamann, 1975). This was supported by Kitamura and Itoh (1987) who evaluated the reaction volume of protonic ionization of buffer agents on predicted models for pressure dependence of pH. The separation of positive and negative charge ions under pressure is driven by a water 'electrostriction' phenomenon: water molecules form condensed layers around the charged

species and have a more compact arrangement. This arrangement results in a change in the dissociation constant and leads to the dissociation of uncharged molecules forming additional H^+ and OH^-, due to dipole-dipole interactions and hydrogen bonding. Bodanszky et al. (1962) reported the reaction: $H_2O=H^++OH^-$ is accompanied by an apparent molar volume decrease of 21.3 ml at 25°C. Thus, the pH of water decreased by 0.2-0.5 pH unit per 100 MPa (Pandey, 2002). Pressure decreases pH more in water than in milk and buffer, and this could responsible for the increased destruction effect in water as compared with milk (protein in milk acting as a buffer).

Compared with our previous kinetic data on *C. sporogenes* 7955 spores in milk, *G. stearothermophilus* spores, the associated D values of *G. stearothermophilus* were lower than those of *C. sporogenes* 7955, which indicated high-pressure resistance of *stearothermophilus* spores were lower than *C. sporogenes* spores.

7.3.3 Pressure resistance and thermal resistance

The variation in D values of *G. stearothermophilus* spores as affected by temperature at a constant pressure was well described by the Z_P value (negative reciprocal slope of the log D vs T curve at constant pressure) (Figure 7.5). The computed Z_P values were 26.2, 18.8, 17.9 °C for 500, 700, 900 MPa, respectively, before temperature correction and 25.3, 18.1, 15.5 °C, respectively, after correction (Table 7.3). The thermal resistance therefore decreased by about 40% when the pressure increased from 500 to 900 MPa. Patazca et al. (2006) also reported that Z_P values of *G. stearothermophilus* spore in water decreased with pressure (58.8, 27.2, 27.4 °C at 500, 600, 700 MPa, respectively). The Z_P values in both water and milk had a decreasing trend with pressure indicating *stearothermophilus* spores to be more sensitive to temperature at higher pressures. On the contrary, *C. sporogenes* spore showed reverse trend with Z_P increasing with pressure.

Table 7.3 Constant pressure Zp value (°C) of *G. stearothermophilus* 10149 spores in HP treated milk

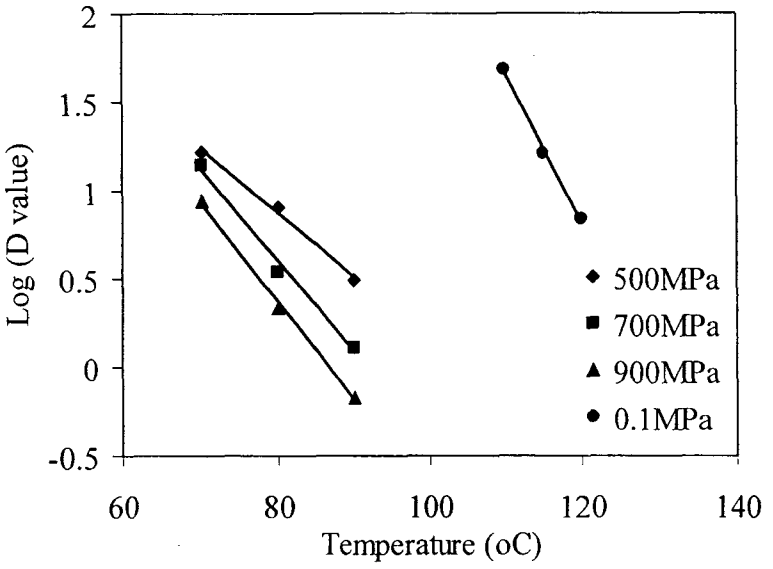
Pressure(MPa)	Uncorrected		Corrected	
	Z_p value	R^2	Z_p value	R^2
500	26.2	0.99	25.3	0.99
700	18.8	0.98	18.1	0.99
900	17.9	0.99	15.5	0.99

Similarly, the pressure resistance of the D values were compared at different constant temperatures to obtain the Z_T values negative reciprocal slopes of log D vs P values (Figure 7.6). Uncorrected and corrected Z_T values were 1450, 710, 600 MPa and 2560, 770, 610 MPa, respectively at 70, 80, 90 °C (Table 7.4). In this case, Z_T values decreased by 75% with as the process temperature increased from 70 to 90 °C. Again, *C. sporogenes* spore showed a reverse trend with Z_T increasing with pressure. This meant that at higher temperature, *stearothermophilus* spores were sensitive to the pressure destruction which was the opposite with respect to *C. sporogenes*. Compared with Z_T value in water 352, 313, 216MP (92, 100, 111 °C) (Patazca et al., 2006), Z_T values of *stearothermophilus* spores in milk were higher, which confirms that the spore kinetics are better carried out in the real media than in water or buffer.

Table 7.4 Constant temperature Z_T value (MPa) of *G. stearothermophilus* 10149 spores in HP treated milk

Temperature(°C)	Uncorrected		Corrected	
	Z_T value	R^2	Z_T value	R^2
70	1450	0.94	2560	0.99
80	710	0.97	770	0.95
90	600	0.99	610	0.99

(a)



(b)

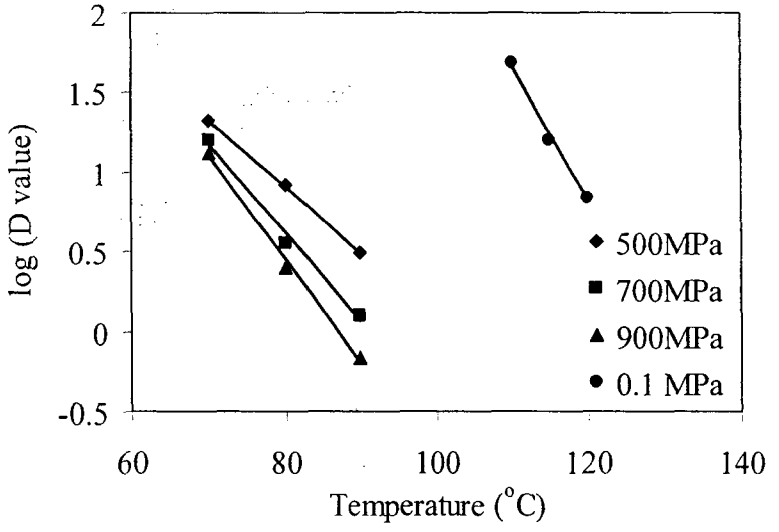
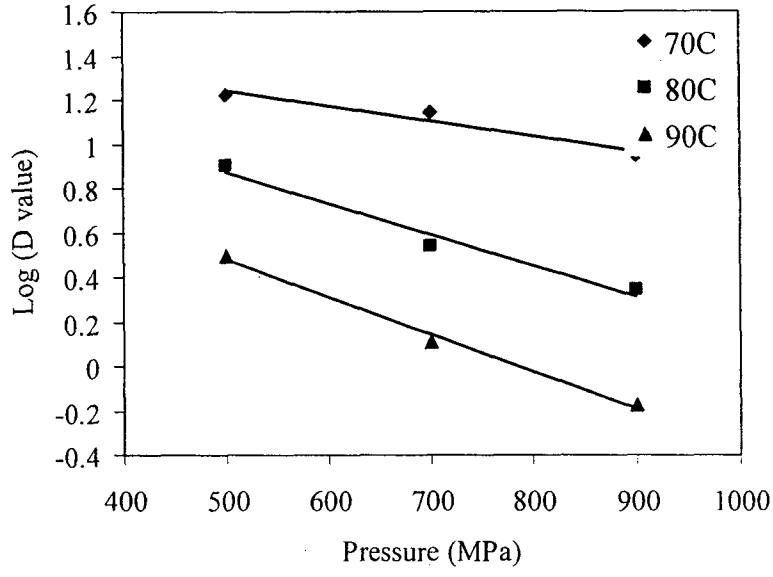


Figure 7.5 Uncorrected (a) and corrected (b) logarithmic D value of *G. stearothemophilus* 10149 spores in HP treated milk ((♦) 500, (■)700, (▲)900 MPa) and thermal treated milk (0.1 MPa) vs. temperature

(a)



(b)

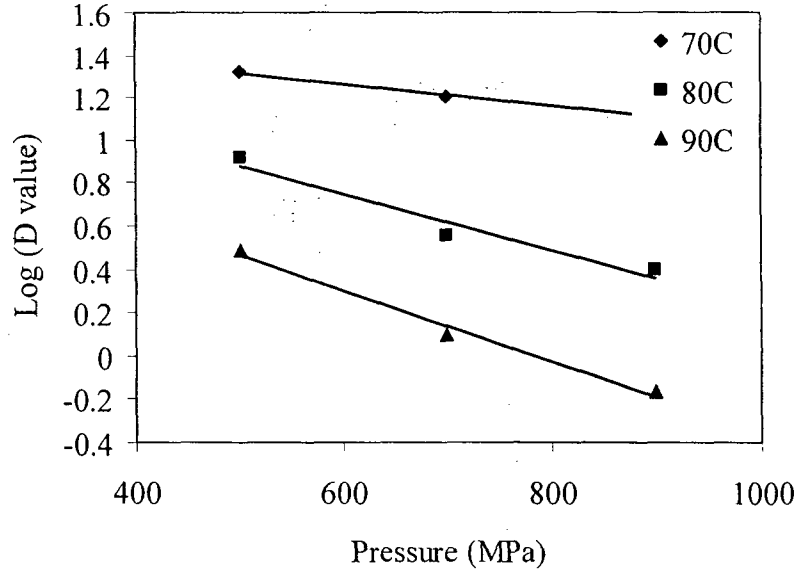


Figure 7.6 Uncorrected (a) and corrected (b) logarithmic D value of *G. stearothermophilus* 10149 spores in HP treated milk vs. pressure at temperature (♦) 70, (■) 80, (▲) 90 °C

7.3.4 Predicted D values at higher temperature

Using the Z_P and Z_T, D values were again computed for an extrapolated temperature of 121°C to compare the thermal and pressure D values at commercial processing temperatures. Figure 7.7 also illustrates the D value curves against temperature and Table 7.5 shows the computed D values at specific temperatures (105, 110, 115, 120 and 121 °C). It can be found that extrapolated HP D values of *G. stearothermophilus* 10149 spores progressively got smaller as the temperature increased all the way up to 121 °C. The reversal trend is not apparent, but that could happen at much higher temperatures (because of the lower thermal z value relative to pressure z values).

Table 7.5 Predicted decimal reduction time (D values) of *G. stearothermophilus* 10149 spore in HP treated milk and *C. sporogenes* spores 7955 in thermal treated milk

Pressure (MPa)	Extrapolated D value (min) at temperature (°C)						
	95	100	105	110	115	120	121
500	1.9	1.2	0.74	0.46	0.28	0.18	0.16
700	0.62	0.33	0.17	0.092	0.049	0.026	0.023
900	0.31	0.15	0.069	0.033	0.016	0.0078	0.0065
0.1	845.6	320.7	121.6	49.4*	16.1*	6.3*	5.5

* real experimental data

Finally, compared with D values of *C. sporogenes* 7955, those of *stearothermophilus* were much lower. It meant that *G. stearothermophilus* is not a good candidate for testing the validity of HPHT processing even though its conventional thermal resistance was much higher. However, *G. stearothermophilus* 10149 could be better candidate for milder HP processes carried out moderate pressures and temperatures of extended shelf-life products.

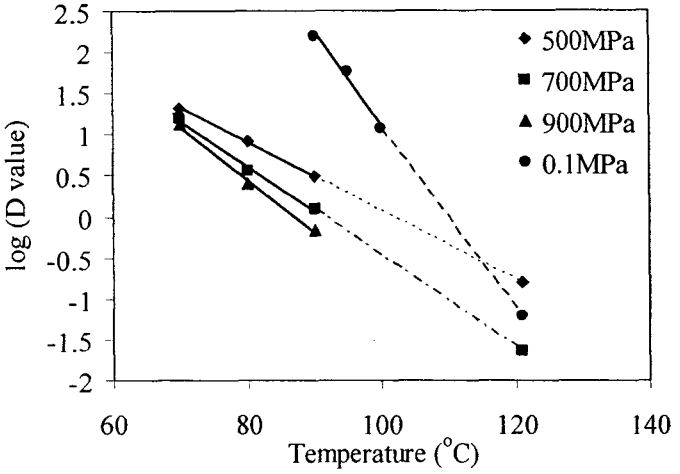


Figure 7.7 Predicted logarithmic D value of *G. stearothemophilus* 10149 spores in HP treated milk ((♦) 500, (■)700, (▲)900 MPa) and *C. sporogenes* 7955 spores(*) in thermal treated milk(0.1 MPa) vs. temperature

7.4 Conclusions

The study showed that, within the range of experimental conditions, again confirmed the obvious: higher pressures and high temperatures always resulted in a faster microbial reduction (smaller D value). *Geobacillus stearothermphilus* pressure resistance did not correlate with its heat resistance, hence thermal resistance and pressure resistance may not go hand in hand for all bacterial spores. Z_P and Z_T values indicated that these spores were relatively more sensitive to pressure and temperature, both acting synergistically contributing to greater rate of destruction at higher temperatures and pressures. *C. sporogenes* 7955(PA3679) spores were more resistant than *G. stearothermphilus* 10149 spores in milk under HPHT treatment conditions. *G. stearothermophilus* 10149 spores are not good candidates as surrogates of *C. botulinum* as conventionally used in thermal processing. So far within the scope of this study, *C. sporogenes* 7955(PA3679) spores are more resistant to HP destruction among those studies. Because of the higher resistance of Clostridial spores to pressure destruction, the evaluation of HP destruction kinetics of *C. botulinum* becomes a necessity even though it is more difficult work with and requires more elaborate experimental set up, safety procedures and special labs for carrying out enumeration. This is the focus of the next phase of the thesis work.

Preface to Chapter 8

In the previous chapters 4-7, the high pressure inactivation kinetics of *Clostridium sporogenes* 11437 and 7955, and *Geobacillus stearothermophilus* 10149 spores in milk and *Clostridium sporogenes* 7955 spores in salmon meat base were investigated as possible non pathogenic surrogates to be used for establishing/verification of high pressure processing of low acid foods at elevated temperatures. The studies were useful in establishing a HP destruction kinetic database for bacterial spores as influenced by pressure, temperature, media and spore types, and revealed many important points as discussed in detail in the previous chapters. Notably, *C. sporogenes* 7955 was the most resistant spore among those studied. Food based media permits some baro-protection and hence yield more meaningful kinetic data than studies in water or buffer. Spores with higher thermal resistance do not necessarily have higher pressure resistance, hence *Clostridium sporogenes* 7955 was found more pressure resistant than *G. stearothermophilus* 10149. It appears the Clostridial spores have greater resistance than Bacillus spores. Since the target for establishing HP processes for low acid foods is still believed to be based on *Clostridium botulinum,* it is imperative that accurate data on the destruction kinetics of these spores are necessary. There have been some sporadic information in literature on destruction kinetics of *Clostridium botulinum.* Most of these have come from different labs, using different procedures, different equipment and different conditions. More data in the area is certainly needed for regulatory approval of the process. These issues have prompted the studies detailed in this and the next chapter. The first step in carrying out such work was to find a proper lab capable of handling *Clostridium botulinum* spores, their collection, preparation, treatment and subsequent enumeration. Contact was established with Health Canada and the work was carried out under the supervision of Dr. John Austin, and his colleagues. All spores were prepared, procured, treated and enumerated by the candidate under the supervision of HC professionals. The first step was to screen a range of different strains of *Clostridium botulinum* available from HC for their pressure resistance (Chapter 8) and then carryout detailed destruction kinetics study with the more resistant strain (Chapter 9).

In the screening study, the spores suspended in a phosphate buffer were pressure treated with two holding times at two pressures (800 and 900 MPa) and two temperatures (90 and 100 °C). Three strains were picked as the most promising among them from pressure resistance point of view (Chapter 8) and studied further for detailed destruction kinetics (Chapter 9).

Part of this study will be submitted for publication(s). This research work was completed by the candidate under the supervision of Dr. H. S. Ramaswamy and Dr. John Austin. Microbiological support was given for spore preparation and enumeration by Mr. Jeff Bussey (Health Canada, Ottawa) during experiments.

Chapter 8

Pressure resistance screening of Group I *Clostridium botulinum* spores at high temperature high pressure processing conditions

Abstract

Twelve strains of *Clostridium botulinum* Group I spores suspended in phosphate buffer (0.1M) at approximately 10^7 CFU/ml concentration were subjected to high pressure treatments at different pressures (800 and 900 MPa), temperatures (90 and 100 °C) and times (0.5 to 15 min). The treatments were chosen to have a wide range of pressure severity to be able to discriminate the spore strains for their pressure resistance. An insulated test chamber was used to achieve temperature stability during treatment. Preliminary studies showed the need for an 8 day anaerobic incubation for enumeration. Strains PA9608B, HO9504A and CK2-A had a higher pressure resistance among 12 strains while Strain 62A was completely inactivated by these combinations. The D values of the more resistant were in the 1.8-0.66 min range at 900 MPa and 100 °C treatment. The temperature sensitivity parameter (Z_P value) at 900 MPa ranged from 10 to 16 °C, and pressure sensitivity (Z_T value) at different temperatures ranged from 340-760 MPa. The strain PA9805B produced the most resistant spores that had higher estimated Z_P value (16.0 °C) and Z_T value (470 MPa).

8.1 Introduction

The importance of high pressure processing as novel and innovative food preservation method has been highlighted in the previous chapters. High pressure high temperature (HPHT) processing is getting recognized as alternative technique to high temperature sterilization because it is able to inactivate highly resistant bacterial spores in low acid (pH 4.5) foods. Adiabatic compression results in quick heating, and has the potential to raise initial product temperatures from 90 °C to sterilizing levels that are generally possible under HTST and UHT techniques. A brief holding at this peak pressure will be sufficient to achieve the desired sterility in the product. A quick pressure

release will reverse the heating effect resulting in almost instantaneous cooling to around 90 °C. Thus the pressure processing can be used to simulate HTST operation in bulk foods which are impossible under conventional heating conditions. Hence the HPHT process has potential to produce the same or better high quality products than possible with HTST and UHT techniques.

In the previous chapters 4-7, the high pressure inactivation kinetics of *Clostridium sporogenes* 11437 and 7955, and *Geobacillus stearothermophilus* 10149 spores in milk and *Clostridium sporogenes* 7955 spores in salmon meat base were investigated as possible non pathogenic surrogates to be used for establishing/verification of high pressure processing of low acid foods at elevated temperatures. The studies were useful in establishing a HP destruction kinetic database for bacterial spores as influenced by pressure, temperature, media and spore types, and revealed many important points as discussed in detail in the previous chapters. Notably, *C. sporogenes* 7955 was the most resistant spore among those studied. Food based media permits some baro-protection and hence yield more meaningful kinetic data than studies in water or buffer. Spores with higher thermal resistance don't necessarily have higher pressure resistance, hence *Clostridium sporogenes* 7955 was found more pressure resistant than *G. stearothermophilus* 10149. It appears the Clostridial spores have greater resistance than Bacillus spores. Since the target for establishing HP processes for low acid foods is still believed to be based on *Clostridium botulinum,* it is imperative that accurate data on the destruction kinetics of these spores be generated. There have been some sporadic information in literature on destruction kinetics of *Clostridium botulinum.* Most of these have come from different labs, using different procedures, different equipment and different conditions. More data in the area is certainly needed for regulatory approval of the process.

Clostridium botulinum has a ubiquitous occurrence in soil or in sediments of lakes and forms seven types of neurotoxins differing in their serological specificities (Peck 2005). Strains of *C. botulinum* are anaerobic and classified into four groups according to physiological differences and the type of toxin formed and most cases of the foodborne botulism are related to Group I and Group II (Austin et al., 2000, 2001). *C. botulinum* group I strains (proteolytic strains forming heat-resistant spores) are of concern in the

safe production in canning industry and Group II (nonproteolytic, psychrotrophic strains forming lower heat resistance spores and forming neurotoxin at 3 °C) are of concern in chilled food safety (Austin et al., 2000, 2001). *C. botulinum* Group I strains form neurotoxins type A, B, F and Group II form neurotoxins type B, E, F (Peck, 2005). In low-acid food (pH > 4.5) spores of *C. botulinum* can germinate and produce neurotoxin. To ensure the safety of low-acid canned food, it has been a common practice to set thermal processes to deliver a heat treatment aimed at reducing the spore concentration of *C. botulinum* by 12-decimal reductions (Stumbo, 1973).

So far, studies on the effect of pressure on bacterial spores were carried out mostly on spores of *Bacillus* spp., such as *Bacillus subtilis* and *G. stearothermophilus.* However, few reports on the pressure resistance of *Clostridium botulinum* spores are available (Reddy et al., 2006; Margosh et al., 2004a). Reddy et al. (1999, 2000, 2003, 2006) have perhaps carried out the most detailed investigation on the effects of high pressure (up to 827 MPa) combined with elevated process temperatures (35-75 °C) on spores of *C. botulinum* type A (2 proteolytic strains), B (4 nonproteolytic strains) and E (2 nonproteolytic strains) in phosphate buffer and crabmeat. Two type A strain (BS-A and 62-A) spores were found to be more resistant than type B and E spores. Type A spores can not be completely inactivated at a high pressure and a moderate temperature (827 MPa/75°C/20min) treatment. Only partial destruction (3 log units) of this type spores was achieved at this combination. In the mean time, *C. botulinum* Type B and E spores can be completely inactivated by this combination. In this report, the process temperature had significant variation during the treatment due to the equipment limitation (Ahn et al., 2007). Margosh et al. (2004a, 2006) evaluated the resistance of 7 *C. botulinum* strains (2 proteolytic type A, 2 proteolytic type B, 2 nonproteolytic type B and 1 nonproteolytic type E) in mashed carrots and Tris-His buffer at the combinations of pressures (0.1-1400 MPa) and temperatures (60-120 °C). Proteolytic type B TMW 2.357 exhibited a greater resistance to pressure than other *C. botulinum* spores. Since less numbers of proteolytic *C. botulinum* strains had been investigated. Thus, *C. botulinum* (group I) strains, which are able to form heat resistant spores, need to be screened and identified under high pressure at isothermal or quasi-isothermal condition.

The objectives of this study were 1) to standardize the experimental procedure for enumerating the *C. botulinum* spores subjected to high pressure treatment 2) to screen the different strains of *C. botulinum* spores (group I) to high pressure resistance 3) to compare the effect of media (milk and buffer) on the pressure resistance of these spores.

8.2 Materials and Methods

8.2.1 Preparation of *C. botulinum* (group I) cultures and spores

Twelve strains of *Clostridium botulinum* group I (Table 8.1) were obtained from the Botulism Reference Service of Health Canada. The spore suspensions of each strain were prepared separately according to the method described by the Bureau of Microbial Hazards, Food Directorate, Health Products and Food Branch (HPB, Health Canada, Ottawa, Ontario). Cultures were grown in Trypicase Peptone Glucose Yeast extract (Difco, Detroit, MI, USA) broth (TPGYB) at 35 °C for 3 days in an atmosphere of 10%H_2, 90%N_2 in an anaerobic chamber (Coy Laboratory products, Inc., Ann Arbor, MI, USA). Cultures (100µl) of each strain were spread-plated on McClung Toabe agar (Daifas et al., 2000) plates at 35 °C for 5 days in the same anaerobic condition. Spores were harvested in 5ml cold sterile distilled water, centrifuged at 10000X g for 10 minutes at 4 °C and washed 8 times with 20 ml of sterile water until no neurotoxin was detected. The spores suspensions were diluted to 10^9 CFU/ml, heat shocked at 75 °C for 20 min and frozen stored at -40 °C until use.

8.2.2 Sample preparation

The prepared spore suspensions were diluted in cold sterile phosphate buffer (0.1 M, pH 7.0) to a final concentration of 10^7 CFU/ml for resistance screening tests. 1.7ml of those suspensions were transferred to sterile polypropylene bottles (Wheaton, Millville, NJ, USA) and filled full without air inside. Each tube was placed in 60mm length ½"width 0.1mm thickness plastic heat shrink tube (Electronic Use, Mode Electronics, Burnaby, B.C. Canada) and sealed by gas burner flame in short time (within 1 s) in order to prevent sample leaking and contamination.

For comparison of resistance in milk and buffer, PA9508B, HO9504A and CK2-A spores were also diluted in milk (commercially sterile UHT, 2% fat, 3.2% protein, 6% carbohydrate, Agrinove, Saint-Claire, Quebec) to a final concentration at 10^7 CFU/ml. Then, sample milk was filled in sterile polypropylene bottles and plastic shrink tubes and sealed by the same procedure above. All of sealed tubes were stored in ice water until processing. The thermal treated samples (80 °C for 15 min) were used as control.

Table 8.1 *C. botulinum* Group I culture collections used in this study

Strain ID	Type	Origin	Location	Year of isolation	Source
62A	A	Virgin soil	USA	1922	Riemann
IB1-B	B	Feces	Peterborough, Canada	1979	BRS
CK2-A	A	Feces	Canada	1974	BRS
MRB	B	Mushrooms	Montreal, Canada	1973	BRS
Langeland					
A6	A	NR	NR	NR	NR
GA0108BEC	B				
PA9508B	B	Pate compagne	Sherbrooke, Canada	1995	BRS
13983B	B				
H461297F	F	Honey	Wisconsin	1998	BRS
GA0101AJO	A	Gastric liquid	Desbiens, Canada	2001	BRS
HO9504A	A				

NR, not recorded
BRS, Botulinum Reference Service for Canada, Health Canada, Ottawa, ON

8.2.3 Insulated Chamber

Same as detailed for *C. sporogenes* 7955 in Chapter 6.

8.2.4 High pressure treatment

Same as detailed for *C. sporogenes* 7955 in Chapter 6.

8.2.5 Enumeration of survivors

Total viable spores of each sample were directly determined by spread plating method. Each sample tube was aseptically opened, and contents were serially diluted in sterile water. Duplicate plates were used for each dilution. The dilutions were spread-plated using McClung Toabe agar with 5% sterile egg yolk plates. These plates were incubated at anaerobic 37°C condition for 8 days before counting.

For optimizing incubation time, a study was carried out before resistance screening tests. PA9508B and HO9504A spore samples were pressure treated (900 MPa/100 °C/3min) and then plated. Plates were incubated at 37 °C anaerobic condition 10 days until no new colonies grew. The results demonstrated 8 days incubation was necessary (Figure 8.2).

8.2.6 Sample temperature control during HP treatments

Same as detailed for *C. sporogenes* 7955 in Chapter 6. The sample temperatures were measured in a dummy tubes without the spores, but located and treated along with the test tubes.

8.2.7 Determination of kinetic inactivation parameters

Same as detailed for *C. sporogenes* 7955 in Chapter 6.

8.2.8 Data analysis

Microsoft Excel 2000 was used to perform mathematical and statistical analysis. Linear regression model was used to evaluate the treatment and strain. Significant mean differences were calculated by ANOVA model at $p<0.05$.

8.3 Results and discussion

8.3.1 Sample temperature control during HP treatments

Typical sample temperature profile during a test run (800 MPa and 90 °C treated for 15 min) is shown in Figure 8.1. Due to the insulation, sample temperature was not

severely affected by the temperature drop in the pressure medium surrounding the insulated chamber which dropped rather quickly. The mean temperatures were generally within a degree of the set point. The mean and standard deviation in sample chambers for the different tests are summarized in Table 2. The longer holding time (12-15 min in this treatment) resulted in a relatively larger temperature drop. For this treatment, we particularly elevated initial temperature 1 °C more to compensate temperature drop; however, this procedure will only shift the mean rather than prevent the temperature drop. In previous Chapters, this was stabilized by increasing the chamber temperature. But for the screening experiments, these were not. Overall, the treatment temperatures were within 1°C except the longer holding time treatments (900 MPa /12min, 800 MPa /15 min) (Table 8.2).

8.3.2 Effect of incubation time on survival count

Two strains of *C. botulinum* spores (PA9508B and HO9504A) were high pressure treated and for enumeration of survivors were plated and incubated at 37 °C under anaerobic condition for 10 days (Figure 8.2). Colonies were counted and recorded everyday. The visible colony numbers increased during incubation but reached a stable level by 8 days of incubation. Compared to conventional 2-3 days of incubation for normal bacterial spores would be misleading in this case as the number of colonies continue to increase with incubation time. Germination and growth of pressure treated spores just began on the 2nd day. On the fourth day, pressure treated spores were just counted a half number of total counts. After 8 days incubation, there was not new colony formed and seen by eye vision (tests were continued beyond two weeks and the colonies grew in size, but no new colonies appeared). This could mean that the high pressure treatment changed spores' viability character, their germination and growth rate or simply the spores needed time to recover from the HP shock. An 8 day incubation was used for all tests carried out in this *C. botulinum* study.

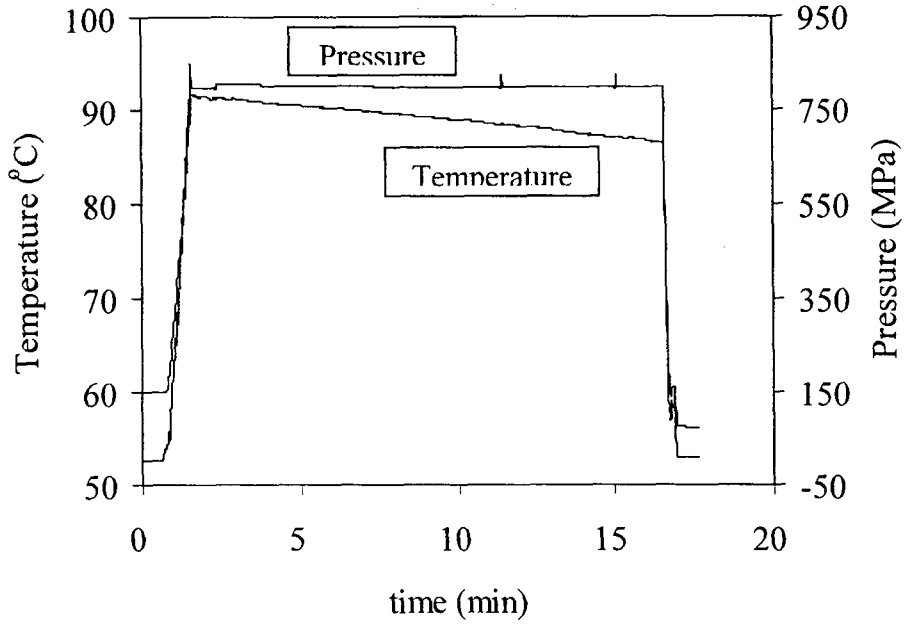


Figure 8.1 Pressure and sample temperature change during a typical test run (800 MPa, 90°C, 15 min)

Table 8.2 Pressure levels, initial temperature, process temperatures (during the holding) and holding times used in this study

	Pressure (MPa)	Initial temperature (°C)	Process temperature (°C)	Time (min)
Phosphate buffer	800	58.3	89.77±0.39	5
	800	58.3	89.14±1.45	15
	900	55.8	89.41±0.33	4
	900	55.8	89.98±0.93	12
	900	64.2	100.49±0.25	0.5
	900	64.2	99.81±0.26	3
Milk	700	70.6	99.68±0.58	8
	800	67.0	99.80±0.13	6
	900	57.5	90.99±0.82	12
	900	65.4	101.18±0.13	3
	900	72.7	110.27±0.12	0.8

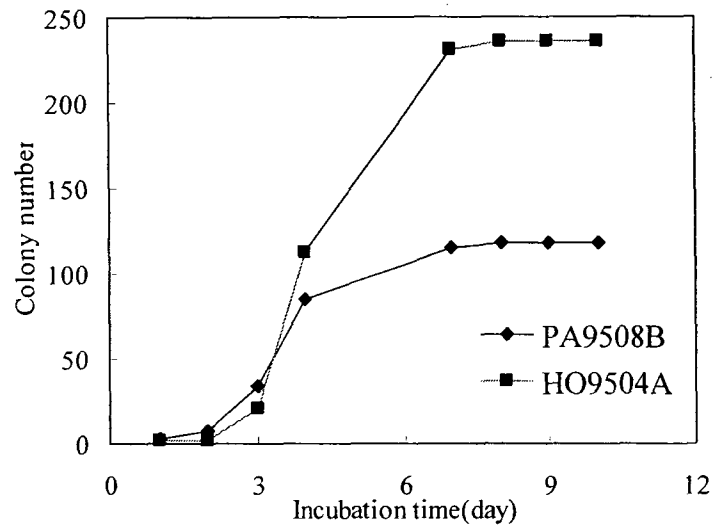


Figure 8.2 *C. botulinum* colony counts during anaerobic incubation following pressure treatment.

8.3.3 High pressure resistance screening of *C. botulinum* (group I) spores

Table 8.2 shows the experimental conditions used in this study. Desired process temperatures of 90 and 100°C were obtained by adjusting the initial temperature and controlled using the insulated setup. The total time of preheating and assembling the insulator was less than 15min and spores counts were not affected by time delay. Table 8.3 shows nominal survivor counts of the different strains of *C. botulinum* spores under the different treatment conditions. All strains showed a decreased in the number of survivors relative to the control (10^7 CFU/ml) and hence were subject to different levels of HP destruction. Higher survival counts (Table 8.3) indicate a higher resistance of spores for pressure destruction.

With each and every HP treatment given, the *C. botulinum* strain 62A was totally destroyed with no viable spore count on the plates. This strain therefore appears to be the most sensitive among those studied for pressure destruction. Next to this are strains Langeland, IB1-B and 13983B (in that order) which were inactivated by one or more of

the treatments, but not all the treatments. The remaining strains were partially inactivated to different extents at each of the treatments, but never completely by any one of them.

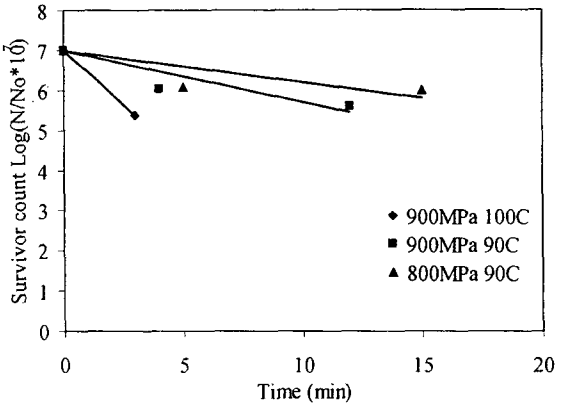
Table 8.3 Nominal survivor counts down from 10^7 CFU/ml (means ±SD, n=2) of *C. botulinum* (group I) spore strains after HP treatments

	Nomial survivor count log(N/No*10^7)(standard deviation, n=2)						
Strain	Control	900 MPa 100°C 3min	900 MPa 100°C 0.5min	900 MPa 90°C 12min	900 MPa 90°C 4min	800 MPa 90°C 15min	800 MPa 90°C 5min
62A	7	N/A	N/A	N/A	N/A	N/A	N/A
IB1-B	7	N/A	3.65±0.04	N/A	3.33±0.01	1.84±0.09	4.41±0.02
CK2-A	7	2.52±0.11	5.98±0.06	5.10±0.03	6.02±0.04	5.41±0.01	5.87±0.02
MRB	7	N/A	4.83±0.01	2.22±0.11	3.59±0.01	2.35±0.13	3.58±0.01
Langeland	7	N/A	3.66±0.02	N/A	2.57±0.05	N/A	2.75±0.07
A6	7	2.54±0.12	6.05±0.01	5.08±0.08	5.93±0.01	5.29±0.03	5.80±0.01
GA0108BEC	7	2.52±0.09	6.07±0.07	4.92±0.07	5.83±0.04	5.15±0.01	5.76±0.01
PA9508B	7	5.37±0.01	7±0.01	5.60±0.04	6.03±0.02	5.99±0.01	6.08±0.01
13983B	7	N/A	5.34±0.08	3.55±0.01	5.64±0.02	3.49±0.02	5.54±0.04
H461297F	7	1.00±0.01	5.49±0.03	3.12±0.02	5.12±0.04	3.67±0.10	5.24±0.01
GA0101AJO	7	2.00±0.01	5.94±0.21	3.63±0.09	5.51±0.04	4.89±0.10	5.45±0.01
HO9504A	7	3.67±0.08	6.04±0.05	5.74±0.01	6.07±0.01	5.91±0.03	5.99±0.02

N/A no viable spores were detected

The observed destruction trends were similar to those commonly observed during the previous studies described in earlier Chapters. An increase in process temperature at a specific treatment pressure, or an increase in treatment pressure at a specific process temperature, resulted in an increase in the rate of destruction during the holding time. Strains PA9608B, HO9504A and CK2-A demonstrated a higher survival count among the 12 strains. Figure 8.4 show the survivor curves for strains PA9608B, HO9504A and CK2-A vs pressure holding time. The nominal survivors demonstrated a good linear trend (1st order inactivation kinetics) at 900 MPa and 100 °C combination treatments because the pressure and temperature conditions were better maintained during these test runs due to the relatively short holding times. However, for the treatments which had longer holding times, the data had a poorer fit (low R^2) because of the larger temperature drop (longer holding time).

(a)



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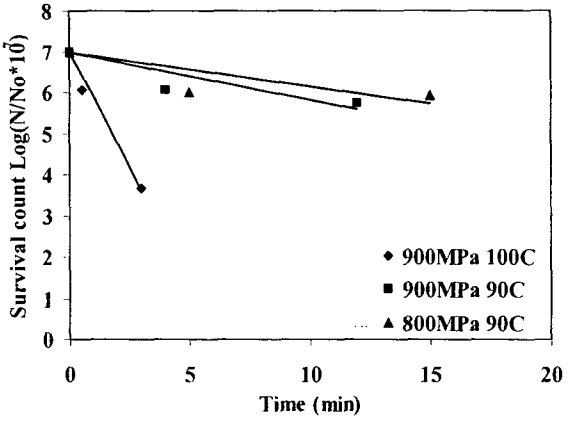

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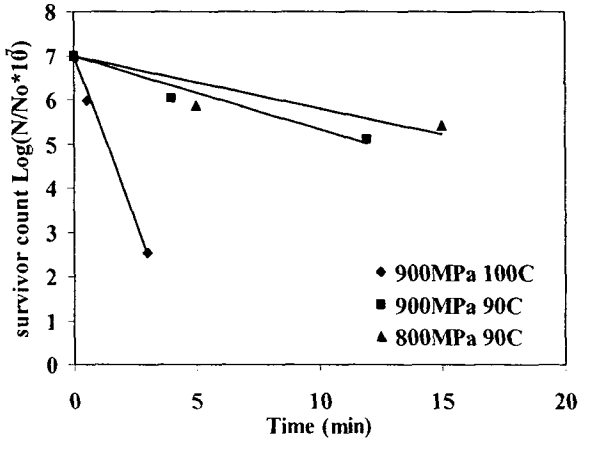


Figure 8.4 Nominal survivors of *C. botulinum* spore strains a) PA 9508B, b), HO9504A and c) CK2-A at 900 MPa+100 °C (♦), 900 MPa+90 °C (■) and 800 MPa+ 90 °C (▲) treated in 0.1M phosphate buffer(n=4 or 6, p-value<0.05)

Table 8.4 summarizes the estimated high pressure decimal reduction times (D values) of *C. botulinum* (group I) spore strains in 0.1M phosphate buffer. The D values for 62A are only estimates because the strain population showed no survivor even at the shortest treatment time of 0.5 min at 900 MPa-100 °C or 4 min at 100 MPa-90 °C or 5min at 800 MPa-90 °C. Assuming a minimum 7 log reductions in these treatments (since there were no counts), the D values were estimated to be lower than 0.5/7, 4/7 and 5/7 min, respectively (i.e., <0.07, <0.57 and <0.7min, respectively). Similar estimates are given for Langeland, IB1-B and 13983B strains at selected condition when the treatments were sufficient to completely kill the spores. For the rest the D values were ranged from 0.5-1.8, 2.3-8.5 and 2.7-11.7 min at these the processing conditions, 900 MPa-100 °C, 100 MPa-90 °C and 800 MPa-90 °C, respectively. These D values indicated that the pressure resistance of the different strains of *C. botlinum* spores varied significantly (P<0.05) and depended on their isolation source and indigenous natural characteristics. In this screening study, experimental data points were limited, often with only two or maximum three points; hence they should only be treated as estimates for the purpose of determining trends in the relative pressure resistance. Kinetic data quantification requires a more detailed study.

Table 8.4 Estimated high pressure decimal reduction times (D values) of *C. botulinum* (group I) spore strains in 0.1M phosphate buffer (pH 7.0)

Strain	900 MPa 100°C	R^2	900 MPa 90°C	R^2	800 MPa 90°C	R^2
62A	<0.07		<0.57		<0.7	
IB1-B	<0.15		<1.1		2.7	0.86
CK2-A	0.66	0.99	6.0	0.94	8.5	0.76
MRB	<0.1	0.93	2.3	0.75	2.9	0.73
Langeland	<0.15		<0,56		<1.9	
A6	0.67	0.99	5.9	0.91	7.9	0.76
GA0108BEC	0.67	0.99	5.4	0.81	7.4	0.81
PA9508B	1.8	0.99	7.7	0.72	12.7	0.51
13983B	<0.1		3.4	0.99	4.2	0.99
H461297F	0.49	0.99	3.0	0.96	4.3	0.93
GA0101AJO	0.60	0.99	3.5	0.98	6.4	0.73
HO9504A	0.88	0.97	8.5	0.72	11.7	0.49

N=4 or 6, p-value<0.05 for all items
N/A no data

The estimated D values of PA9508B, HO9504A and CK2-A were 1.8 and 12.7 min, 0.88 and 11.7 min, and 0.66 and 8.5 min at 900 MPa/100°C and 800 MPa/90°C treatment conditions, respectively (Table 8.4). These values were higher than those of others, and PA9508B was the most resistant strain followed by HO9504A and CK2-A. Overall, it was observed that pressure resistance of the spore was not correlated with neurotoxin type (A, B etc as indicated at the end of the spore strain identifier; HO9504A is A type and PA9508B is B type). Reddy et al. (1999, 2000) reported that *C. botulinum* type A spores were more resistant to pressure than type B and E. BS-A and 62-A spores in phosphate buffer were reported to have 3 log unit reduction after HP treatment 827 MPa 20 min at 75°C. *C. botulinum* spore TMW2.357 (type B) in Tris-His buffer (THB) was reported to exhibit 2.4 log unit reduction after 800 MPa / 20min at average 87 °C (Margosch et al., 2004). Due to equipment limitation, both studies had significant process temperature variations (Ahn et al., 2007). In contrast, this study showed the 62A spores were had the lowest resistance.

Extrapolated D values of *C. sporogenes* 7955 spores for these three treatment conditions, 900 MPa/100 °C, 900 MPa/90 °C, 800 MPa/90 °C, were 1.95, 4.6, 5.3 min, respectively, in a buffer (Koutchma, et al., 2005). These values in milk as reported in Chapter 5 were 1.3, 7.0 and 10.7 min, respectively. It can therefore be observed that the most resistant PA9508B spores in buffer tested in this study had D values higher than *C. sporogenes* 7955, although the media were different (buffer vs milk). Therefore, further studies may be required to carryout more detailed evaluation of on high pressure kinetics of *C. botulinum* PA9508B spores in other low acid foods. The process developed must meet the sterility criterion with respect to the most resistant *C. botulinum* spore strain.

8.3.4 Pressure and temperature sensitivity of *C. botulinum* (group I) spores

Z_T (thermal sensitivity at different pressures) and Z_T (pressure sensitivity parameter at different temperatures) are normally calculated from D value vs temperature at specific pressures and D value vs pressure plot at different temperatures, as negative reciprocal slopes. In this set of screening experiments, to get estimates of both Z_P and Z_T, experiments were carried out at 100 and 90 °C process temperatures at a pressure level of

900 MPa and at two pressures 900 and 800 MPa at a constant temperature of 90 °C. Since only two pressures and two temperatures were used, Z_P and Z_T values were obtained using the simplified slope equation:

$$Z_P = (100\text{-}90)^\circ C \,/\, [\log (D_{90^\circ C}/D_{100^\circ C})] \text{ and}$$

$$Z_T = (900\text{-}800)\text{MPa} \,/[\log (D_{800}/D_{900})].$$

These results are tabulated in Table 8.5. Z_P values for different strains varied from 10.5 to 16C. These values could not be calculated for the four strains which were completely killed by one of the treatment given (N/C in Table 8.5). Higher Z_P value means higher temperature resistance at a given pressure. By comparing thermal treatment z value (10 °C), it can be recognized that some *C. botulinum* spores had similar or higher temperature resistance under the high pressure processing conditions than in conventional thermal processing. Among the strains, PA9805B had the maximum Z_P value (16 °C). In previous chapters it was observed that *C. sporogenes* 7955 had a Z_P value of 20.2°C in milk and 16.4 °C in salmon at 900 MPa, and Koutchma et al. (2005) reported a value of 23.7 °C for the same in buffer. Relative to these, PA9805B appears to have about the same or a little lower resistance to temperature change than *C. sporogenes* 7955.

Table 8.5 Estimated temperature and pressure sensitivity parameters of *C. botulinum* (group I) spores in 0.1 M sodium phosphate buffer (pH 7.0)

Strain	Z_P value (°C) (at 900 MPa)	Z_T value (MPa) (at 90 °C)
62A	N/C	N/C
IB1-B	N/C	N/C
CK2-A	10.5	670
MRB	N/C	N/C
Langeland	N/C	N/C
A6	10.6	770
GA0108BEC	11.0	740
PA9508B	16.0	470
13983B	N/C	N/C
H461297F	12.9	630
GA0101AJO	13.1	380
HO9504A	11.7	720

NC: Not calculated due to complete destruction of spore population

Z_T values for the different strains in this study varied significantly from 380 to 770 MPa (Table 8.5) at 90 °C. Higher Z_T value means higher pressure resistance at a given temperature. Again the values could not be calculated for four strains due complete inactivation under the treatment conditions. Five strains (CK2-A, A6, 13983B, GA0108BEC, H461297F and HO9504A) had higher Z_T values higher than 600 MPa, and GA0101AJO had the least 380 MPa. The most pressure resistant strain, PA9508B, had a mid range Z_T value of 470 MPa. Rovere (1996) reported that Z_T values of *C. sporogenes* spore were 725 MPa at 90 °C, 962 MPa at 100 °C, 752 MPa at 108 °C and Reddy (1999) pointed out that Z_T value of *C. botulinum* Type A was 1524 MPa at 75 °C. In the previous chapters, it was observed that the Z_T values of *C. sporogenes* 7955 at 90 °C were 690 MPa in milk and 535 MPa in salmon, within the range observed for some strains of *C. botulinum*.

8.3.5 Effects of substrate milk and phosphate buffer on spore resistance

The more resistant spores (PA9508B, HO9504A and CK2-A) found in the previous section were inoculated in milk and 0.1 M sodium phosphate buffer (pH 7.0) and were subjected to selected high pressure and high temperature combination treatment to compare if their pressure resistance would be different. The nominal log reductions (Table 8.6) were compared with each other by ANOVA statistic analysis. Among these three spores, PA9508B always showed the highest survival count and therefore the highest resistance pressure destruction while CK2-A showed the least. The same trends were observed earlier in the resistance screening study.

For the milk and buffer comparison, PA9508B and CK2-A spores showed higher resistance trend when suspended in milk. HO9504B was more stable in buffer in some treatments but the difference in the extent of their survival between milk and buffer was not high. This confirms that the suspension media has an effect on the destruction kinetics as seen earlier with *C. sporogenes* in milk and salmon.

Table 8.6 Nominal log reductions (standard deviation n=4) of HP inactivation of three resistant *C. botulinum* group I strains in milk and 0.1M sodium phosphate buffer (pH 7.0)

HP Treatment*	Type of substrate	Type of strain		
		PA9508B	HO9504A	CK2-A
900 MPa 90 °C 12min	Milk	1.02±0.19	1.40±0.05	1.87±0.08
	Buffer	1.82±0.09	1.84±0.09	2.38±0.21
700 MPa 100 °C 8min	Milk	2.24±0.03	4.00±0.17	4.66±0.47
	Buffer	2.31±0.08	3.31±0.16	4.40±0.23
800 MPa 100 °C 6min	Milk	0.74±0.07	1.90±0.04	4.53±0.09
	Buffer	1.56±0.04	1.72±0.05	4.68±0.11
900 MPa 100 °C 4min	Milk	0.93±0.09	1.88±0.11	3.27±0.06
	Buffer	1.51±0.08	1.76±0.06	4.02±0.14
900 MPa 110 °C 0.8min	Milk	1.75±0.04	3.54±0.18	4.95±0.30
	Buffer	1.96±0.05	3.48±0.17	5.57±0.16

* Data can be compared within the same treatment condition

8.4 Conclusions

Preliminary studies indicated the need for at least 8 days of anaerobic incubation for survival counts of *C. botulinum* spores subjected to high pressure treatment to make sure all viable colonies are well formed. This incubation time was probably necessary to allow spores to recover from the HP shock which might delay their growth. The 12 group I strains varied in their pressure resistance and four of them were completely inactivated during the treatment. Among the remaining, PA9508B, HO9504A and CK2-A were the more resistant strains. There was no correlation between the pressure resistance and neurotoxin type because not all of A type strains or B type strains of group I were high resistant. PA9508B and CK2-A spores showed more resistance in milk than in phosphate buffer. The highest pressure resistance was with PA9508B and it might be prudent to do a more detailed HP destruction kinetics study on the three strains, PA9508B, HO9504A and CK2-A, or at least PA9508B.

Preface to Chapter 9

In the previous Chapter 8, twelve strains of *C. botulinum* group I spores were screened for high-pressure resistance, and strains PA9508B, HO9405A and CK2-A were found to be more HP resistant with PA9508B demonstrating the most. Experiments were only designed to be able to screen the pressure resistance rather than a detailed evaluation of destruction kinetics for all the strains. This chapter describes the detailed evaluation HP destruction kinetics of PA9508B strain. HP destruction kinetics of *C. botulinum* PA9508B spores suspended in milk were studied and compared to literature data as well as our previous on *Clostridium sporogenes* and *Geobacillus stearothermophilus*. This work would partially fulfill the second and the third objectives. The results obtained in this study will provide new data and useful information for establishing high-pressure sterilization processing of low acid foods.

Part of this study will be submitted for publication. This research work was completed by the candidate under the supervision of Dr. H. S. Ramaswamy and Dr. John Austin. Microbiological support was given on spore preparation and enumeration by Mr. Jeff Bussey (Health Canada, Ottawa) during experiments.

Chapter 9

High pressure destruction kinetics of *Clostridium botulinum* PA9508B spores in milk at elevated temperatures

Abstract

Milk (2% fat, commercially sterile) was inoculated with *C. botulinum* PA9508B spore stock suspension was to give a cell concentration of 10^7/ml and subjected to high pressure (HP) treatments (700-900 MPa; 90-100 °C and 0-24 min). Samples in 1.7 mL plastic vials were placed in a specially constructed insulated chamber to prevent temperature drop during the treatment. Survivor counts in the "zero" min holding time treatment were lower than the control, but they did not demonstrate significant reductions, which meant high pressure come-up time did not play an important role in those reductions. The pulse effect was therefore less obvious in this case. During holding time period, the pressure destruction was well described by the first order model (R^2 > 0.87). Destruction rates (D value), pressure and temperature sensitivity parameters (Z_P and Z_T values) were evaluated by the first order reaction rate model. Higher pressures and temperatures resulted in an accelerated spore destruction rate. For 900 MPa treatments, the D values were 14.5, 1.8, 0.35 at 90, 100, 110 °C respectively. Temperature sensitivity Z_P values (at constant pressure) were 11.2, 12.3, 12.4 °C at 700, 800, 900 MPa respectively, with z value increasing with pressure and were much higher than the thermal treatment z value 7.8 °C. The pressure sensitivity parameter Z_T values (at constant temperature) were 470, 630, 800 MPa at 90, 100, 110 °C, respectively, and with the z values increasing with temperature. By comparison of the Z_T and Z_P, it appears the spore was relatively more sensitive to temperature than to pressure. Predicted D value at 100-121°C demonstrated that pressure inactivation effect decreased as the temperature increased. At temperatures beyond 115 °C, heat was essentially the principle mode of spore destruction.

9.1 Introduction

The importance of high pressure processing as novel and innovative food preservation method has been highlighted in the previous chapters. High pressure high temperature (HPHT) processing is getting recognized as alternative technique to high temperature sterilization because it is able to inactivate highly resistant bacterial spores in low acid (pH 4.5) foods. HPHT process has potential to produce the same or better high quality products than possible with HTST and UHT techniques.

An IFT expert panel had pointed out the lack of kinetic data for various bacterial spores suspended in food products under various pressure–thermal conditions so far (FDA, 2000). The pressure process establishment required inactivation kinetic data of resistant bacterial spores. The use of the log-linear model allowed the comparison of the rates of conventional heat inactivation with high-pressure and high temperature treatments (Koutchma et al., 2005; Patazca et al., 2006). In the studies of HP inactivation kinetics of spores, most of the survivor curves showed a shoulder or a tail during pressure holding. It was impossible to apply the first order modeling to calculate D and z values. The problem of analysis of kinetic data by the first order log-linear model was the temperature large variation due to HP adiabatic heating and heat loss. Maintaining constant process temperature was particularly important to get linear survivor curves like in thermal inactivation experiments (Patazca et al., 2006). This has also been demonstrated in our studies as detailed in the previous chapters.

Reddy et al. (1999, 2000, 2003 and 2006) have studied HP in combination with elevated temperatures on spores of *C. botulinum* type A (proteolytic BS-A and 62-A), type B (nonproteolytic 2-B, 17-B, KAP8-B, and KAP9-B) and type E (Alaska and Beluga) and found *Clostridium botulinum* had greater inactivation when the temperature was increased from 35 to 75 °C at 827 MPa. Type A spores were more resistant than others because they just achieved 3-log unit reduction for 827 MPa/20min at maximum 75 °C. Margosch et al. (2006) reported that proteolytic TMW2.357(*C. botulinum* types B) and TMW 2.479(*B. amyloliquefaciens*) exhibited a greater resistance to pressure than

bacterial spores (*Bacillus* spp. and *C. botulinum* spp.). However, both experiments had temperature control limitations.

In the previous Chapter 8, twelve strains of *C. botulinum* group I spores were screened for high-pressure resistance, and strains PA9508B, HO9405A and CK2-A were found to be more HP resistant with PA9508B demonstrating the most. In the above studies, experiments were only designed to be able to screen the pressure resistance rather than a detailed evaluation of destruction kinetics for all the strains. This chapter describes the detailed evaluation HP destruction kinetics of PA9508B strain.

The objectives of this study were (1) to evaluate the HP destruction kinetics of *C. botulinum* PA9508B spores and compare them with other pressure resistant spores and (2) to investigate the pressure and thermal sensitivity of these bacterial spores.

9.2 Materials and Methods

9.2.1 *Clostridium botulinum* PA9508B culture and spores preparation

Same as detailed for *C. botulinum* in Chapter 8.

9.2.2 Sample inoculation and packing

Same as detailed for *C. sporogenes* 11437 in Chapter 4.

9.2.3 High pressure equipment

Same as detailed for *C. sporogenes* 11437 in Chapter 4.

9.2.4 Plastic POM (Polyoxymethylene) insulator

Same as detailed for *C. sporogenes* 11437 in Chapter 4.

9.2.5 High pressure treatment

Same as detailed for *C. sporogenes* 11437 in Chapter 4.

9.2.6 Sample temperature control during HP treatments

Same as detailed for *C. sporogenes* 11437 in Chapter 4.

9.2.7 Thermal process treatment

Same as detailed for *C. sporogenes* 11437 in Chapter 4.

9.2.8 Enumeration of survival spores

Same as detailed for *C. botulinum* in Chapter 8.

9.2.9 Kinetic data analysis

Same as detailed for *C. sporogenes* 11437 in Chapter 4.

9.2.10 Kinetic data temperature calibration

Same as detailed for *C. sporogenes* 11437 in Chapter 4.

9.3 Results and discussion

9.3.1 Thermal destruction kinetic of *C. botulinum* PA9508B spores

Figure 9.1 illustrates the nominal survival counts against time for destruction of *C. botulinum* PA9508B spores in milk by thermal treatment. Inactivation at "zero' min holding was not observed by thermal treatment come-up time because the treatment temperature (90-100 °C) was not high enough. However, thermal destruction was observed during holding time. The survivor curves were well described by first order reaction (log linear regression) model with the correlation coefficient (R^2) which varied from 0.90 to 0.97. The D values were 156, 57.5, 12.1 min at 90, 95, 100 °C respectively (Table 9.1). The z value was 7.8 °C with R^2 value 0.99.

9.3.2 High pressure destruction kinetics of *C. botulinum* PA9508B spores

Figure 9.2 shows a specific HP treatment example (900 MPa/90°C), which had the longest treatment holding time (24 min) in this study. In this treatment, the milk sample initial temperature (preheated) and chamber medium temperature were 55.9 and 67.2 °C respectively.

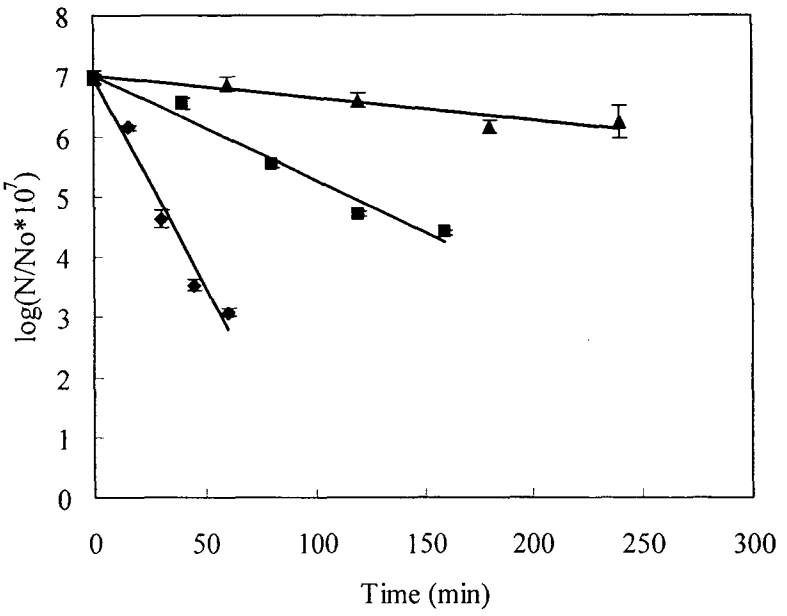


Figure 9.1 Nominal survivors of *C. botulinum* PA9508B spores in thermal treated milk (1 atm) at temperature (▲) 90, (■) 95, (♦) 100 °C

Table 9.1 Decimal reduction time (D values) of *C. botulinum* PA9508B spores in milk subjected to thermal treatment

Pressure (MPa)	Temperature (°C)	D value (min)	R^2	Z value (°C)
0.1	90	273.9	0.90	7.8
	95	57.5	0.97	
	100	14.4	0.97	$R^2 = 0.99$

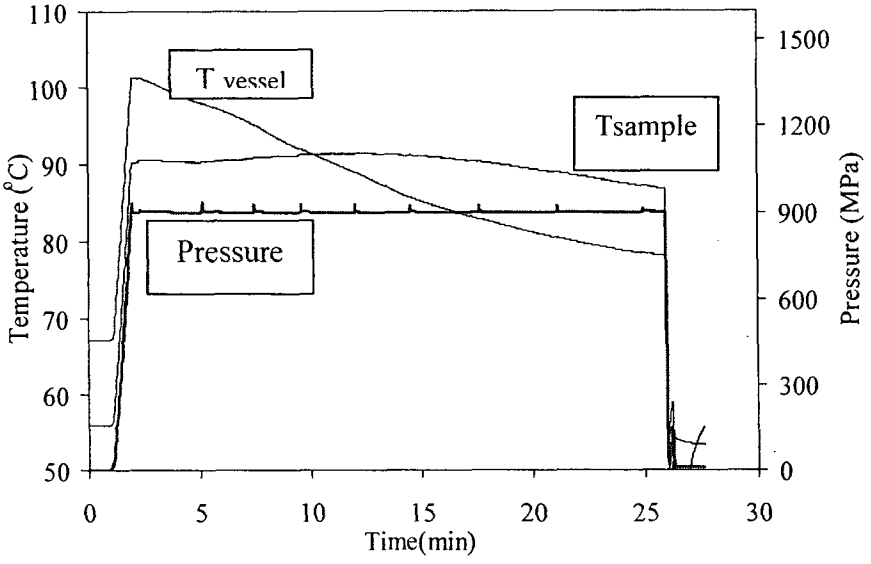


Figure 9.2 Typical pressure and temperature curves observed high pressure processing at 900 MPa/90°C. The time for this treatment was come-up time (56 s), process time (24min) and depressurization time (15 s)

When pressure reached 900 MPa, milk temperature rose to 90.2°C and chamber medium temperature became 101.1 °C. During pressure holding, sample temperature remained at the same level within the first 6 min and then increased to maximum 91.4 °C at the 10th min holding and decreased to 86.7°C at 24 min holding end. The temperature increased from the 6^{th} min to the 14^{th} min holding due to the heating from high temperature vessel medium. In the meantime, vessel medium temperature decreased steadily from 101.1 to 78.0°C. By comparison of vessel temperature and sample temperature variation, it was concluded that plastic insulator and temperature control method could be helpful for destruction kinetic experiments under constant pressure and quasi-isothermal conditions. However, sample temperature variations were still there, original kinetic data needed to be calibrated to eliminate temperature minor errors later.

Figures 9.3-9.5(a captions) illustrate *C. botulinum* spores original survivor curves under various pressures (700, 800, 900 MPa) and different temperatures (90, 100, 110 °C) during pressure holding. No inactivation occurred by come-up period of any high pressure and high temperature combinations because these bacterial spores were greatly pressure resistant.

During holding time, all survivor curves confirmed that the destruction effect was associated with higher pressure and higher temperature combination, which resulted in higher spore reductions. These survivors were well described by the first order model ($R^2 > 0.87$). In each figure, 110 °C HP treatments resulted in more reductions than in 100 and 90 °C HP treatments. Treatment at 700, 808, 900 MPa achieved 5.65±0.21, 5.12 ±0.10 and 5.27±0.62 log reductions within 4, 2.4 and 1.8 min holding at 110 °C. No tailings were observed in this study. Margosch et al. (2006) reported that the inactivation reduction of the most resistant *C. botulinum* TW 2.357 spores in THB (Tris-His buffer) were 1.8, 2.2 log units at 100°C/4 min for 800, 900 MPa treatments. In 110 °C HP treatment, 2.9 and 2.8 log unit reductions were achieved by 800 MPa/2min and 900 MPa/1min. These results proved both *C. botulinum* PA9508B and TW2.357 spores had similar resistance.

For microbial destruction kinetic study, 4 to 5 log unit reduction was achieved in most 100 and 110 °C treatments. Log linear regression analysis were used to compute the D values listed in Table 9.2 and these D values indicated bacterial spore's resistance responded to specific pressure and temperature combinations. An increasing in pressure and temperature resulted in a decrease in D value or an acceleration in destruction of bacterial spores rate. For example, the uncorrected D values were 32.7, 18.2, 14.2 min and 0.67, 0.51, 0.35 min for 700 – 900 MPa HP treatment at 90, 110 °C respectively (Table 9.2).

For accurately evaluating bacterial spore resistance, original temperature profiles of each treatment were used to obtain effective holding times and then the corrected nominal survivor curves (Figure 9.3-9.5 b captions) and corrected D values (Table 9.2).

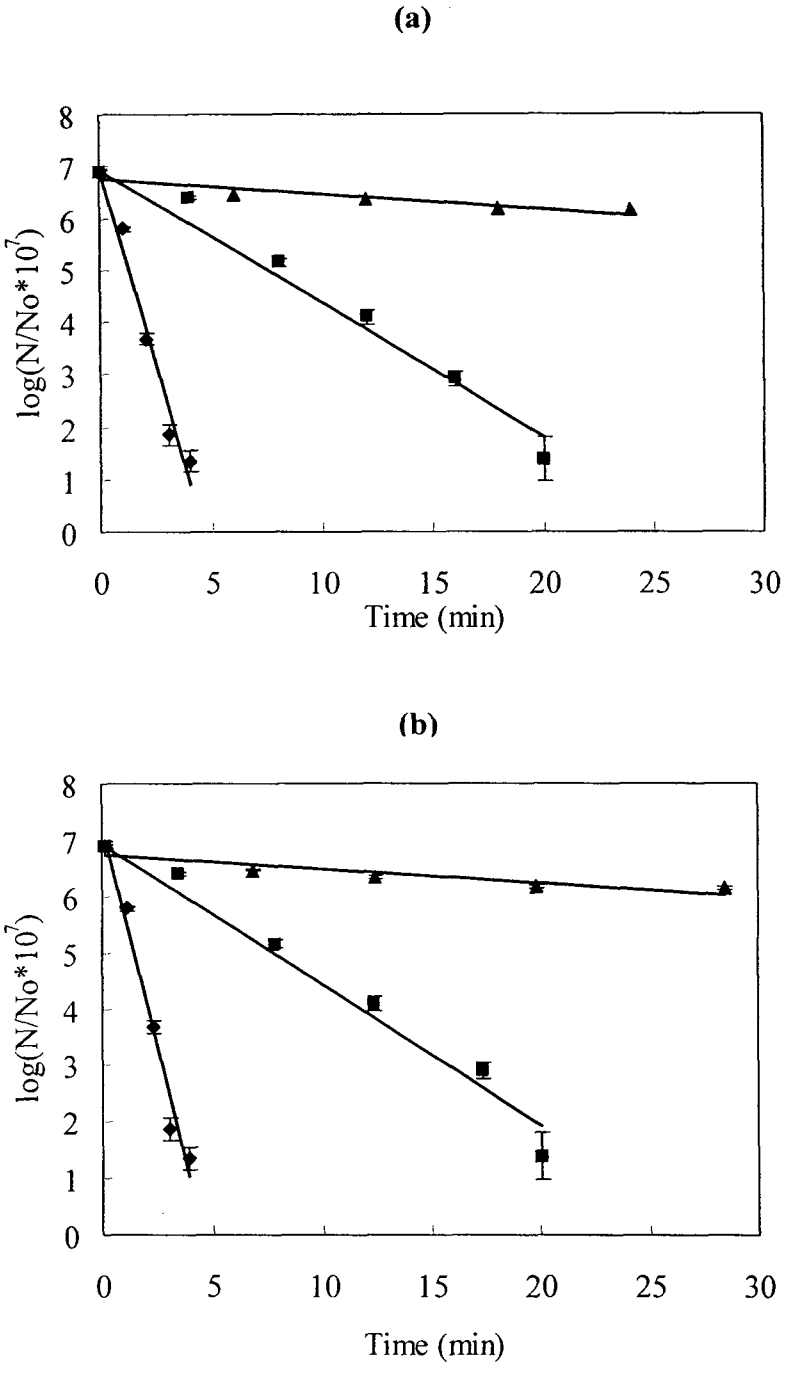
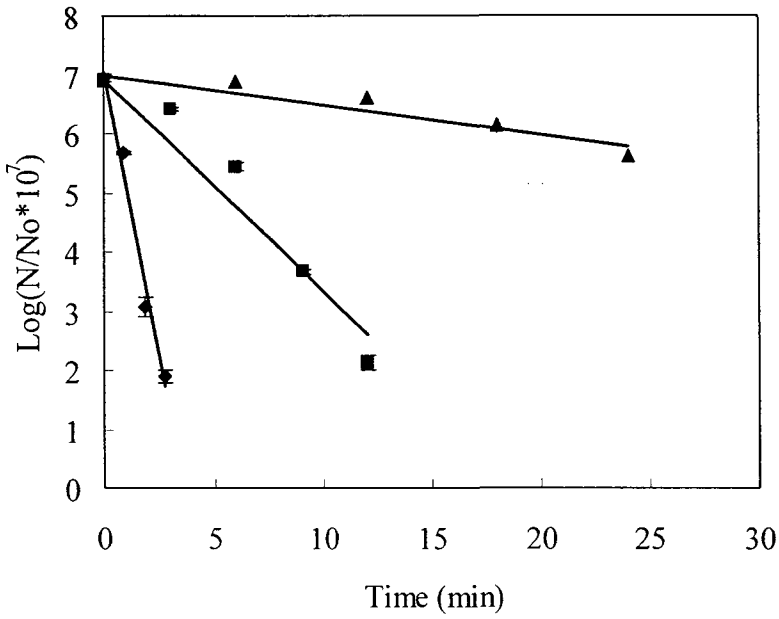


Figure 9.3 Mathematic uncorrected (a) and corrected (b) nominal survivors of *C. botulinum* PA9508B spores in 700 MPa high pressure treated milk at temperature (▲) 90, (■)100, (♦) 110 °C

(a)



(b)

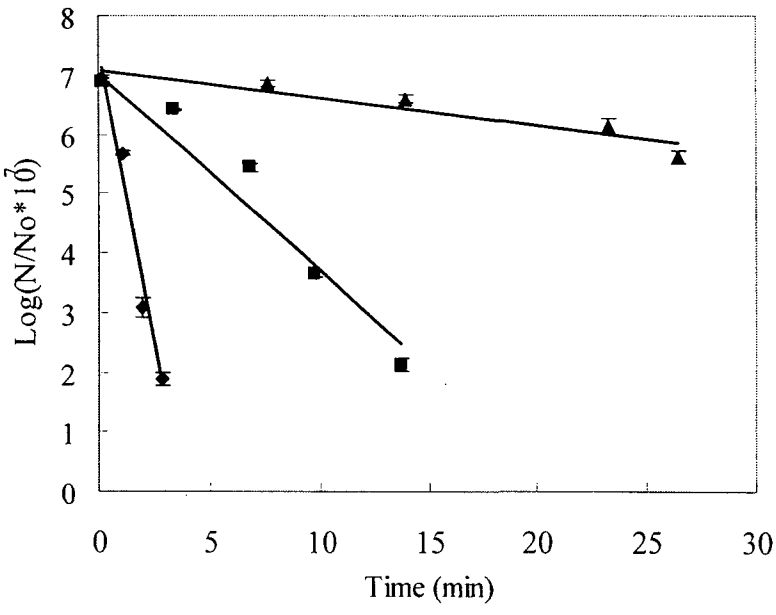
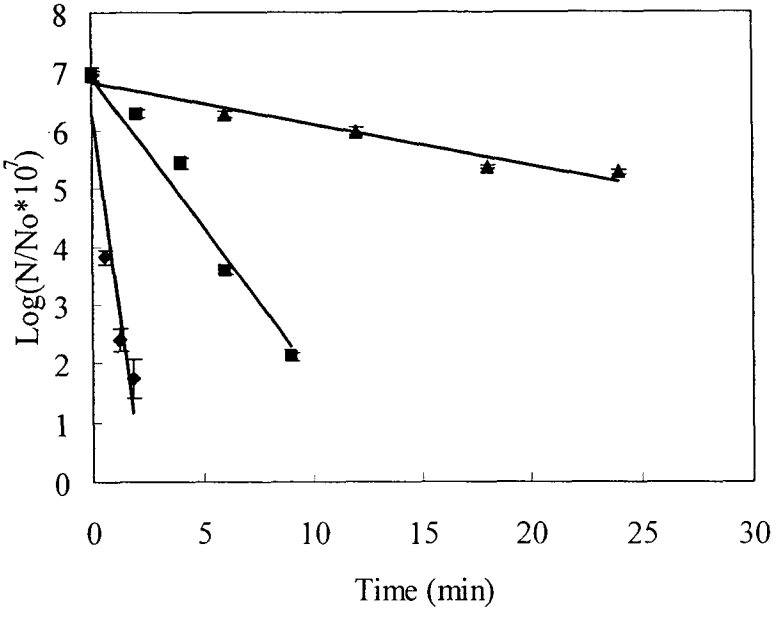


Figure 9.4 Mathematic uncorrected (a) and corrected (b) nominal survivors of *C. botulinum* PA9508B spores in 800 MPa high pressure treated milk at temperature (▲) 90, (■)100, (♦) 110 °C

(a)



(b)

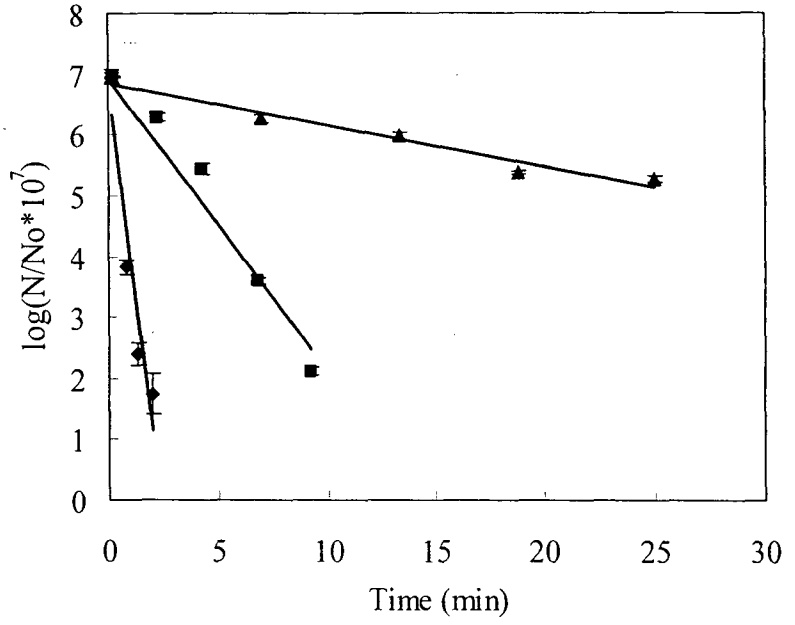


Figure 9.5 Mathematic uncorrected (a) and corrected (b) nominal survivors of *C. botulinum* PA9508B spores in 900 MPa high pressure treated milk at temperature (▲) 90, (■)100, (♦) 110 °C

After temperature correction, the D values were 38.9, 21.3, 14.5 min and 0.63, 0.51, 0.35 min for 700 – 900 MPa HP treatment at 90, 110 °C respectively (Table 9.2). At 110 °C, uncorrected and corrected D values were almost the same due to the short holding time and accurate temperature control (insulation). In this study, the treatment at lower temperature (90 °C) needed longer holding time and temperature fluctuation was observed to occur more often, so corrected D value changed accordingly.

Table 9.2 Decimal reduction time (D values) of *C. botulinum* PA9508B spores in milk subjected to HP treatment

Pressure (MPa)	Temperature (°C)	Uncorrected		Corrected	
		D value (min)	R^2	D value (min)	R^2
700	90	32.67	0.87	38.92	0.85
	100	3.59	0.98	3.75	0.98
	110	0.67	0.97	0.63	0.98
800	90	18.16	0.92	21.29	0.88
	100	2.44	0.96	2.73	0.96
	110	0.51	0.98	0.51	0.98
900	90	14.16	0.96	14.52	0.96
	100	1.77	0.98	1.81	0.98
	110	0.35	0.90	0.35	0.91

At the same temperature, pressure was more efficient and resulted in significant decrease of D values (Table 9.2). D values were 274 and 14.4 min for thermal treatment and 14.2, 1.8 min for 900 MPa pressure treatment at 90, 100 °C respectively. Treatment at 900 MPa pressure usually resulted in almost one twentieth and one eighth of thermal treatment D values. This indicated that high pressure combined with high temperature could inactivate bacterial spores and effectively shorten the processing time. In the future, the high pressure sterilization processing should be established at higher temperature (T_P>110°C) for shortening processing time.

The D values of *C. sporogenes* 7955 spores in buffer were 2.83, 2.3 min and 0.97, 0.82min for 700, 800 MPa at 100, 108 °C, respectively (Koutchma et al., 2005). Compared to *C. sporogenes* 7955 in buffer, PA9508B spores were more resistant at 100 °C but less resistant at 110 °C. The D values of *C. sporognes* 7955 in milk were 13.6,

11.4, 7.0 min and 2.4, 1.7, 1.2 min for 700,800, 900 MPa at 90, 100°C respectively (Chapter 5). It observed that the D values of *C. botulinum* PA9508B were higher than those of *C. sporogenes* in milk when the temperature was lower 105°C.

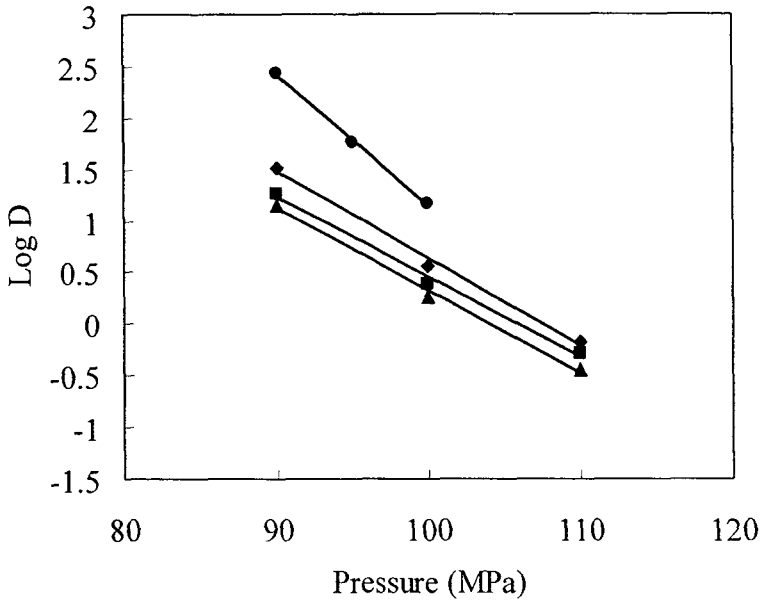
The D values of *G. stearothermophilus* 10149 spore in milk were 3.6, 1.3 min and 2.5, 0.6 min for 700, 900 MPa at 80, 90°C, respectively (Chapter 7). By contrast, D values of *C. botulinum* PA9508B were almost 10 times higher than those of *stearothermophilus* 10149 under pressure. This showed that *C. botulinum* PA9508B spores had more resistance than *G. stearothermophilus* 10149 spores under high pressure. However *G. stearothermophilus* 10149 spores were more heat resistant than those of *C. botulinum* and *C. sporogenes* in thermal treatment (pressure 0.1 MPa), whose its D values in milk were 49.3, 16.1, 6.3 min at 110, 115, 120°C respectively.

9.3.3 Pressure and temperature dependency of the kinetic parameters

Z_T and Z_P of *C. botulinum* PA9508B spores suspended in milk were analyzed from the respective D value curves either as a function of pressure or temperature. Thermal sensitivity (at constant pressure) of D values was described by the Z_p value (Figure 9.6). The Z_P were 11.8, 12.9, 12.5 °C for 700, 800, 900 MPa respectively, before correction and 11.2, 12.3, 12.4°C for 700, 800, 900 MPa, respectively after correction (Figure 9.6b, Table 9.3). Higher Z_P value meant lower thermal sensitivity. These Z_P values increased by 10.7% when the pressure increased from 700 to 900 MPa. Although PA9508B spore strain had conventional thermal D values much higher than under pressure at the temperatures tested (Tables 9.1 and 9.2), its sensitivity to temperature change was much lower (z value, 7.8 °C)(Table 9.1). Like PA9508B, *C. sporogenes* spores had a similar thermal sensitivity trend.

Similarly, the pressure resistance (Z_T) value was obtained from the linear relationship at each constant temperature (Figure 9.7). Corrected Z_T values *C. botulinum* PA9508B 7955 in milk were 470, 630, 800 MPa at 90, 100, 110°C, respectively (Figure 9.7b, Table 9.4), which indicated that the pressure resistance increased with process temperature. This indicated that pressure resistance increased by 70% with temperature increasing from 90 to 110°C.

(a)



(b)

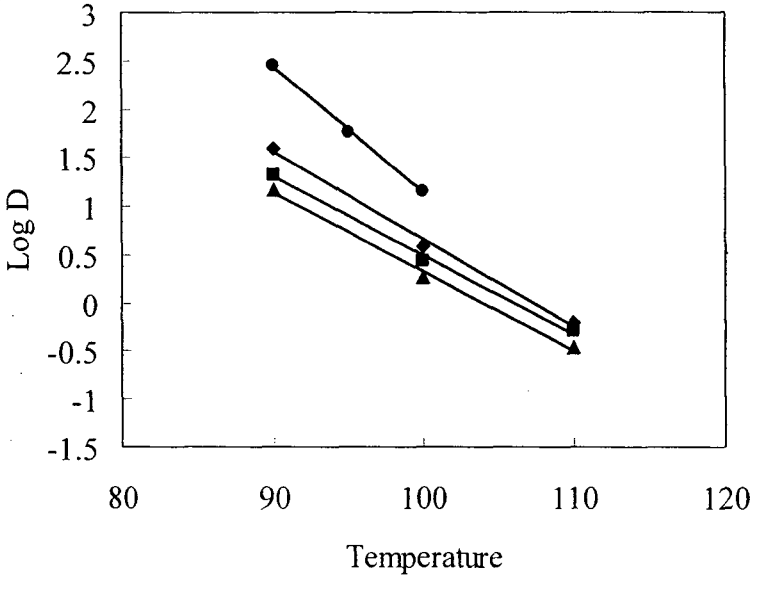
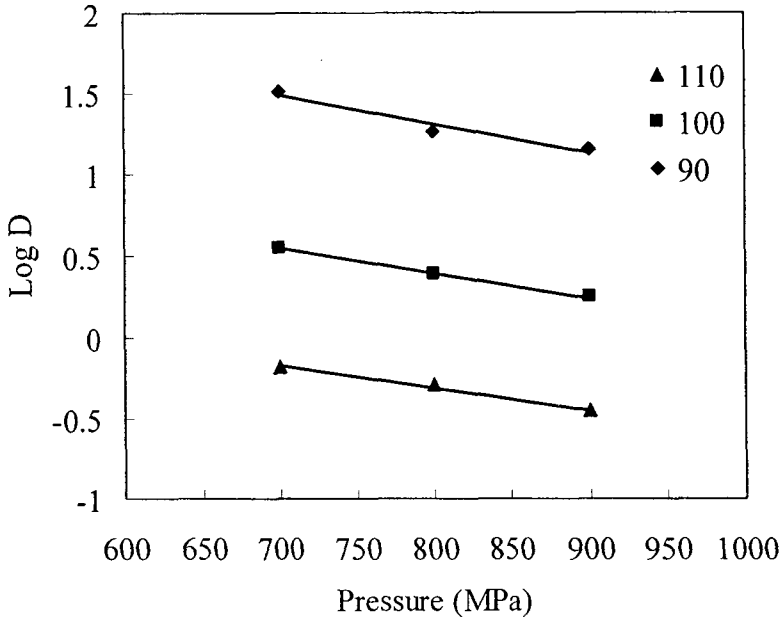


Figure 9.6 Uncorrected (a) and corrected (b) logarithmic D value of *C. botulinum* PA9508B spores in HP treated and thermal treated milk and temperature at pressure (♦) 700, (■)800, (▲)900 MPa, (●) 0.1 MPa

(a)



(b)

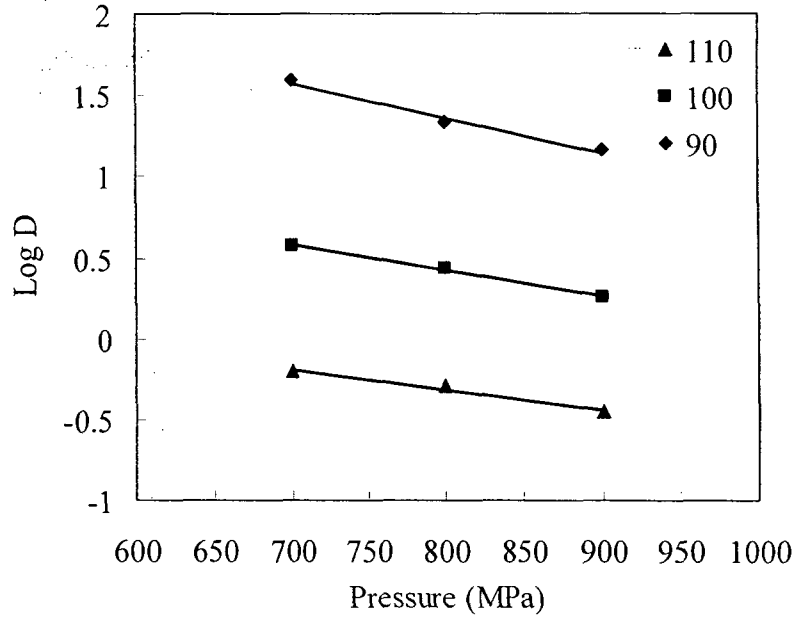


Figure 9.7 Uncorrected (a) and corrected (b) logarithmic D value of *C. botulinum* PA9508B spores in HP treated milk vs. pressure at temperature (♦) 90, (■) 100, (▲)110 °C

Table 9.3 HP constant pressure z-value (z_P) (°C) of *C. botulinum* PA9508B spores in HP treated milk and thermal treated milk

Pressure (MPa)	Uncorrected		Corrected	
	z_p value	R^2	z_p value	R^2
700	11.8	0.99	11.2	0.99
800	12.9	0.99	12.3	0.99
900	12.5	0.99	12.4	0.99
0.1	7.8	0.99		

Table 9.4 HP constant temperature z-value (z_T) (MPa) of *C. botulinum* PA9508B spores in HP treated milk

Temperature (°C)	Uncorrected		Corrected	
	z_T value	R^2	z_T value	R^2
90	550	0.95	470	0.98
100	660	0.99	630	0.99
110	720	0.99	800	0.98

From the Z_P and Z_T value evaluation, one can conclude that the *C. botulinum* spores were relatively more sensitive to temperature than to pressure. These Z_T values of *C. sporogenes* 7955 spores showed a similar trend. On the contrary, *G. stearothermophilus* spores were more sensitive to pressure than to temperature (Patazca et al., 2006).

9.3.4 Predicted high pressure D value at high temperatures

Predicted log D values and D values at higher temperature were calculated from logarithmic linear regression extension of known thermal or high-pressure kinetic parameters. Figure 9.8 illustrate log D values against temperature and Table 9.4 shows the computed D values at specific temperatures (105, 110, 115, 120 and 121 °C). We can find that predicted D values under pressure processing (HTHP) were higher than predicted thermal treated D value around 121°C because high-pressure spore had higher Z_P values. It meant that at the temperature close to or higher than 121°C high pressure can reduce the enhancement of destruction and even lower the inactivation rate. Such conditions would need to rely on inactivation effects caused by heat rather than pressure.

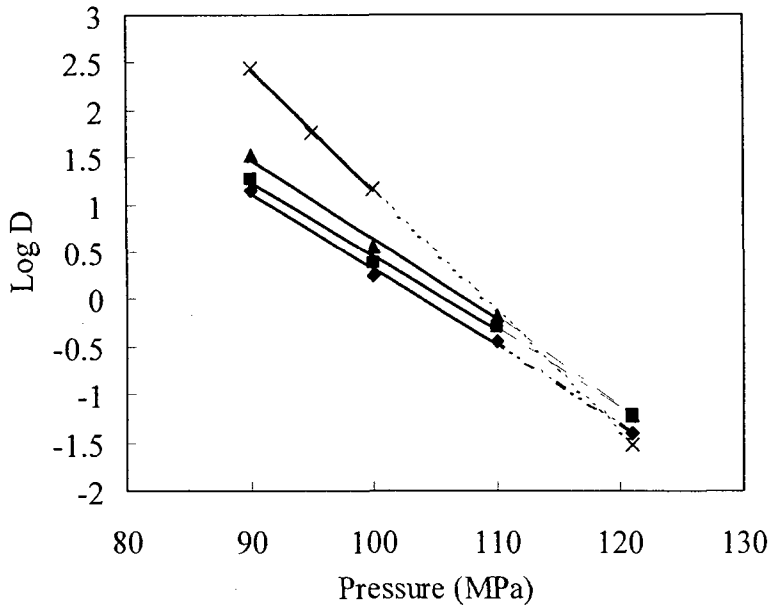


Figure 9.8 Predicted decimal reduction time (D values) and predicted decimal reduction time (predicted D values) of *C. botulinum* PA9508B spores in milk against temperature

Table 9.5 Predicted decimal reduction time (D values) of *C. botulinum* PA9508B spore in HP treated and thermal treated milk at higher temperature

Pressure (MPa)	Extrapolated D value (min) at temperature (°C)				
	105	110	115	120	121
700	1.61	0.67*	0.20	0.07	0.06
800	1.21	0.51*	0.18	0.07	0.06
900	0.83	0.35*	0.13	0.05	0.04
0.1	3.24	0.75	0.17	0.04	0.03

* real experimental values

Margosch et al. (2006) observed that a pressure-mediated protection happened on *B. amyloliquefaciens* TMW 2.479 spore at 120 °C under 800 to 1200 MPa. Comparing with predicted D values of *C. sporogenes* 7955, 11437 and *G. stearothermophilus* 10149 spores in milk, we find that predicted D values of *C. sporogenes* 7955 at 121°C were higher than those of PA9508B and 11437 while *C. sporogenes* 11437 and *C. botulinum* PA9508B had similar resistance. This indicated *C. sporogenes* 7955 spores were more

resistant than *C. botulinum* PA9508B spores at 121°C in HPHT treatments as well in thermal treatments. *C. sporogenes* and *C. botulinum* had their own Z_P and s_T values which were quite different, which explained why *botulinum* spores were more resistant than *C. sporogenes* spores (Tp < 105°C) and *C. sporogenes* spores were more resistant than *botulinum* spores (Tp > 105°C). *C. sporogenes* 7955 spores were suitable as a reference for high pressure processing validation. *C. sporogenes*11437 spore was also good to work at 121°C. However, *G. stearothermophilus* 10149 spores were far less resistant than *C. botulinum* and not suitable for validation work.

9.4 Conclusions

C. botulinum PA9508B spore was the most resistant pathogenic spores among those studied. This was also more resistant compared with *C. sporogenes* 7955 spore and *Geobacillus stearothermphilus* 10149 spore at 80-105°C temperature range. Due to differences in the Z values, *C. sporogenes* 7955 spores showed higher resistant than PA9508B spores at temperature higher than 110°C. It suggested that *C. botulinum* PA9508B spore inactivation kinetics be used as a reference base unless other strains of higher resistance were discovered.

Chapter 10

General conclusions

High pressure sterilization of low acid foods has been recognized to be a novel potential alternative process technique to thermal sterilization. Before its establishment and approval, high-pressure resistance of target pathogenic bacterial spores and non-pathogenic surrogate spores need to be identified and high-pressure inactivation kinetic data need to be evaluated. Focused on this main topic, this research work was carried out and relevant results were obtained. The following are the general conclusions of the study:

(1) Polyoxymethylene (POM) is a high performance high-density polymer with low thermal conductivity, widely used in high hydrostatic conditions at room temperature. Our insulation set-up designed for the kinetic studies demonstrated that POM was a good choice as an insulating material for constructing insulated test chambers for HP high temperature kinetic studies. The compression heating characteristics of the material also matched somewhat that of the liquid pressure medium. With its good insulation characteristics, POM insulator was able to take the advantage of adiabatic compression heating and delay heat loss and maintain a stable internal temperature for kinetic studies.

(2) Adiabatic compression heating behavior of milk was accurately evaluated with in a POM insulator. The relationship of adiabatic temperature rise, initial temperature and pressure was found to be: $\Delta T_P = -0.306 + 0.0224T_i + 0.0423P + 4.49x10^{-4}T_i^2 + 1.31x10^{-4}T_iP - 1.24x10^{-5}P^2$ (R^2 =0.999, n = 50, SE = 0.20°C, $p<0.05$). The milk adiabatic temperature rise increased with increasing of initial temperature and pressure. Initial temperatures at 83.5, 91.7, 95.8°C and 80.7, 88.9, 93.0°C at 800, 900MPa, respectively, resulted in process temperatures 121, 130, 135°C, respectively. Compression adiabatic heating and decompression cooling rate depended on pressurization and depressurization speed, and were faster than

any conventional thermal processing heating and cooling processes. Thus HTHP processes should be able to simulate HTST processing technique for bulk foods.

(3) Using pressure inactivation of *C. sporogenes* 7955 as an example, the fabricate set-up was shown to be adequate for carrying out high pressure inactivation kinetics at nearly isobaric and isothermal conditions.

(4) *Clostridium sporogenes* ATCC 11437 spores are widely used as standard for thermal sterility reference testing in the pharmaceutical industry. High pressure combined with elevated temperature was able to relatively easily destroy these spores in milk. Hence, *C. sporogenes* 11437 is not a high pressure resistant spore and not a good candidate as surrogate for HPHT processing.

(5) Temperature corrections are necessary to get isothermal and isobaric kinetic data in situation where the pressure and temperatures are not stable during the treatment. The pressure in most cases (except during the come-up period) is kept constant because the HP system can pump in more fluid to compensate for any loss in the system pressure. However, there could be temperature instability in spite of using insulated chambers. In such cases, appropriate mathematical procedures need to be used to get temperature specific destruction kinetic data. Such a procedure was standardized and implemented for gathering accurate kinetic data. The limitation of this calibration was that the correction error tended to become larger when the temperature varied a lot. Therefore, application of insulation kinetic study was the key to have better final results.

(6) *Clostridium sporogenes* 7955 (PA3679) is a common surrogate strain used for *C. botulinum* in thermal processing validation. The HP resistance of this strain was much more than *C. sporogenes* 11437 and hence it would be a better candidate as surrogate in HTHP studies. D values of *C. sporogenes* 7955 in both thermal and pressure treatments were higher than those of *C. sporogenes* 11437, hence there was a correlation between thermal and pressure resistance. *C. sporogenes* 7955

spores had a lower pressure sensitivity at higher temperatures and lower thermal sensitivities under high pressures. The pressure resistance in general was higher than the temperature resistance. This strain has been used in thermal processing validation and could serve as a useful reference in high pressure sterilization for processing establishment and validation. At 121°C, pressure had no enhanced effect on spore inactivation and high temperature was the key player. At lower temperatures, however, pressure enhanced the destruction kinetics of the *C. sporogenes* 7955 spores.

(7) *C. sporogenes* 7955 spore destruction kinetics as well as its pressure and thermal resistances in salmon fish and milk were similar. However, the spore suspension in fish meat had a lower resistance than in milk. This indicated food matrix has influence on the pressure as well as thermal resistance of the spores.

(8) *Geobacillus stearothermophilus* 10149 spores are traditionally more resistant *C. sporogenes* to thermal destruction. Since a correspondence was observed between thermal and pressure resistances in the previous studies with *C. sporogenes*, HP destruction studies of *Geobacillus stearothermophilus* 10149 spores in milk were evaluated. However, it was found that these spores have higher resistance in thermal processing but lower resistance in HTHP processing. These spores were more easily destroyed by high pressure at high temperature than *C. sporogenes* spores and therefore *Geobacillus stearothermophilus* was not considered a useful candidate as surrogate in HTHP work. These spore destruction was also more sensitive both temperature and pressure, thus the process was synergistic.

(9) In studies with *C. botulinum* spores, an anaerobic incubation of at least 8 days was found necessary to allow complete development of colonies for counting. This could be due to possible injury/shock to spores during the HP treatment. Screening of twelve strains of *C. botulinum* spores of different sero-types for HP resistance showed PA9508B, HO9504A and CK2-A to be more pressure resistant than the others. Neurotoxin types did not reveal any correlation with pressure

resistance. *C. botulinum* spores had a lower pressure sensitivity at higher temperature and a lower temperature sensitivity under higher pressures. PA9508B and CK2-A spores showed more resistance in milk than in phosphate buffer.

(10) High-pressure destruction kinetic data on *C. botulinum* PA9508B spores showed a higher pressure combined with high temperature always resulted in a faster microbial reduction (smaller D value). However, the magnitude of difference in D values between thermal and pressure processing condition decreased at higher pressures. The results demonstrated that *C. botulinum* PA9508B spores were the more resistant than *C. sporogenes* 7955 spores and *Geobacillus stearothermphilus* 10149 spores. It suggested that *C. botulinum* PA9508B as a pathogenic spore former, should be a considered target for establishing HP sterilization processing, unless more resistant strains are exposed.

Chapter 11

Contributions to knowledge and future recommendations

The major contribution to knowledge of this research project is evaluating the high pressure high temperature destruction kinetics data for several potential surrogate high resistant bacterial spores as well as for spores of *Clostridium botulinum*. In order to do so it was necessary to develop and standardize a methodology for temperature control in the high pressure equipment so that meaningful kinetic studies could be carried out. Finally, the minor temperature variation that still existed needed to be corrected using an iterative technique to get temperature specific kinetic data. The following are the specific contribution to literature and knowledge:

(1) A thick wall insulated chamber using a high density polymer (polyoxymethylene) was fabricated and tested in high-pressure chamber to take the advantage of adiabatic compression heating, delay heat loss and maintain temperature under high temperature high pressure processing conditions. Experimental evaluation spore inactivation kinetics require such accessories in order to get meaningful kinetic data.

(2) A mathematical procedure was used to compensate for temperature deviations during the kinetic studies so that accurate data on isothermal and isobaric inactivation kinetic data could be generated from the study.

(3) Kinetic studies on several potential surrogate spores, traditionally considered to be good candidates for thermal resistance, were evaluated for HP processing work. These include several nonpathogenic spores, *C. sporogenes* 11437; *C. sporogenes* 7955; *G. stearothermophilus* 10149 suspended in milk or salmon fish meat base slurry. Their destruction kinetic behavior were characterized and kinetic parameters describing temperature and pressure resistance were computed to identify the most resistant spores and provide data for process validations. Among those three strain spores, *C. sporogenes* 7955 had the highest resistance in thermal processing and as well as HPHT processing. *G. stearothermophilus* 10149 had the highest resistance in thermal processing, but had the lowest in pressure processing. The temperature

and pressure sensitivity of the spores were species dependent. *C. sporogenes* spores were relatively more sensitive to temperature than to pressure. Its pressure resistance was correlated with heat resistance. However, *G. stearothermophilus* was relatively more sensitive to pressure than to temperature and its pressure resistance did not correlated with its heat resistance. Among those studied, *C. sporogenes* 7955 spores is the best candidate as a surrogate.

(4) High pressure destruction kinetics studies on *Clostridium botulinum* are valuable and limited in literature. Extensive studies were carried out in cooperation with Bureau of Microbial Hazards, Health Canada (Ottawa, Ontario) on HP destruction of these spores. Since it has been recognized that the resistance of *Clostridium botulinum* to HP destruction is strain dependent, a screening was carried out to identify the high pressure resistant *C. botulinum* group I spores in buffer. PA9508B, HO9405A and CK2-A were found to be more resistant spores in *C. botulinum* group I. There was no correlation between HP destruction and sero-type. The strain PA9508B spores was found to be the most HP resistant with lower pressure and thermal sensitivities. The work was confirmed in both buffer and milk media.

(5) The HP destruction kinetic studies tend to indicate that at process temperatures traditionally used in thermal processing the *Clostridium botulinum* spores the high pressure likely to loose its inactivation enhancement ability which is so apparent at lower process temperatures. It is necessary to recognize this fact especially because conventional notion implies that HTST techniques are better for quality retention.

Recommendations for Future Research

This research work has demonstrated several important findings. Meanwhile it also showed some interesting areas for future research and development, which could be summarized as follows:

(1) Further HP destruction kinetics of *C. sporogenes* 7955 and *C. botulinum* PA9508B spores should be carried out using other low acid foods (such as pork meat, meshed potato, carrot) to confirm kinetic parameters under combinations of high-pressure and high-temperature. It is important for validation of high pressure sterilization processing and process validation.

(2) Some literature data show *B. amyloliquefaciens* to have higher pressure resistance under high-pressure at elevated temperatures. It could be better surrogate bacterial spore for high pressure sterilization validation, if found to consistently have higher pressure resistance than *Clostridium sporogenes. botulinum.* Only further studies can yield better insight in to this since the published destruction kinetics studies in literature have a lot of variability.

(3) *C. botulinum* HO9504A and CK2-A spores in milk had a higher resistance, so they should also be explored further.

(4) It would be useful to extend the process temperatures to beyond those employed in the study to 120-135°C range. Since the process times are likely to be shorter, the same insulating set-up developed in this study may be sufficient.

(5) It would be interesting to explore the influence of food components such as proteins, fats and carbohydrates on HP destruction kinetics of bacterial spores.

(6) POM demonstrated good insulation under high-pressure and high-temperature application. Looking for new high polymer materials with lower thermal conductivity and high mechanical performance would be helpful for equipment design and high pressure engineering.

(7) Evaluation quality change in high-pressure high-temperature treated foods quality would provide more information for new product development.

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iii) The protocol for decontaminating spills

Spills seldom occur in our laboratory since good house keeping is encouraged and monitored on a regular basis to prevent such events. However, in the event of a spill, the protocol for decontaminating spills of type 2 microorganisms is as outlined in both the McGill and Department laboratory safety manuals. If the spill occurs on the bench, it is absorbed by the white paper bench cloth routinely placed on each bench. The cloth is removed and the "contaminated" area is soaked with 1% hypochlorite solution for ~ 30 minutes and then wiped dry. Then the area wiped up with paper towels soaked in 70% ethanol. If spill occurs in the safety cabinet, the spill is wiped up and then the area is soaked with 70% ethanol for ~ 30 minutes, dried with clean paper towels and the U.V light left on for ~ 1 hour prior to resumption of work. Students/ staff do not enter into the lab for at least 1 hour after a spill. All towels, contaminated paper clothes etc, lab coats, masks are placed in autoclavable bags and decontaminated by sterilization. All bags are clearly labeled "Autoclaved and Sterilized".

If a spill occurs on the body, the clothing is removed and sterilized. Splashes to the face are washed with germicidal soap and hot water. All spills are reported to the laboratory supervisor and a follow up session is done with the students to go over the cause(s) of the spill and to re-enforce preventive measures.

Copies of the McGill and departmental safety protocols are in the lab all the times for perusal by the students. Furthermore most of the work done in our laboratory is under the constant supervision of Mr. Bernard Cayouette.

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7. **Does the protocol present conditions (e.g. handling of large volumes or high concentrations of pathogens) which could increase the hazards of the infectious agent(s)?**
   No, Cultures are prepared in 10 ml amounts, in triplicate, for use in the inoculation studies.

8. **Do the specific procedures to be employed involving genetically engineered organisms have a history of safe use?**
   N/A

9. **What precautions are being taken to reduce production of infectious droplets and aerosols?**
   All inoculation/enumeration procedure are done under the strictest of aseptic conditions in a biological safety cabinet. All students/ staff wear appropriate protective clothing when handling cultures or inoculated foods.

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10. **List the biological safety cabinets to be used.**

Building	Room No.	Manufacturer	Model No.	Serial No.	Date Certified
Macdonald Stewart	1-055	Labconco	36205-04	247196	Nov 21/03 11/09/01