Process Considerations on the Application of High Pressure Treatment at Elevated Temperature Levels for Food Preservation

vorgelegt von
Ing. Adriano Ardia

von der Fakultät III – Prozesswissenschaften
der Technischen Universität Berlin
zur Erlangung des akademischen Grades

Doktor der Ingenieurwissenschaften
- Dr.-Ing. -

genehmigte Dissertation

Promotionsausschuss:
Vorsitzender: Prof. Dr. Roland Tressl
Berichter: Prof. Dr. Dipl.-Ing. Dietrich Knorr
Berichter: Prof. Dr. Gustavo Barbosa-Canovas

Tag der wissenschaftlichen Aussprache: 27.05.2004

Berlin 2004
D 83

To those who I love….

INDEX

	Page
Abstract	III
1 Introduction	1
1.1 High pressure technology and microbial inactivation: general aspects	1
1.2 Foodborne organisms	3
1.2.1 Pathogens under pressure	3
1.2.2 Spores under pressure	6
1.3 Pressure build-up and adiabatic heat of compression of liquid systems	15
1.4 Objectives of the work	19
2 Material & methods	20
2.1 High pressure Equipments	20
2.1.1 Multivessel Model U111	20
2.1.2 SIG-Simonazzi LAB50 unit	22
2.1.3 Micro-System unit up to 1400 MPa	23
2.1.3.1 Technical aspects	27
2.2 Microbial production & high pressure treatments	31
2.2.1 Vegetative cells	31
2.2.1.1 *Lactobacillus rhamnosus* GG ATCC 53103	31
2.2.1.2 Enumeration techniques for low concentration levels	32
2.2.2 Spore forming bacteria preparation	33
2.2.2.1 *Alicyclobacillus acidoterrestris* DMS 2498	33
2.2.2.2 *Bacillus stearothermophilus* ATCC 7953	34

2.3 HHP treatment of sucrose solutions and orange juice 35

2.4 Regression analysis 37

2.4.1 Modelling the adiabatic heating of compression of fluid systems 37

2.4.1.1 Water & sucrose solutions 37

2.4.1.1.1 Mixing rules 37

2.4.1.1.2 Mathematical routine 38

2.4.1.2 Di-2-ethyl-hexil sebacate & n-hexane 39

2.4.2 Modelling the pressure-temperature impact on the microbial inactivation 40

3 Results & discussion 42

3.1 Adiabatic heat of compression of fluid systems 42

3.1.1 Sucrose solutions and orange juice 42

3.1.2 Di-2-ethyl-hexil sebacate and n-hexane 44

3.2 Microbial inactivation & modeling 47

3.2.1 *Lactobacillus rhamnosus* GG ATCC 53103 in orange juice 47

3.2.2 *Alicyclobacillus acidoterrestris* DMS 2498 in orange juice 50

3.2.3 *Bacillus stearothermophilus* ATCC 7953 in buffer solutions 54

4 Process optimization & technical aspects 66

4.1 Numerical modelling on the heat transfer during HHP treatments 66

4.2 High pressure unit design for ultra-high pressure treatments 70

5 Conclusions 73

Annex 77

References 83

Abstract

Among the new emerging technologies which are proposed to allow quality retention or minor quality damages in food products, High Hydrostatic Pressure (HPP) is considered to be a real and efficient alternative process to the most used and common industrial techniques for food preservation since it offers a chance of producing food of high quality, greater safety and increased shelf-life.

It is the aim of this work to explore ways in which high pressure can be used to achieve sterilization of most resistant microbial species in foods in which conventional thermal processing fails to obtain competitive high-quality products.

In order to match these requirements, considerations on the process itself and on possible process improvements have been developed which take into account for the physics of the pressure build-up phase. Studies on modelling the adiabatic heat of compression of basic fluids and on the inactivation of vegetative cells and spore-forming bacteria in different media at low pressure levels (700 MPa) and within short compression times have been done.

In order to proof that a gradient of temperature may result in a non-uniform spatial inactivation in the product sample, a mathematical model, which was developed to follow the thermal increase during the build-up phase of fluid systems, was implemented into a self-made software which was performed to predict the microbial inactivation of vegetative cells and spore-forming bacteria as function of the spatial temperature distribution in the product during the whole pressure treatment.

On the base of results which were obtained at low pressure conditions (700 MPa), an innovative high pressure unit was designed which allowed the generation of higher pressure levels (1400MPa) in short times (less than 20 s) and in adiabatic conditions.

The set-up of the ultra-high pressure unit was performed, by which it was possible to carry out investigations on the adiabatic heat of compression of different fluids, in a wide range of pressure and temperature, and it was possible to perform studies on the inactivation kinetics of bacterial spores at higher processing conditions, in order to clarify the mechanism of inactivation of highly resistant organisms.

In addition, a separate section for process optimization and suggestions on technical solutions is presented.

Zusammenfassung

Unter den neuartigen, nicht-thermischen Verfahren stellt vor allem die Anwendung von hohem hydrostatischem Druck (HPP) eine effektive und erfolgversprechende Alternative zu konventionellen industriellen Prozessen der Lebensmittelverarbeitung dar, um Lebensmittel mit verlängerter Haltbarkeit bei hoher Produktqualität und –sicherheit zu erzeugen .

Ziel dieser Arbeit ist die Bewertung des Potentials einer Hochdruckanwendung zur Sterilisation auch von widerstandsfähigen Mikroorganismen in Lebensmitteln, bei denen thermische Verfahren nicht geeignet sind, hochqualitative Produkte zu erzeugen. Um dieser Anforderung gerecht zu werden, wurde, unter Berücksichtigung der physikalischen Grundlagen während der Druckaufbauphase die Anwendung von Hochdruck untersucht sowie Möglichkeiten zur Optimierung des Verfahrens entwickelt. Die adiabate Erwärmung während der Kompression von Modellfluiden sowie die Inaktivierung von vegetativen Zellen und sporenbildenden Bakterien in unterschiedlichen Medien bei niedrigem Druckniveau (700 MPa) und schnellem Druckaufbau wurde untersucht und mathematisch modelliert. Um das Vorliegen eines Temperaturgradienten nachzuweisen, der zu einer inhomogenen Verteilung der Behandlungsintensität führt, wurde eine mathematische Modellierung des Temperaturverlaufs während des Druckaufbaus in flüssigen Systemen durchgeführt. Durch Implementation in eine selbst entwickelte Software konnte die resultierende Inaktivierung von vegetativen Zellen sowie Sporenbildnern vorhergesagt werden. Basierend auf den bei niedrigem Druckniveau (700 MPa) erhaltenen Ergebnissen wurde eine neuartige, innovative Anlage zur Anwendung hoher hydrostatischer Drücke entwickelt, die das Erreichen eines höheren Drucks (1400 MPa) innerhalb sehr kurzer Zeit (kleiner als 20 s) unter adiabaten Bedingungen ermöglicht. Durch den Bau dieser Ultra-Hochdruckanlage konnten Untersuchungen zur adiabaten Erwärmung während der Kompression unterschiedlicher Fluide in einem weiten Druckbereich durchgeführt werden. Darüber hinaus wurde die Inaktivierungskinetik bakterieller Sporen bei höheren Drücken untersucht, um die zugrunde liegenden Mechanismen der Inaktivierung extrem resistenter Organismen aufzuklären. In einem weiteren Teil der Arbeit werden Vorschläge zur Optimierung des Prozesses sowie technische Lösungsansätze für entsprechende Anlagen vorgestellt.

1 Introduction

1.1 High pressure technology and microbial inactivation: general aspects

High pressure technology is now entering North American and European markets since it is gaining in popularity because of its great potential to improve the quality of existing products and to put forward novel developments. Great interest for this technology comes from beverage industries.

HPP is a processing tool that has recently reached industrial relevance with industrial sized pressure units available up to 800 MPa (Ardia, 2003).

High pressure treatment has been used for sterilization or pasteurization of foods although sterilization has been limited to small scale processing.

Compared with conventional thermal processes, several benefits of HHP treatment can be identified.

High pressure is instantaneously and uniformly applied all over the product and the pressure transmission is practically without delay.

One of the main problems related to heat treatments is the difficulty to achieve uniform distribution of temperature in the product without the risk of over-processing: the heat flux generated by the gradient of the temperature between the coldest point of the product, the centre, and the warmest point of the system, the heat source external to the product, may produce an undesirable over-processing effect which is mainly due to the low heat conductivity of the product. In order to reach the desirable value of temperature in the centre of the product, long treatment times are required and as a consequence, less quality of the final product is achieved.

Indeed, hydrostatic pressure is uniformly distributed all over the sample and a uniform pressure distribution in the product is realized within seconds.

Like high temperatures, high pressure levels can result in gelatinization of starch granules, in denaturation of proteins (i. e. enzymes) and in the reduction of the microbial population; on the other side, high pressure does not affect the molecular covalent bonds and inhibits chemical reactions, like Maillard reactions, off-flavours production, vitamins destruction and enzymatic browning.

Microbial inactivation is one of the main task for the application of high pressure

technology.

Since pressure is uniformly applied in the product, uniform microbial inactivation can be achieved all over the product.

In general, vegetative cells are inactivated at low pressure levels, around 400-600 MPa, while more resistant bacterial spores can survive pressures higher than 1000 MPa (Patterson et al., 1995).

High pressure affects the permeability of the cellular membrane, which is responsible for the nutrient and respiration transport mechanisms of the cell. The modified permeability of the cellular wall results in a disturbed transport mechanism which produces a loss of nutrients and complete death of the cell.

The inactivation level which follows high pressure treatment depends of several factors which are related through them: number and type of present microbes, pressure level and duration of the treatment (holding time), temperature and composition of the product.

Sensibility to pressure can change depending on the growth phase of the microbe.

In general, cells in the exponential phase of growth are less resistant to pressure than cells which are in a stationary phase of growth (Earnshaw et al., 1995).

Temperature may have a significant effect on the microbial inactivation.

Knorr (1993) observed a synergistic effect in the combination of thermal and hyperbaric treatments on the inactivation of microbes, demonstrating that an increase of temperature could result in higher and more efficient microbial reductions.

It has been demonstrated that bacterial spores require more extreme conditions of pressure and temperature and longer treatment times to be inactivated (Sale et al., 1970).

Up-to-date, high pressure technology has been limited to pasteurization processes, since the presence of quite high pressure resistant organisms and also the achievable pressure limit in industrial plants seem to be the real limit of this application in food industry.

1.2 Foodborne organisms

1.2.1 Pathogens under pressure

Foods can be vehicles for transmission of infection.

Some foodborne diseases are well recognized while some foodborne pathogens are considered to be emerging because they are new microorganisms or because the role of food in their transmission has been recognized only recently.

Outbreaks of salmonellosis have been reported for decades, but within the past 25 years the disease has increased in incidence on many continents. In the Western hemisphere and in Europe, *Salmonella serotype Enteritidis* (SE) has become the predominant strain. Investigations of outbreaks indicate that its emergence is largely related to consumption of poultry or eggs.

Yuste et al. (2000) subjected poultry sausages inoculated with *Salmonella enteritidis* to HPP at 500 MPa by combining thermal treatments at 50, 60 and 70°C for 10 and 30 minutes. Most of the pressure treatments generated statistically higher reductions than the corresponding heat treatments alone. A reduction of 7.5 log cycles was obtained, suggesting that high pressure processing is a suitable alternative process for preservation.

Masschalack et al. (2001) studied the inactivation of six gram-negative bacteria by high hydrostatic pressure treatment in the presence of hen egg-white lysozyme, denaturated lysozyme and lysozyme-derived peptides. Under pressure, all bacteria except two *Salmonella* species showed higher inactivation in the presence of 100 μg/ml of lysozyme than without additive, indicating that pressure sensitized the bacteria to lysozyme.

Alpas et al. (1999) investigated the pressure resistance of six *Salmonella* strains at 345 MPa for 5 to 15 minutes at 25 and 50°C. The lower temperature value yielded a reduction of 5.5-8.3 log cycles while the higher temperature level resulted in more than 8 log-cycles within 5 minutes.

Tholozan et al. (2000) studied the physiological effects of HPP on *Salmonella typhimurium*.

Pressure treatments were performed at room temperature for 10 minutes in sodium citrate (pH 5.6) and sodium phosphate (pH 7.0) suspension buffers. Higher acidity

was found to increase the pressure sensitivity and exponential decrease of cell counts was observed increasing pressure levels.

Infection with *Escherichia coli* serotype O157:H7 (EHEC) was first described in 1982. Subsequently, it has emerged rapidly as a major cause of bloody diarrhoea and acute renal failure. Outbreaks of infection, generally associated with beef, have been reported in Australia, Canada, Japan, United States, in various European countries, and in southern Africa. In 1996, an outbreak of *Escherichia coli* O157:H7 in Japan affected over 6,300 school children and resulted in 2 deaths. This is the largest outbreak ever recorded for this pathogen.

Alpas et al. (1999) investigated the pressure resistance of six *E. coli O157:H7* strains at 345 MPa for 5 to 15 minutes at 25 and 50°C. The higher temperature level yielded more than 8 log- cycles reduction within 5 minutes.

Benito et al. (1999) observed that most pressure resistant strains of *E. coli O157:H7* were also more resistant to mild heat, acid, oxidative agents and osmotic stresses, concluding that the inactivation of resistant strains can be achieved through a combination of mild processes.

García-Graells et al. (2000) combined high pressure treatments with lactoperoxidase systems to investigate the inactivation of 4 *E. coli* strains in milk. The use of lactoperoxidase did not improve the inactivation when combined with high pressure. It was observed that milk had a protecting effect against pressure.

Listeria monocytogenes is considered emerging because the role of food in its transmission has only recently been recognized. The disease caused this pathogen by is most often associated with consumption of foods such as soft cheese and processed meat products that are kept refrigerated for a long time because *Listeria* can grow at low temperatures. Outbreaks of listeriosis have been reported from many countries, including Australia, Switzerland, France and the United States. Two recent outbreaks *of Listeria monocytogenes* in France in 2000 and in the USA in 1999 were caused by contaminated pork tongue and hot dogs, respectively.

Morgan et al. (2000) investigated the combined effect of high pressure treatments and lacticin 3147 on *Listeria innocua* DPC1770 in milk. The combination of 250 MPa and lacticin 3147 resulted in more than 6 log-cycles reduction.

Karatzas et al. (2001) observed a synergistic effect of high pressure treatments

combined with the antimicrobial action of the plant-derived volatile carvacrol at different temperatures on *Listeria monocytogenes Scott A*. Lower temperatures resulted in more effective antimicrobial effects.

Ritz et al. (2001) investigated the damage inflicted to *Listeria monocytogenes* cells treated by high pressure for 10 minutes at 400 MPa in pH 5.6 citrate buffer. No cell growth occurred after 48 hours on plate count agar.

Chen and Hoover (2003) obtained 8 log-cycles inactivation of *Listeria monocytogenes Scott A* in UHT milk combining 500 MPa and 50°C for 5 minutes. The same inactivation level was reached at 22°C after 35 minutes. The authors concluded that treatment temperatures of 40-50°C would allow the use of lower pressure or shorter treatment times.

Viruses have emerged as important causes of foodborne and waterborne diseases in recent years, with numerous outbreaks associated with Norwalk viruses, genus *Norovirus*. Norovirus-associated gastroenteritis is transmitted by the fecal-oral route.

Humans of all ages are susceptible to rotavirus infection. Infantile diarrhea, winter diarrhea, acute nonbacterial infectious gastroenteritis, and acute viral gastroenteritis are names applied to the infection caused by the most common and widespread group A rotavirus.

Water, shellfish, and salads are the most frequent sources for infection by *Hepatitis A*, which can be transmitted through fecal contamination of food or water.

Khadre et al. (2002) subjected human *rotavirus* to high pressure treatment at 300 MPa and 25°C for 2 minutes. A reduction of 8 log-cycles was obtained but a small fraction of the virus population remained resistant to pressure treatments at 800 MPa for 10 minutes.

Kingsley et al. (2002) investigated the impact of HPP on the inactivation of *hepatitis A* virus (HAV), *poliovirus* and a *Norwalk* virus surrogate. The authors observed complete inactivation of HAV after 5 minutes at 450 MPa, while *poliovirus* was unaffected by a treatment at 600 MPa for 5 minutes. The feline calcivirus was completely inactivated after 5 minutes at 275 MPa.

The five *Vibrio* species which pose a foodborne health risk to humans are *V. cholerae*, *V. parahaemolyticus*, *V. vulnificus*, *V. mimicus* and *V. hollisae*. All five species have been implicated in food-poisoning outbreaks associated with shellfish.

Berlin et al. (1999) obtained complete inactivation when subjected six different pathogenic *Vibrio* strains to 200 and 300 MPa for 5-15 minutes at 25°C. The authors suggested the use of elevated temperatures during HPP to ensure complete inactivation of *Vibrio* in seafood.

1.2.2 Spores under pressure

Bacterial spores are not by themselves a hazard to the food industry.

It is the eventual germination, outgrowth, and proliferation of the organism which results in spoilage, causing sometimes problems of off-flavour and browning to the product.

It is not always possible to detect spoilage caused by bacteria which can multiply without changing the appearance, odour or taste of the food.

Temperature may have a significant effect on the microbial inactivation.

Spoilage must be expected within a wide temperature range. The various types of microorganisms as well as the genera, species and strains vary in their temperature as well as food requirements, thus the bacterial flora of a spoiled food will vary greatly.

Factors affecting endospore formation include temperature of growth, pH of the medium, aeration, the presence of minerals, especially Mn^{2+} and Ca^{2+}, the presence of certain carbon or nitrogen compounds, and their concentrations (Bergère, 1991).

Some microorganisms respond to starvation or environmental stress not only in the often observed way of producing antibodies or stress proteins (Stevens, 1992) but by starting a process of structural differentiation that results in highly compartmentalized cells with an extremely low metabolic activity. In the dormant state, spores undergo no detectable metabolism and exhibit a higher degree of resistance to inactivation (Heinz and Knorr, 2001). During metabolic inactivity, spores are still capable to monitor the nutritional status of their surroundings and can respond rapidly to the presence of nutrients by germinating and resuming vegetative growth (Nicholson, 2000). Thus, spore formation represents a strategy by which the bacterial cell escapes temporally from unfavourable nutritional conditions.

Most of the considerations of how spores resist thermal inactivation are closely linked to the spore ultra-structure (Tipper et al., 1972; Warth, 1978).

During vegetative form, bacteria multiply by symmetric division of single organisms into two daughter cells with identical morphological and genetic characteristics. For spore-forming bacteria, the occurrence of an unequal cell division can be regarded as the first morphological manifestation of the transition from vegetative to dormant state. At this stage, the formation of a septum divides the cell into two compartments of unequal size but with an identical set of chromosomes. From this stage onward, the membrane of the mother cell grows around the forespore compartment, which is already surrounded by an intact membrane. Finally the three membranes enclose the forespore compartment, which will later develop to the central protoplast of the spore.
At this stage the cell is dehydrated: loss of ions like potassium and decrease in pH by approximately 1 unit mark the beginning of water removal.
At the same time, accumulation of peptidoglycan between the inner and the outer membrane initiates the formation of the cortex and the germ cell wall.
In addition, small acid-soluble proteins (SASPs), which seem to play an important role in the spore resistance, are detected. During maturation, the uptake of considerable quantities of divalent cations such as Ca^{++}, Mg^{++} or Mn^{++} occurs. Simultaneously, the mother cell synthesizes the protoplasmatic accumulation of dipicolinic acid (DPA). Due to the progressive dehydration of the core, the final DPA concentration exceeds its saturation level, and it is assumed that DPA-Ca^{++} complexes are formed. The high degree of dehydration causes the spore to have a bright appearance when observed microscopically in the phase contrast illumination.
The release of the spore occurs when lysis of the mother cell is initiated by endogenous lytic enzymes.
After sporulation, the resulting organism is extremely resistant to external attack by physical or chemical means. This resistance is due mainly to the spore's high degree of compartmentalization (Fig.1.2.2.1).
High hydrostatic pressure is capable to inactivate bacterial spores more effectively when combined with elevated temperatures (Sale et al., 1970; Hayakawa et al., 1994; Ananta et al., 2001).
The most important bacteria for food preservation are *Bacillus* and *Clostridium*.
Studies have shown that the antimicrobial effect of HPP against spores is enhanced when used in combination with other treatments including heat (Okazaki, 1996),

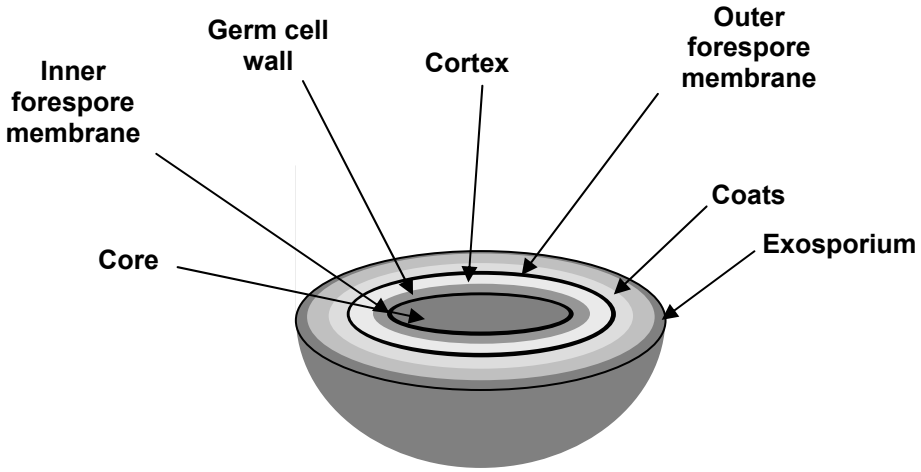


Fig.1.2.2.1 Simplified structure of bacterial spore.

acidification (Roberts, 1996, Murakami, 1998), carbon dioxide (Haas, 1989) and antimicrobial substances such as nisin (Yamazaki, 2000) and sucrose palmitic acid ester (Hayakawa, 1994).

The acidification of foods is often used to prevent the growth of some heat-resistant microbes, because the low pH can reduce the thermal resistance of spores and because many spore-forming bacteria cannot germinate below a certain value of pH.

In each case, no pressure treatment, alone or in combination with other treatments, have been found adequate to inhibit or inactivate bacterial spores in foods.

Yamazaki et al (2000) studied the sensitivity of *Alicyclobacillus acidoterrestris* spores to nisin.

They observed that spores were more sensitive to nisin than vegetative cells and that the inhibition of the outgrowth increased at low values of pH. The authors concluded that nisin addition is a suitable application for preventing the spoilage caused by *A. acidoterrestris* in most acid drinks.

Lee et al. (2002) investigated the effectiveness of combined pressure and heat treatment without use of chemicals for killing spores of *Alicyclobacillus acidoterrestris* in apple juice. The authors observed that spore viability was not appreciably reduced by high pressure alone at room temperature while it was significantly reduced by combined treatments with 45, 71 and 90°C. Treatments at 90°C combined with 414 or 621 MPa for 1 minute reduced the number of viable spores by >5.5 logs to

undetectable levels (<1CFU/ml).

Okazaki et al. (2000) studied the effect of thermal and hyperbaric pressure on the inactivation of four different strains of *Bacillus*. They observed that even if the bacterial spores have the common nature of producing heat resistant spores, each type revealed different survival behaviour by thermal treatment combined with HPP for unknown reasons. For instances, survival curves of *B. subtilis* and *B. stearothermophilus* spores became convex at 400 MPa, 65°C and 120°C for 50 minutes, respectively, while survival curves of *B. coagulans* and *C. sporogenes* spores were linear in a temperature range of 50-110°C and a pressure range of 0.1-400 MPa, thus demonstrating the different pressure-temperature resistance of the four *Bacillus* strains.

Nahayama et al. (1996) reported the resistance of *Bacillus* strains to pressures of 981 MPa for 40 minutes and of 588 MPa for 120 minutes. Spores of all the strains showed the same reaction to the two pressure treatments. Spores of *B. stearothermophilus* IAM12043, *B. subtilis* IAM12118 and *B. licheniformis* IAM13417 were not affected by both pressure treatments.

Gola et al. (1996) reported that combined high pressure and temperature treatments were very effective in reducing *B. stearothermophilus* spore count in phosphate buffer (pH 7.0). A 5-log reduction was achieved at various high pressure, high temperature and treatment times (700 MPa for 5 min or 700 MPa for 3 min, both at 70°C).

Taki et al. (1991) indicated that *B. licheniformis* spores suspended in a pH 7.0 buffer medium could also be inactivated by combining 600 MPa, moderate temperature of 60°C and adequate holding time among 20 and 60 minutes.

Reddy et al. (1999) reported the inactivation of spores of *Clostridium botulinum type E Alaska* at pressures of 758 and 827 MPa. The authors found a 4.5-log reduction at 758 MPa and 50°C for 5-10 minutes of treatment time. A reduction of temperature to 35°C leaded to a 3-log inactivation while an increase of pressure to 827 MPa and a temperature of 40°C for 10 minutes resulted in a 5-log reduction. It was concluded that a processing time of 5 minutes or more is required at these pressures and temperatures to obtain a significant microbial reduction of Alaska spores.

Hayakawa et al. (1994) observed that pressure-treated spores were vulnerable when

exposed to sucrose palmitic acid esters. It was hypothesized that the application of high pressure produced the deposition of sucrose esters on the spore coat and thereby changed the surface hydrophobicity and water permeability during pressurization

Shearer et al. (2000) affirmed that the combination of HPP, mild heat and sucrose laurate was necessary for the inactivation and inhibition of spores of *Bacillus* and suggested a combined treatment to be a valid alternative to conventional heat processing.

The impact of the initial microbial population on spore inactivation has been studied by Furukawa et al. (2002). On the basis of previous works (Furukawa et al., 2001), it was indicated that spore clumps in the initial spore suspension decreased the inactivation rate by hydrostatic pressure treatment. It was observed that the inactivation rate decreased as the initial bacterial concentration increased. Tsuchido et al. (1985) suggested that the surface hydrophobicity of cells was increased by inactivation through heat treatment and that in the vegetative cell suspension, cell clumps were formed as a result of the increase of the hydrophobicity of cells during pressurization. Hayakawa et al (1994) suggested that the surface hydrophobicity of spores was increased by the inactivation through pressurization and that spore clumps were formed through pressurization. Furukawa et al. (2002) concluded that, even if spore clumps were present in the initial suspension, the increase of the inactivation rate of bacteria as their concentration decreases is caused by the bacterial clumps which were formed through pressurization. They suggested that in practical application of hydrostatic pressure treatments, the initial concentration of bacteria should be as low as possible and the initial spore clumps should be removed or prevented by the aim of surface-active agents.

It is well known that pressure may induce germination of bacterial spores.

Wuytack et al. (2000) investigated the *Bacillus subtilis* germination pathways triggered by nutrients and pressure. They observed that a pressure of 100 MPa triggers the germination cascades that are induced by nutrient germinant alanine (Ala) and by a mixture of asparagines, glucose, fructose and potassium ions (AGFK), by activating the receptors for alanine and asparagine, GerA and GerB, respectively. The presence of spore cations, which was found to be important for germination

induced at 100 MPa, appeared to play a minor role for germination induced at 600 MPa, suggesting the existence of a different pathway for germination at 600 MPa.

Setlow et al. (2001) confirmed that the process of germination could be consisting of two Stages. Stage I is characterized by activation of the germinant receptors and release of dipicolinic acid while completion of Stage II requires cortex hydrolysis and the associated swelling and water uptake by the spore core.

Paidhungat et al. (2002) suggested that a pressure of 100 MPa induces spore germination by activating the germinant receptors and that a pressure of 550 MPa opens channels for release of dipicolinic acid from the spore coat, which leads to the later steps in spore germination. They suggest that spores treated at pressures higher that 550 MPa are in Stage I of germination which will be completed in Stage II during the incubation period at temperatures used to assess spore viability on plates.

Setlow et al. (2003) studied the effect of dodecylamine on the germination of spores of *Bacillus subtilis*. Germination was accompanied by almost complete loss of dipicolinic acid, degradation of the spore's peptidoglycan cortex, release of the spore's pool of free adenine nucleotides and the killing of the spores. No metabolism was initiated by spores. The authors suggested that dodecylamine may trigger spore germination by directly or indirectly activating the release of dipicolinic acid from the spore core , through the opening of channels for dipicolinic acid in the spore's inner membrane.

Young et al. (2003) investigated the mechanism of killing spores of *Bacillus subtilis* by hypochlorite and chlorine dioxide observing that spores were not killed by DNA damage and that the major factor in spore resistance appeared to be the spore coat.

Similar results were obtained by Setlow et al. (2002) when spores of *Bacillus subtilis* were exposed to the effect of ethanol, strong acid and strong alkali. Spore's killing was not through DNA damage and the spore coats did not protect spores against these agents. It was suggested that spore killing by ethanol and strong acid involves the disruption of a spore permeability barrier while spore killing by strong alkali is due to the inactivation of spore cortex lytic enzymes.

Lee et al. (2003) studied the effect of acid shock on sporulating *Bacillus subtilis* cells. Wild-type spores and mutant cells were acid-shocked with HCl at 30°C for 30 minutes and 1 hour. An increase of the heat resistance was observed at pH 5 which

was attributed to alteration of spore structure.

Oh et al. (2003) investigated the inactivation of *Bacillus cereus* spores by high pressure treatments at different temperatures. The effect of pH on the initiation of germination was studied. Increasing processing temperature during HPP enhanced the effect of sporulation and consequently the inactivation.

Inactivation of *Bacillus cereus* spores inoculated in model cheese from raw milk by high pressure was tested by López-Pedemonte et al. (2003) in presence of nisin or lysozyme. Following a germination cycle at 60 MPa and 30°C, spores were subjected to two different pressure treatments at 300 or 400 MPa for 15 minutes at 30°C. The combination improved the efficiency of the whole treatment.

Hayakawa et al. (1998) investigated the effect of rapid decompression rates on the inactivation of *B. stearothermophilus* IFO12550. By the assumption that the impact of force upon rapid decompression is much stronger than the force caused by the pressurization alone and that when the stress exceeds the threshold limit of the spore coat strength, the spore is subsequently killed, the authors proposed that the required pressure for sterilization of *B. stearothermophilus* spores could be decreased form 500-600 MPa to 100-200 MPa. The authors claimed to reach sterility at 200 MPa and 75°C for 60 minutes. A very slight difference in decompression rate (0.05 ms) lead to a sharp increase in the magnitude of spore destruction.

This type of mechanism is not yet proven and it is still objective of study.

It has been observed (Wuytack et al., 1998) the inactivation of spores of *Bacillus subtilis* to be more efficient at moderate pressure (200 to 500 MPa) than at higher pressure (>500 MPa). This was explained by finding that pressure can induce spore germination and lost in the thermal and pressure resistance and by assumption that this germination is less efficient at high pressure (Clouston et al, 1969; Gould, 1970; Sale et al., 1970; Sojka, 1994). This leaded to the conclusion that a significant reduction in spore survival could be obtained by application of a cyclic process alternating between low and high pressure at moderate temperatures (40 to 70°C) (Butz et al., 1990; Hayakawa et al., 1994; Sojka, 1994).

Sojka and Ludwig (1997) tested pulsed high pressure on *Bacillus subtilis* ATCC9372 with the first pressure set at 60 MPa and an initial temperature of 70°C for 1 minute, followed by 500 MPa for 1 minute. The cycle was repeated 10 more times and after

10-day incubation period at 37°C in test tubes, no growth was observed.

Oscillatory pressurization was found more effective than continuous pressurization also by other authors (Yuste at al., 1998; Ponce et al., 1998, 1999; Capellas et al., 2000).

Yuste et al. (2001) observed that oscillatory pressurization did not offer better results than continuous pressurization when working with mechanically recovered poultry meat (MRPN). The authors attributed this phenomenon to the fact that microbial resistance to pressure may be increased in food products (Cheftel and Culioli, 1997) and that indigenous microbiota, instead of inoculated laboratory collection strains, should grow more readily even after being severely stressed (Cheftel 1992).

Furukawa et al. (2003) investigated the effect of reciprocal pressurization (RP) on the inactivation of spores of *Bacillus subtilis*. Spores were subjected to pressures form 200 to 400MPa combined with thermal treatments at 25, 35, 45 and 55°C. Scanning electron microscopy and transmission electron microscopy observation showed that spores treated by RP treatment were more morphologically and structurally changed than spores treated by continues pressurization. Significant differences in the release of dipicolinic acid were observed and it was concluded that the core fraction was released into the spore suspension.

The acidification of foods is often used to prevent the growth of some heat-resistant microbes, because the low pH can reduce the thermal resistance of spores and because many spore-forming bacteria cannot germinate below a certain value of pH.

Wuytack et al. (2001) subjected spores of *Bacillus subtilis* to pressure treatments at 100 and 600 MPa and over a pH range from 3 to 8. The inactivation was maximally 80% and was not increased at low pH while higher inactivation levels were obtained when spores were first treated at neutral pH and then exposed for 1 hour to low pH. This was explained by the fact that pressure-induced spore germination, which is known to occur at neutral pH, was inhibited at low pH (<5).The author suggested that acidification of the food after the pressure treatment, instead of before, could improve the total inactivation since an acidification at pH 3 will kill all spores germinated at 100 MPa and a large fraction of the spores germinated at 600 MPa, even without the need for a thermal treatment. The results were in agreement with other literature statements (Ando and Tsuzuki, 1983; Bender and Marquis, 1985; Marquis and

Bender, 1985) where it was observed that at low pH, spore cations like Ca^{2+}, Na^{+}, K^{+}, Mg^{2+} and Mn^{2+} are exchanged with protons, turning the native spores into so-called H-spores which are more sensitive to high temperature and do not germinate at moderate pressure even after a readjustment of the pH to neutrality.

1.3 Pressure build-up and adiabatic heat of compression of liquid systems

The benefits of high pressure technology can be maximized when the adiabatic heat of compression which occurs during the pressure build-up is considered, since microbial inactivation by high pressure is improved at higher temperature levels.

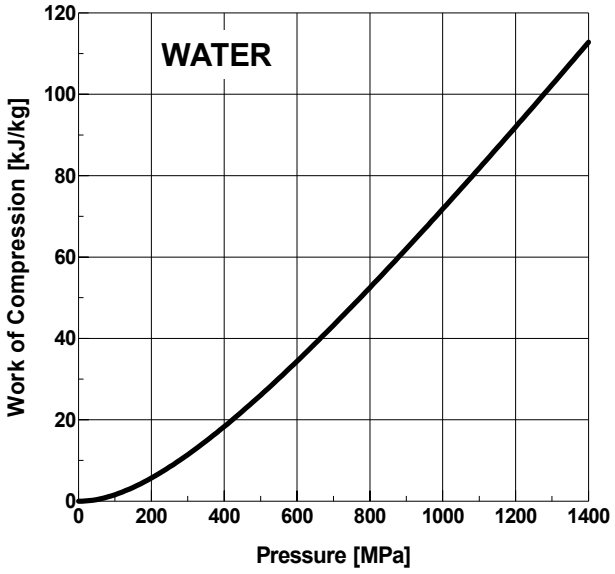


Fig.1.3.1 Work of compression for water expressed in kJ/kg, as function of the pressure level.

All compressible materials change temperature during physical compression, depending on their compressibility and specific heat (Ting et al., 2002)

In water, W_{compr} -the specific work of compression- which can be expressed as

$$W_{compr} = -\int V \cdot dp \qquad \text{Eqn.1}$$

is not higher than approximately 55 kJ in the case when 1 kg of water is compressed up to 1400 MPa.

This value has been obtained upon integration of equation (1) for isothermal situations using the functional relation V=f(p) for pure water given by the NIST formulation (NIST).

In Fig.1.3.1 the specific work of compression W_{compr} is presented for pure water up to 800 MPa.

Following the first and the second laws of thermodynamic and by re-arrangement of the Maxwell equations (Perry, 1984), the temperature change- heating during the

compression and cooling during the decompression- can be described as a function of the thermo-physical properties of the compressible product.

The following Maxwell equation can be used:

$$\left(\frac{\partial T}{\partial p}\right)_S = \left(\frac{\partial V}{\partial S}\right)_p \qquad \text{Eqn.2}$$

where T, p, V, S denotes the temperature, pressure, volume and entropy, respectively.

By mathematical considerations, the right-hand side of equation (1) can be re-written as:

$$\left(\frac{\partial V}{\partial S}\right)_p = \left(\frac{\partial V}{\partial T}\right)_p \cdot \left(\frac{\partial T}{\partial S}\right)_p \qquad \text{Eqn.3}$$

Making use of the definition of the thermal expansion coefficient β as function of temperature T and specific volume V,

$$\beta = \frac{1}{V}\left(\frac{\partial V}{\partial T}\right)_p \qquad \text{Eqn.4}$$

Eqn.3 can be modified as:

$$\left(\frac{\partial V}{\partial S}\right)_p = \beta \cdot V \cdot \left(\frac{\partial T}{\partial S}\right)_p = \frac{\beta}{\rho} \cdot \left(\frac{\partial T}{\partial S}\right)_p \qquad \text{Eqn.5}$$

From the second law of thermodynamic, a relation for enthalpy can be derived:

$$\left(\frac{\partial H}{\partial T}\right)_p = T \cdot \left(\frac{\partial S}{\partial T}\right)_p \qquad \text{Eqn.6}$$

where the left-hand side of equation (6) is defined as the specific heat at constant pressure:

$$Cp = \left(\frac{\partial H}{\partial T}\right)_p \qquad \text{Eqn.7}$$

By combining equation (5), (6) and (7) a general expression for the temperature increase upon compression in adiabatic- isentropic- situations is obtained:

$$\left(\frac{\partial T}{\partial p}\right)_S = \frac{\beta}{\rho \cdot Cp} \cdot T \qquad \text{Eqn.8}$$

where β, ρ and C_p are pressure-temperature dependent.

When the pressure-temperature behaviour of the thermo-physical properties (expansivity, density and specific heat) is known for the product and for the pressure transmitting medium, it is possible to reproduce the thermal profile in the product during the compression phase.

In Fig.1.3.2 the thermal behaviour for water is plotted as function of pressure in dependence of the initial value of temperature in the product.

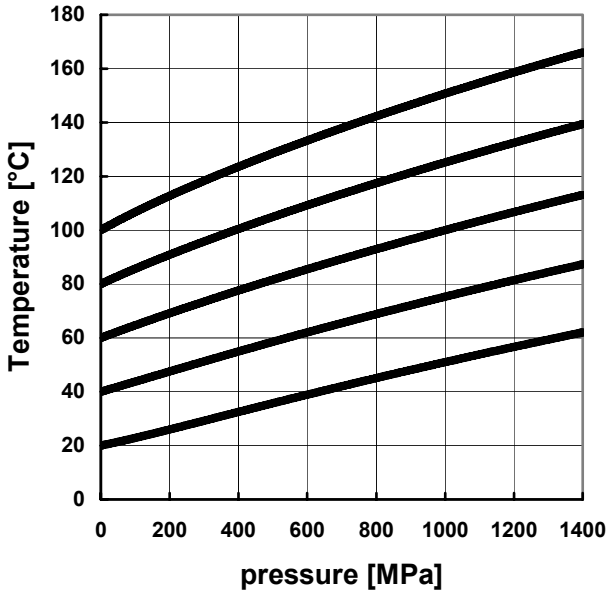


Fig.1.3.2 Thermal behaviour for water as function of p and T prior to compression.

After compression, the product is warmed to a value of temperature which depends on the geometry of the system and on the required time for the compression phase.

Immediately after the pressure release, the product returns to its initial temperature, or even to a lower value, as a consequence of the thermal equilibration which happens during the holding time.

The high cooling capacity is of most interest for the production of high quality foods (Meyer et al., 2000).

The higher is the compression rate, the higher is the absolute value of temperature

which follows the pressure build-up.

Since momentum transport in liquids and solids happens practically without delay, each volume element of the product is characterized by the same pressure level and, in adiabatic situations, by the same temperature. When there is a heat flux across the boundary of the system, the transient temperature field which occurs in the product must be taken into account (Ardia et al., 2004).

In close-to-adiabatic conditions, thermal equilibration occurs in the product during the holding time and even during the pressure build-up, due to the gradient of temperature between the warmest point (the centre of the product) and the coldest point of the system (the metal high pressure vessel), which can lead to non uniform microbial inactivation.

The lower is the compression speed the higher is the effect of thermal equilibration and the lower is the temperature level which is reached by compression. On the other side, the higher is the compression rate, the more homogeneous is the temperature distribution inside of the product, since less time is given to thermal equilibrium. In each case, in non-isothermal conditions, the product is subjected to cooling soon after compression as a consequence of the thermal gradient.

In order to prevent or to reduce this phenomenon and to stabilize the temperature during the holding time, it is possible to choose a sample container which has insulation properties and which provides a barrier to the heat flux, simulating an adiabatic transformation.

In this case, a sterilizing end-temperature can be achieved through the instantaneous adiabatic heating, hence, high pressure levels combined with appropriate mild temperatures before compression and fast compression rates can lead to the inactivation of highly resistant microbial agents.

Coupling pressure and temperature can result in a new approach to food sterilization with a significant improvement in product quality.

1.4 Objectives of the work

High pressure treatments, combined with mild heating have been applied to investigate the inactivation of *Alicyclobacillus acidoterrestris* spores in orange juice.

The effect of short processing times and extreme quick compression and decompression rates on the inactivation of lactic acid bacteria in orange juice was investigated, in order to achieve an extension of the shelf-life of the product, avoiding production off-flavour and browning reactions which strongly reduce the quality of the juice.

The adiabatic heat of compression of liquid fluids was modelled and used within simple mixing rules, simulating the thermal increase which occurs in model food systems during the pressure build-up.

For transient situations like in high pressure vessels, fluctuations in microbial reduction during the treatment should be anticipated.

On the base of results obtained for the inactivation of *Alicyclobacillus acidoterrestris* spores and lactic acid bacteria in orange juice, the impact of a thermal gradient and a not-uniform temperature distribution in the product during the whole pressure treatment was investigated.

In combination with numerical modelling of the transient temperature field and the p-T relation of microbial inactivation, a valuable tool for process design and process control was created.

Following the temperature field simulation, which was then related to the not-uniform microbial inactivation in the pressurized product, an innovative high pressure unit was designed for studying the adiabatic heat of compression of liquid fluids at extreme pressure levels and investigating the impact of extreme pressure-temperature conditions on the microbial reduction of highly resistant organisms, in adiabatic situations.

Spores of *Bacillus stearothermophilus*, which are known to be highly temperature-pressure resistant, were subjected to ultra-high pressure treatments in combination with mild heating, in order find out the optimal operative conditions which could result in a qualitative stable product free of contaminants.

The optimization of high pressure processing which also includes suggestions for possible technical solutions is discussed.

2. Material & methods

2.1 High pressure Equipments

2.1.1 Multivessel Model U111

The high pressure equipment (Model U111, Unipress, Warsaw, PL) consisted of five pressure vessels, completely immersed in a thermostatic bath filled with silicon oil, and connected to a pressure intensifier through capillary tubes (Fig.2.1.1).

This design allowed a simultaneous treatment of five different samples in one pressure build-up at close to isothermal conditions and the possibility to perform reproducible inactivation kinetics up to pressures of 700 MPa and over a wide temperature range between -40°C and 120°C.

Each pressure-chamber, separately connected to an oil-driven intensifier through high pressure valves, was equipped with a K-type thermocouple and a pressure sensor to monitor the temperature and pressure history of the sample during the treatment cycle.

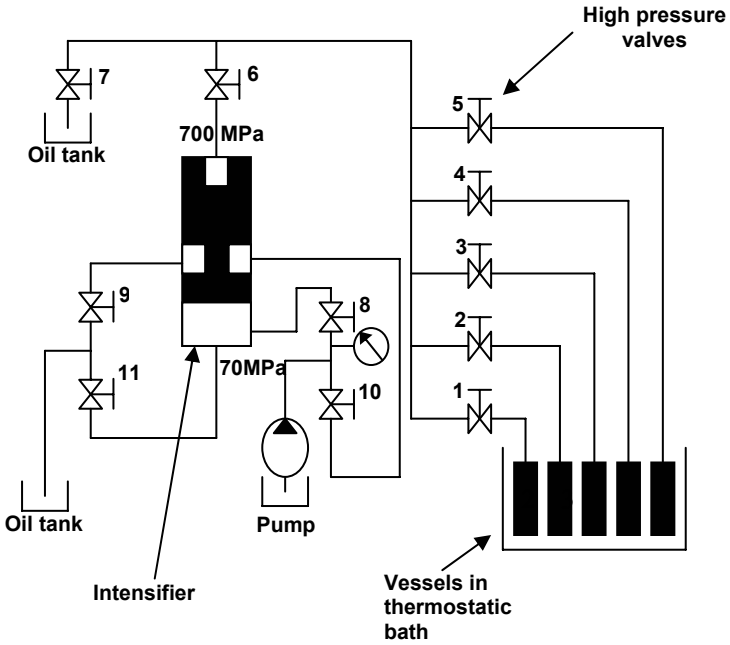


Fig.2.1.1 Schematic hydraulic diagram of multivessel high pressure apparatus U111. The intensifier is connected with the pressure vessels through high pressure valves (1-5). The multiplication factor (~11) of the intensifier leads to a maximum pressure of 700 MPa. Valves 6-11 are used for loading and unloading the pressure medium (silicon oil).

A hydraulic pump (Mannesman Rexroth Polska Ltd, Warszawa, Poland) produced a pressure of 70 MPa in the low pressure part of the intensifier while, due to the section reduction in the intensifier (multiplying factor ≈11), it was possible to reach 700 MPa on the high pressure side.

In all experiments, the compression rate was set constant to reach a pressure level of 600 MPa in 24 seconds.

Making use of a multimeter (Keithley, Multimeter 7001) and a computer acquisition program, the pressure and temperature readings were registered during the pressure build-up with a rate of 3.5 scans per second.

Fig.2.1.2 shows the Multivessel Model U111 set-up.

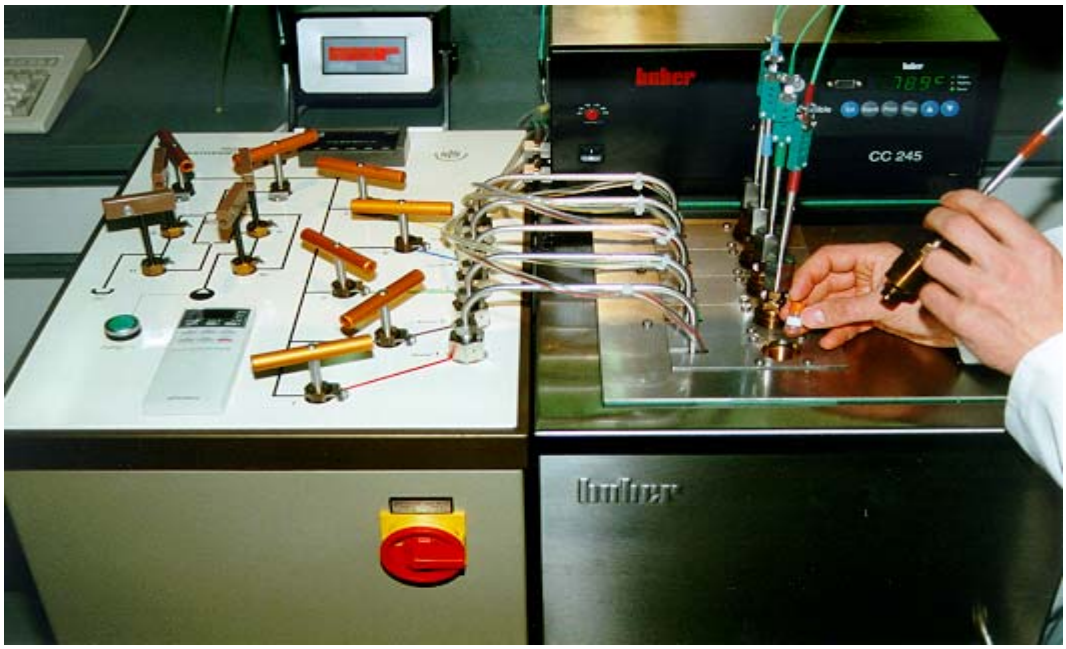


Fig.2.1.2 Multivessel Model set-up.

2.1.2 SIG-Simonazzi LAB50 unit

The LAB 50 single processor machine (SIG Simonazzi, Parma, Italy) is a laboratory system developed to test short time microbiological stabilization of foods and beverages by high pressure treatment (figure 2.1.2.). It is a scaled down version of a bigger industrial unit with the same performances so that the tested processes can straightforwardly be translated to production environments.

Fig.2.1.2 LAB50 unit (SIG Simonazzi, Italy).

The high pressure unit consists of a high pressure processor/intensifier, a hydraulic power unit, an electric cabinet and a PC based control/interface unit

The vessel, made of highest strength steel, is filled with a compression liquid (water-glycol mixture) in order to isostatically transfer the high pressure.

Pressure is generated by an automated hydraulic power unit where a pump loads gas hydraulic accumulators in order to have the amount of oil in pressure already available when needed for very short pressure build up times of a few seconds.

Two pressure multipliers with ratio about 1:3 and 1:10 rise pressure to a maximum of about 600 MPa. The high pressure side of the second multiplier is integrated within the processor and constitutes the processing vessel itself so that all the remaining devices are standard industrial components. New design dynamic seals allow several

thousand cycles before replacement.

This technical design allows fast compression and decompression rates: a processing pressure level of 600 MPa is generated in about 3-4 seconds, while complete release of pressure is realized in less than 1 second.

The instantaneous decompression phase is obtained by discharging the pressure medium in the low pressure side of the internal intensifier (around 60-70 MPa).

The whole pressure generation system is fully automated, a proportional valve and pressure feedback allow tuneable and very reproducible pressure cycles of a few seconds.

Selection of pressure-time profiles as well as diagnostic information and data acquisition are available at the PC interface.

2.1.3 Micro-System unit up to 1400 MPa

The high pressure equipment (Micro-system, Unipress, Warsaw, PL) consisted of one pressure vessel (Fig.2.1.3.1), placed into a heating-cooling block and connected to a high pressure intensifier through a capillary whose diameter was 0.5 mm (Fig. 2.1.3.2).

The heating-cooling block (Fig.2.1.3.3), manufactured in copper-beryllium, allowed quick changes in the temperature of the pressure vessel. Indeed, it was possible to warm the high pressure cell during the compression phase, reproducing the increase of temperature produced by the adiabatic heat of compression of the product sample. This option permitted to realize compression phases in adiabatic conditions, since the gradient of temperature between the sample and the vessel was minimized .

Di-2-ethyl-hexyl sebacate (Sigma-Aldrich Chemie GmbH, Germany) was used as pressure transmitting medium. In ultra-high pressure-temperature ranges, no phase-transition was observed at 1400 MPa and 20°C.

The pressure vessel, realized in stainless steel with a volume of approximately 150 μl, had a conical shape in the lower and higher sections.

Tightness was obtained without the use of sealing parts: metal face-to-face connection between vessel and feed-injection system was enough to ensure stable pressure conditions during the whole treatment.

Pressure was generated by the aid of a step motor (Unipress, PL) which permitted a

pressure build-up to 1400 MPa in less than 20 seconds.

Pressure release was realized by moving the piston back into the high pressure intensifier, always occurring in the time-conditions required for pressure generation.

Both piston and high pressure intensifier were manufactured in a strong alloy matrix.

Tightness between piston and intensifier was obtained through a special packing design.

Fig.2.1.3.4 shows the whole high pressure unit set-up.

The temperature of the cooling medium- silicon oil (Huber, Germany)- was controlled by a thermostatic bath (Huber, Germany), while the temperature of the pressure vessel was controlled through a pressure resistant shielded K-type thermocouple which was installed at the internal surface of the heating-cooling block, directly in contact with the pressure cell.

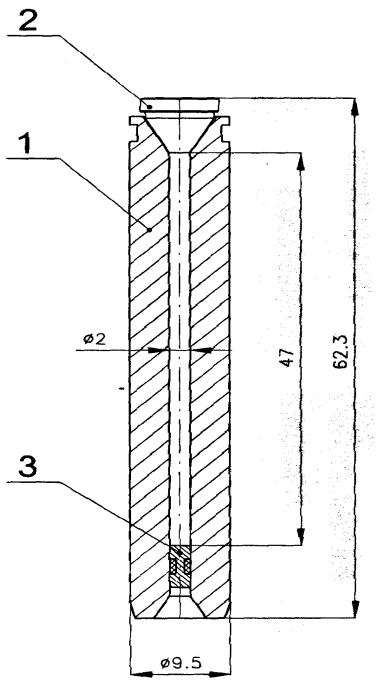
The circuit for the cooling medium was regulated through an electrical valve (Burkert, Germany). Pressure was measured through two different devices: a dynamometer (Unipress, PL) was installed in correspondence of the step motor, while a pressure transducer (EBM, Germany) up to 1400 MPa was connected to the pressure cell.

Fig.2.1.3.5 shows the flow-chart for the temperature and pressure control. On the base of the adiabatic heating profiles of water, which were used to calculate the processing and the initial temperature values as function of the treatment pressure, the control allowed rapid changes of pressure and temperature to realize adiabatic compressions and isothermal holding times.

The two pressure measurements were in agreement. In order to follow the thermal evolution during the pressure treatment in the product, an auxiliary shielded K-type thermocouple was mounted on the top of the pressure-chamber, replacing the clamp screw. The whole pressure treatment was fully automated: a pressure feedback allowed tuneable and very reproducible pressure cycles.

A self-written software, realized with TestPoint (Capital Equipment Corporation, Massachusetts, USA)for pressure-temperature control, was performed, by which it was possible to optimize the processing conditions to realize adiabatic pressure build-up phases and isothermal holding times.

Selection of pressure-time profiles, as well as temperature conditions, diagnostic information and data acquisition, were available at the PC interface.



1. High-pressure vessel
2. Upper closure
3. Floating piston

Fig. 2.1.3.1 Schematic view of high pressure vessel (Handbook, Unipress). The function of the floating piston is to separate the pressure transmitting medium from the product sample.

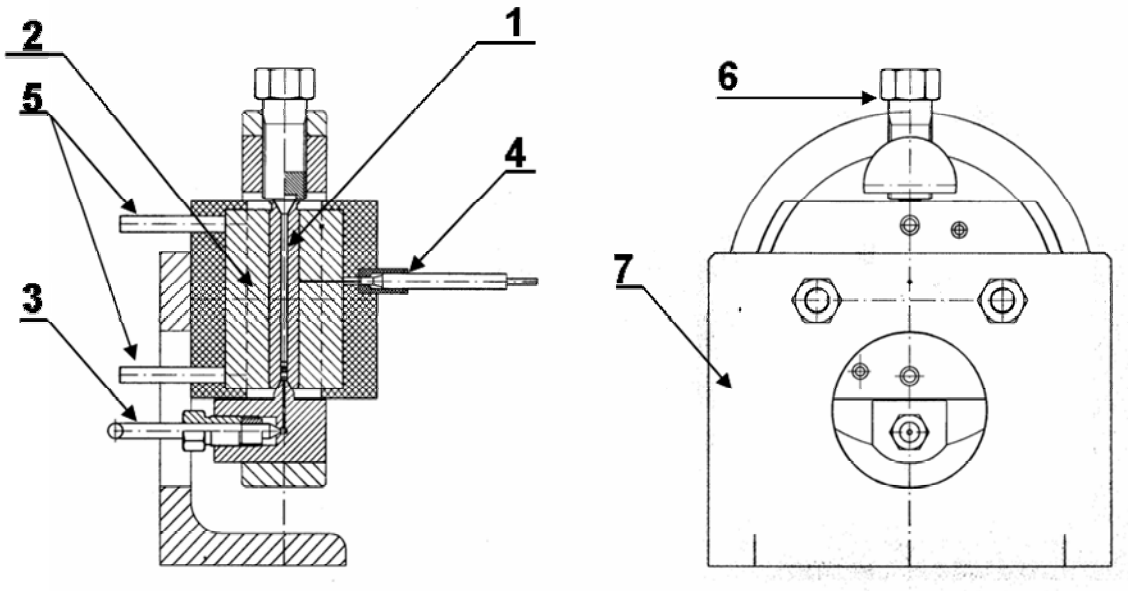


Fig. 2.1.3.2 Schematic view of high pressure vessel and heating-cooling block (Handbook, Unipress). 1. high pressure vessel. 2. heating-cooling block. 3. pressure connector. 4. thermocouple fitting. 5. cooling medium circuit. 6. clamp screw for pressure tightness. 7. Plate for sample cell holding.

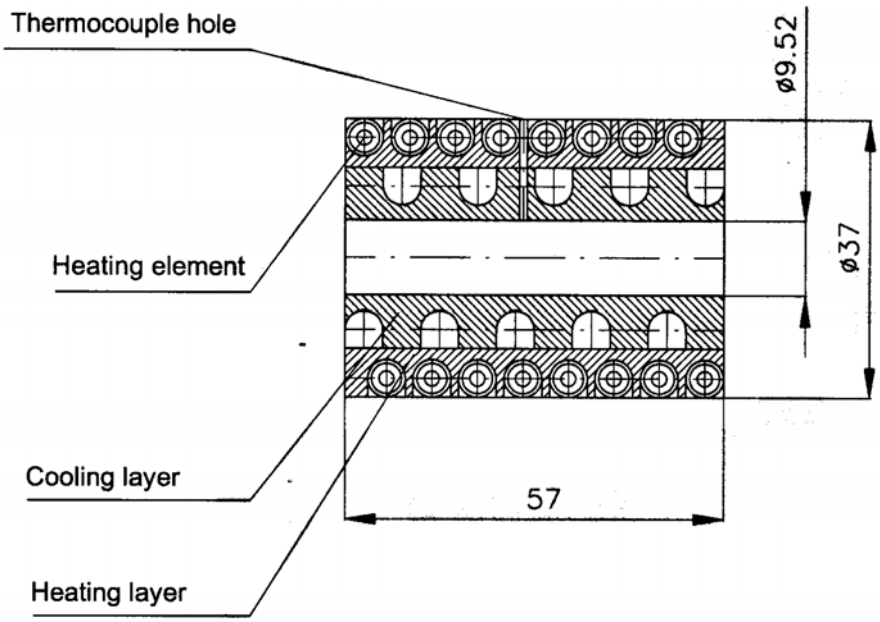


Fig. 2.1.3.3 Schematic view of the heating-cooling block (Handbook, Unipress). The cooling channel is realized in the section closer to the sample cell in order to stabilize temperature during the whole pressure treatment.

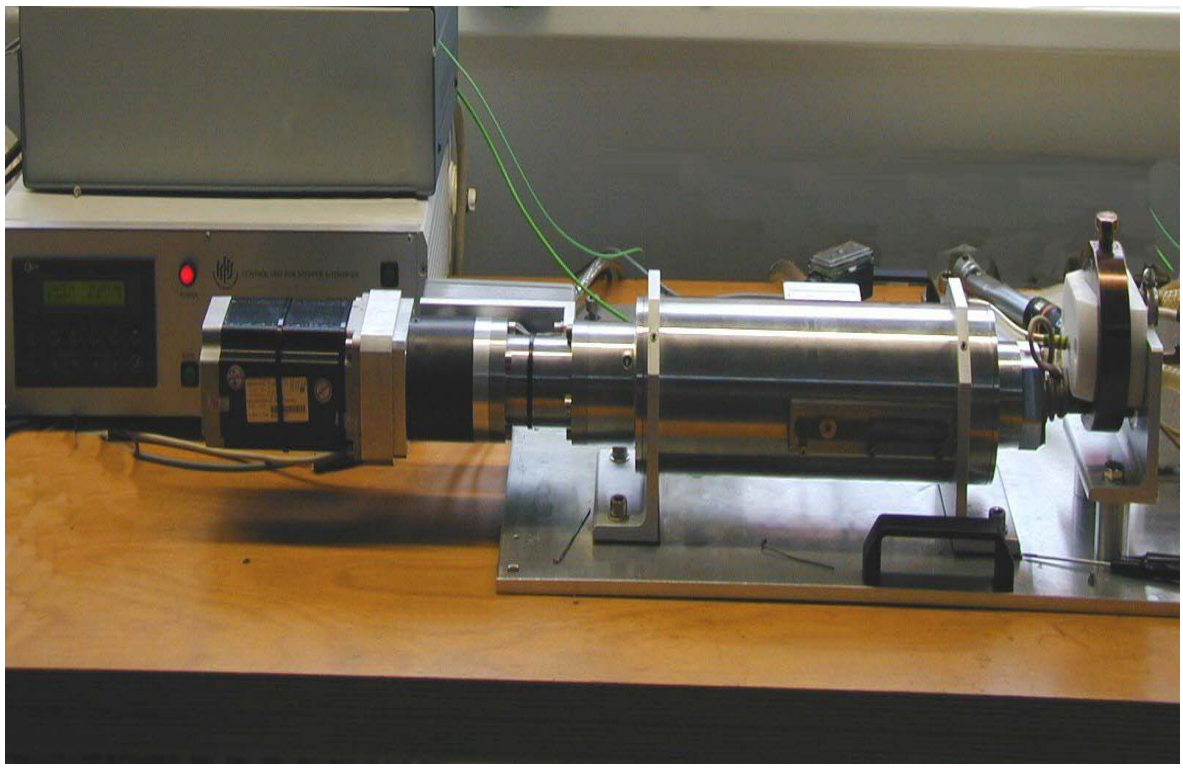


Fig. 2.1.3.4 Set-up of high pressure unit up to 1400 MPa.

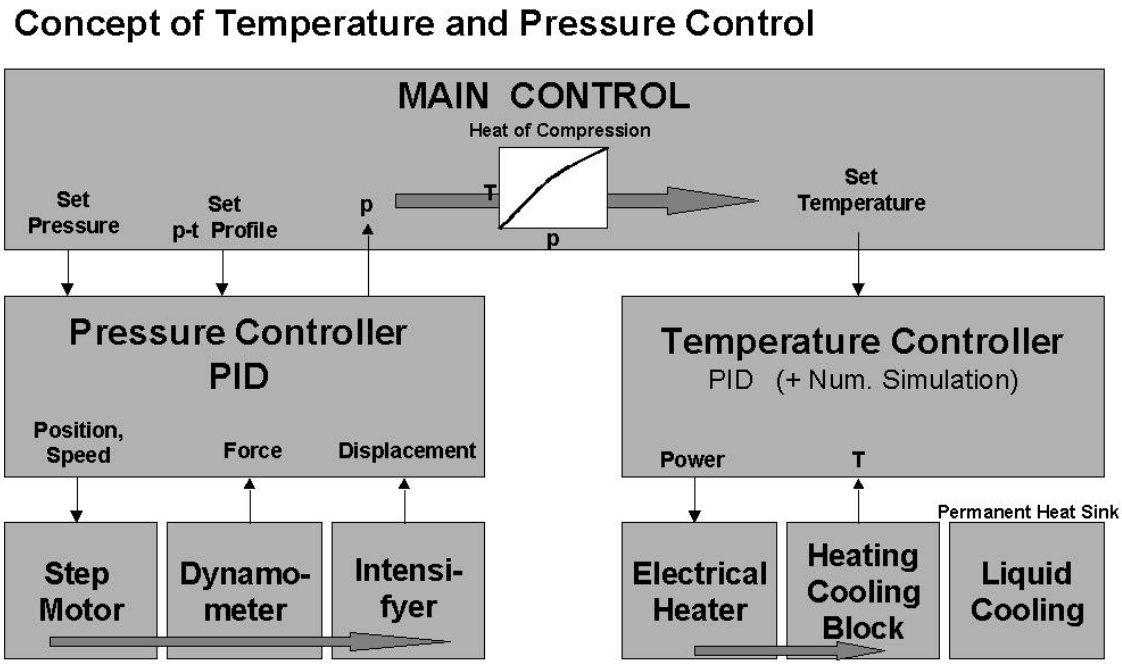


Fig. 2.1.3.5 Schematic representation for the temperature and pressure control.

2.1.3.1 Technical aspects

During the experimental set-up of the ultra-high pressure unit (micro-system), some difficulties were encountered.

Hence, further improvements were necessary to optimize the pressure process.

Plastic deformation of the sample-cell occurred after a certain number of cycles, due to excessive torque required for pressure tightness between upper stopper and clamp screw.

In order to avoid the undesirable phenomenon, an optimum value of the torque was chosen in correspondence of 50 Nm.

By further inspections it was decided to reduce the outer diameter of the sample cell from 9.5 mm to 9.1 mm, which allowed the performance of an acceptable number of pressure cycles.

After the last modification, an improvement of the heat flux between sample cell and heating-cooling block was necessary to avoid convection phenomena. Copper-conductive paste was used to fill the gap between the two metal parts.

One of the main problems related to the geometry and to the mechanical design of the system was represented by the high pressure intensifier, external to the sample-

cell and always kept at room temperature.

After inspections on tightness between piston and intensifier, it was recognized that the original pressure-transmitting medium- mixture of petroleum and extraction naphtha in the ratio 1:1- was producing a leakage in back part of the piston, causing pressure-drop during the compression phase and consequent loss of the pressure-transmitting medium.

Different mixtures of petroleum and extraction naphtha were tested, varying the percentage ratio to produce a more viscous medium which, in any case, could allow rapid pressure transmission.

Also hexane, pentane and ethanol were tested as pure liquids or in mixture with petroleum.

The more adequate medium resulted to be di-2-ethyl-hexyl sebacate which is currently used for calibration procedures of pressure transducer devices.

Literature research (Woo et al, 2002) on this middle-chain hydrocarbon showed no phase-transition occurring in the pressure range of interest (1400 MPa) and at low temperature (around 20°C).

Di-2-ethyl-hexil sebacate resulted to be an acceptable pressure transmission medium and a better stabilizer for the packing system of the piston.

As anticipated, the intensifier stays always at room temperature, with the meaning that during compression and mainly after that the high pressure level is reached, rapid thermal equilibration occurs, cooling the pressure transmitting medium to the initial temperature value.

The use of a fluid which changes phase at room temperature and high pressure levels would not be feasible for ultra-high pressure treatments, causing a delay in the pressure transmission and blockage of the transmission connections.

To reduce the heat flux through the capillary and stabilize the temperature of the pressure-transmitting medium, an external controlled heating system (Isopad GmbH, Germany) was applied on the capillary connection between the intensifier and the sample-cell.

In this way, it was possible to realize high compression rates in the respect of the physic state of the pressure medium.

The change of viscosity of the pressure medium, at ultra-high pressure levels, was

responsible of a pressure-relaxation phenomenon through the capillary tube, causing a delay in reaching the desired pressure value.

For instances, relaxation appeared in a pressure range from approximately 1100 MPa to 1400 MPa.

In order to overcome this difficulty, the temperature of the external controlled heater was set to a value which could account for the adiabatic heat of compression of the pressure medium.

Following the results which will be shown in Fig.3.1.2.1 (chapter 3), it was estimated that the compression of sebacate could result in a thermal increase of approximately 80°C when the starting temperature was set to 20°C.

Hence, pressure treatments at pressure levels higher than 1200 MPa were performed by heating the capillary connector at 100°C during the pressure build-up.

Fig.2.1.3.1 (a) shows the relaxation effect for a pressure cycle up to 1400 MPa.

In particular, the difference between the two pressure signals showed a delay of approximately 30 seconds to reach the maximum pressure.

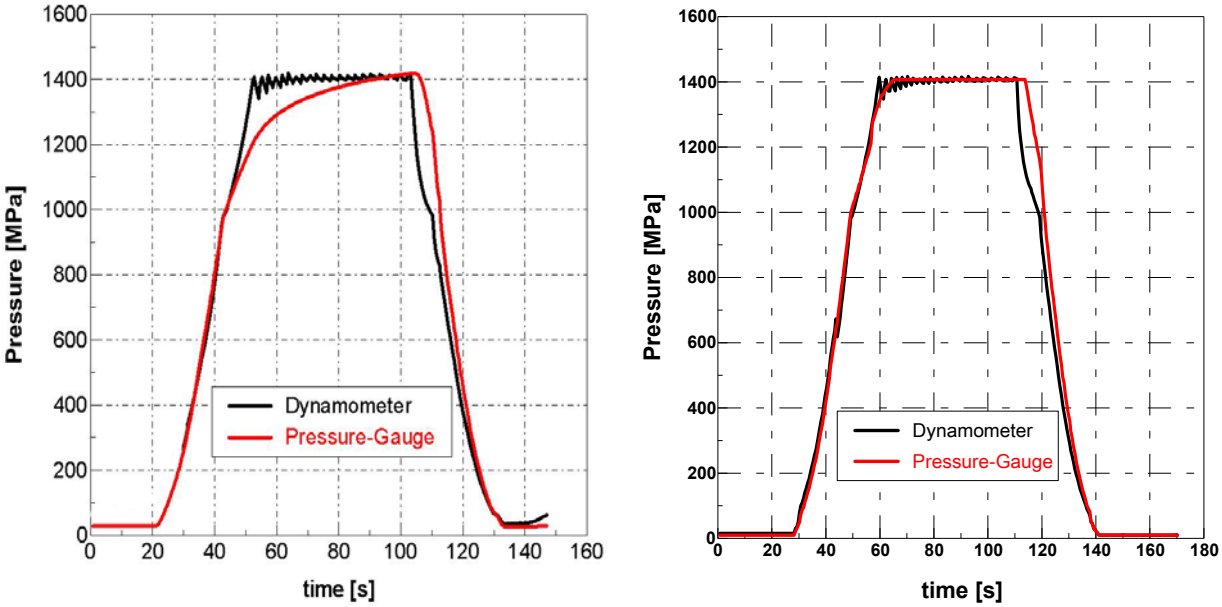


Fig.2.1.3.1 Difference between the two pressure signals, depending on the rapid thermal equilibration in the capillary connection. The red line accounts for the pressure-transducer measurement while the black line is referred to the measurement provided by the dynamometer.

Fig.2.1.3.1 (b) shows the two pressure signals when the capillary connector was heated at 100°C during pressure build-up. Relaxation phenomena were strongly reduced.

In both the graphs it is possible to note the oscillating behaviour for the signal of the dynamometer, which was adopted to control and stabilize the pressure level.

In this way, it was possible to generate acceptable and reproducible pressure cycles.

As shown, choosing for an appropriate pressure transmitting medium could result in an improvement of the combined pressure-temperature treatment and in a strong reduction of treatment times, enhancing the product quality and allowing high production layout.

Depending on a specific product processing, the use of a pressure-transmitting medium which presents an adiabatic heat of compression, comparable or slightly higher than one generated by the compression of the product, would reduce the thermal gradient and produce a more uniform temperature distribution in the sample.

The additional use of an insulating material- for example PET- as the inner layer of the high pressure vessel would strongly enhance the temperature stabilization.

2.2 Microbial production & high pressure treatments

2.2.1 Vegetative cells

2.2.1.1 *Lactobacillus rhamnosus* GG ATCC 53103

Lactobacillus rhamnosus GG ATCC 53103 (*LGG*) was obtained from VTT culture collection (Technical Research Centre of Finland).

From the cell suspension, 5 ml were dropped in Roti®-Store cryo-vials (Carl-Roth, Karlsruhe, Germany).

The bacteria were attached to the beads and properly protected. The contaminated vials were stored in a -80°C freezer (Model U101, New Brunswick Scientific, Nürtingen, Germany).

The growth medium used was MRS broth (Oxoid, Basingstoke, UK). Plate count enumeration was performed using MRS agar (Oxoid, Basingstoke, UK). All media were prepared and sterilized according to the manufacture's instructions. Commercially available orange juice (Hohes C, Eckes-Granini Deutschland GmbH, Germany, pH 3.8) was used for the inactivation experiments.

One bead from deep-frozen *LGG* culture was used to inoculate MRS broth when the starting concentration was adjusted to 10^3 CFU/ml. The culture was incubated at 37°C for 24 hours in order to reach stationary growth phase. After centrifugation at 4923 g for 10 minutes the bacteria were re-suspended in orange juice while a starting concentration of approximately 10^8 CFU/ml was obtained.

HP treatments were carried out in the LAB50 unit at pressure levels from 300 to 600 MPa with holding times from 0.5 to 60 seconds.

Each sample, with a volume of approximately 160 ml, was pressure treated at 40°C taking into account the adiabatic heat of compression which occurs during the pressurization of orange juice. The compression of orange juice up to 600 MPa leaded to a thermal increase of almost 20°C when the sample was kept at room temperature prior pressure treatment.

For lower pressure levels a pre-heating of the samples, prior to compression, was

necessary (e.g. to reach 40°C at 300 MPa the sample was kept at 31°C).

After HP treatment the samples were stored on ice-water until the last treatment cycles were performed. All samples were serially diluted in ringer solution and plated in duplicate on MRS agar. The viable cell number was determined after 48h of incubation at 37°C.

2.2.1.2 Enumeration techniques for low concentration levels

In order to detect low concentrations of *LGG*, the *Most Probable Number Method* (MPN) was applied (Garthright, W. E., 1998).

After pressurization, the samples were diluted in tubes with MRS broth (pH 6.0) and incubated at 37°C for 96 h. From the number of tubes which resulted positive to microbial growth by optical detection of turbidity, the concentration of bacteria (CFU/ml) was calculated.

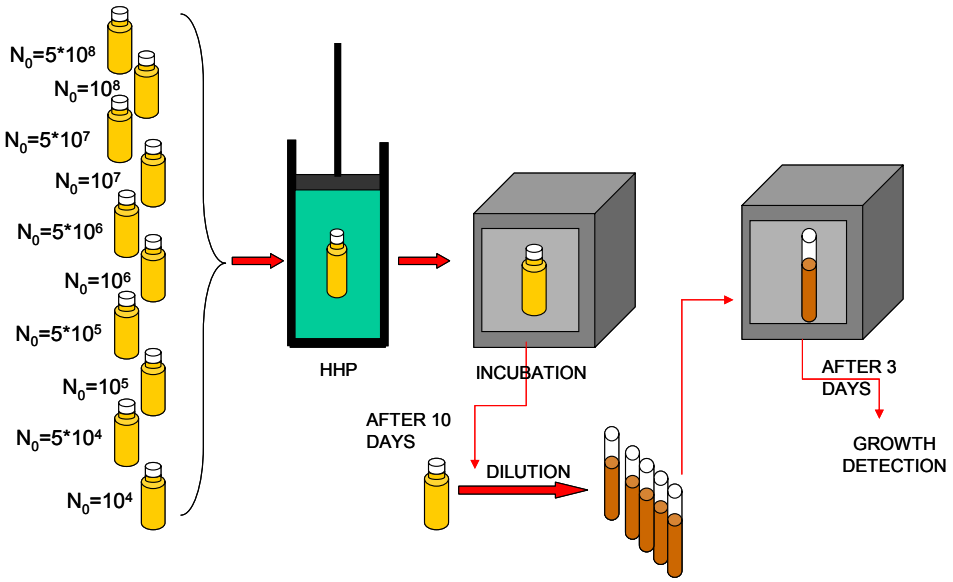


Fig.2.2.1.2 Schematic representation of the applied modified MPN method. Samples were stored at 37°c for 10 days after pressure treatment. After the storage period, 1 ml from each sample was transferred into MRS broth and again incubated at 37°C for 3 days.

In contrast to the standard MPN method, samples containing different initial concentrations of *LGG*, from 10^2 to $5*10^8$ CFU/ml, were pressurized at 600 MPa for 15, 30, 45 an 60 seconds and then stored for 2 weeks at 30°C.

This procedure was adopted to simulate serial dilution prior to compression.
After the storage period, 1 ml from each sample was transferred into MRS broth (pH 4.0) and incubated again for 3 days at 37°C. The pH of the broth was lowered to rule out the germination of bacterial spores which could have survived the HP cycle.
Fig. 2.2.1.2 shows a schematic representation of the applied modified MPN method.

2.2.2 Spore forming bacteria preparation & treatment

2.2.2.1 *Alicyclobacillus acidoterrestris* DMS 2498

Alicyclobacillus acidoterrestris DMS 2498 spore suspensions (provided by ECKES-GRANINI, Nieder-Olm, Germany) with initial counts of $4x10^9$ to $8x10^9$ CFU(colony-forming unit)/ml were incubated for 24 h in 20 ml standard 1 nutrient broth (Merck, Darmstadt, D) at 55°C.
After incubation, 3 ml of the suspension was spread-plated in petri dishes on selected agar (DSMZ- Deutsche Sammlung Von Mikroorganismen Und Zellkulturen- nr 402) supplemented with 10 mg/l $MnSO_4xH_2O$.
After 4-5 days of incubation at 55°C, the percentage of sporulated cells was assessed by phase contrast microscopy.
When 90% of the cells were sporulated, the incubation was terminated. Spore and vegetative cells were separated by centrifugation at 4923 g for 20 min, followed by a treatment with 70% EtOH and subsequent centrifugation. Finally, the pellet was re-suspended in sterile distilled water, placed in cryogenic vials (Nalgene, Rochester, NY, USA) with a total volume of 2 ml and subsequently frozen at -20°C.
For the dilution phase Ringer solution was used.
Colonies were counted after incubation at 55°C for 2 days.
For the thermal and also combined thermal-pressure treatments, the spore were inoculated directly in commercially available orange juice in the concentration of 1:10.
For the thermal treatments the samples were filled in special glass containers, hermetically closed, in order to prevent the evaporation of the solution due to the long processing time at high temperature.
For the combined temperature-pressure treatments the samples were filled in special

containers (Nunc Cryo Tubes Nr. 375299, Nunc A/S, Roskilde, Denmark) with a volume of 1.8 ml.

Spore inactivation by thermal treatment was investigated by using a thermostatic bath filled with silicon oil at a starting temperature of 80, 85, 90 and 95°C, while combined temperature-pressure treatments were carried out by the high pressure unit Model U111 (previously described at paragraph 2.1.1) at 100, 200, 300, 500, 600 and 700 MPa and the same set of temperatures.

2.2.2.2 *Bacillus stearothermophilus* ATCC 7953

Spore of *Bacillus stearothermophilus* ATCC 7953 (Merck, Darmstadt, Germany) with an initial count of approximately 2 $x10^8$ CFU/ml were incubated for 24 hours in standard-1-nutrient broth (Merck, Germany) at 55°C.

At the end of the incubation period, 3 ml of the suspension were spread-plated in petri dishes on nutrient agar (Merck, Germany) supplemented with 10 mg/l $MnSO_4$.H2O. After 4-5 days of incubation at 55°C, the percentage of sporulated cells was assessed by phase contrast microscopy. When the 90% of the cells were sporulated, the incubation period was terminated.

Spores and vegetative cells were separated by centrifugation at 5000 g for 20 minutes followed by a treatment with 70% EtOH and subsequent centrifugation at 5000 g for 20 minutes. Finally, the pellet was re-suspended in sterile, distilled water and placed in cryogenic vials (Nalgene, Rochester, NY) with a total volume of 2 ml and subsequently frozen at -20°C.

The spore concentration remained constant at approximately 2 $x10^8$ CFU/ml throughout the storage period.

Prior to the pressure, thermal or combined pressure-thermal treatment, the initial spore suspension was centrifuged at 5000 g for 10 minutes and then re-suspended into an equal volume of treatment medium.

Inactivation studies were performed in buffer solutions.

In order to investigate the impact of different acidity levels on the microbial reduction, 0.05 mol phosphate buffer pH 7.0 and Aces buffer pH 7.0- 6.0 (Sigma-Aldrich Chemie GmbH, Germany) were used.

For the evaluation of the impact of sole thermal treatments on the inactivation of *B.*

stearothermophilus spores, the bacterial suspension was filled into special glass containers with a volume of approximately 2 ml and hermetically closed to prevent evaporation of the suspension due to the long processing time at high temperature levels.

A thermostatic bath (Huber, Germany), filled with silicon oil, was used to investigate thermal inactivation kinetics at 90, 100, 110, 120 and 130°C.

Pressure treatment were performed in the ultra-high pressure unit up to 1400 MPa (Micro-system, Unipress, Poland).

Prior to the pressure treatments, the suspension was filled into shrinking tubes (1.6/0.8, RS Components GmbH, Germany) with an inner diameter of 1.6 mm and an outer diameter of 2.2 mm, fitting into the high pressure vessel.

Hence, the tubes were hermetically sealed (Hawo, Gerätebau GmbH, Germany) and stored on ice before treatment.

The samples were inserted into the pressure vessel and treated in a pressure range from 800 MPa to 1400 MPa in adiabatic conditions.

Prior to the compression phase, the samples were warmed at initial temperature which results in the desired processing temperatures of 90, 100, 110, 120 and 130°C after compression.

The whole vessel was heated during the pressure build-up, simulating the adiabatic heat of compression of the product. It was assumed negligible the difference between the adiabatic heating of a buffer solution and pure water.

Once that the pressure level was reached, the temperature of the system was stabilized at the processing value along the holding time.

In order to prevent delays in pressure transmission at high pressure levels, the tests at 1200 and 1400 MPa were performed by heating the capillary connection at 100°C (paragraph 4.2).

2.3 HHP treatment of sucrose solutions and orange juice

Starting point for the setup of a mathematical model for the prediction of the adiabatic heat of compression of sucrose solutions was the performance of a set of experiments by which it was possible the record the increase of temperature which

happens in the centre of the sample during the compression phase. Sucrose solutions were prepared by mixing pure water and sugar in 4 different weight percentages of solid content, 10%, 20%, 40% and 60%.

Since Cp, β and ρ change with pressure and temperature, it was necessary to use different initial temperatures to be able to investigate a large range of the independent variables (p: 0-600 MPa; T:5-90°C).

Distilled water was considered as a sucrose solution with a sugar content of 0% and included in the model calculations.

The samples were filled in special containers (Nunc Cryo Tubes Nr. 375299, Nunc A/S, Roskilde, DK) with a volume of 1.8 ml and equilibrated to the starting temperature (5 to 90°C). The temperature in the centre of the sample was measured using a pressure resistant shielded K-type thermocouple (1 mm diameter and 0.45 s response time, Unipress, Warsaw, Pl).

High pressure treatment were performed in the Multivessel Model U111 by using only one pressure chamber.

Orange juice (Hohes C, Eckes-Granini, Deutschland GmbH, pH 3.8) with a sugar content of 9% was pressurized up to 600 MPa starting from different initial temperatures and the thermal profile in the centre of the sample was recorded.

After numerical modelling analysis, the adiabating heat of compression of orange juice was simulated with a sucrose solution of 9% of solid content.

2.4 Regression analysis

2.4.1 Modelling the adiabatic heat of compression of fluid systems

2.4.1.1 Water & sucrose solutions

2.4.1.1.1 Mixing rules

In order to create a model to predict the adiabating heating of sucrose solutions for different concentrations, the following mixing rules were used to estimate the thermo-physical properties needed for the calculation of *dT/dp* (equation (8)).

Basically, the equations for estimating physical properties of mixtures of pure substances at ambient pressure were used (Lewis, 1987).

[W] and [S] denotes the amount of water and solid, expressed in percentage, respectively:

$$\rho_{mixture} = \left[\frac{[W]}{\rho_{water}} + \frac{[S]}{\rho_{solid}} \right]^{-1} \qquad \text{Eqn.9}$$

$$Cp_{mixture} = [W] \cdot Cp_{water} + [S] \cdot Cp_{solid} \qquad \text{Eqn.10}$$

$$\rho_{solid} = \frac{1587.9}{1 + 0.000107 \cdot (T - 15)} \quad [\text{kg/m}^3] \qquad \text{Eqn.11}$$

$$Cp_{solid} = \xi \cdot (1622 + 7.125 \cdot T) \quad [\text{kJ/kg°C}] \qquad \text{Eqn.12}$$

where density and specific heat of solid sucrose are reported as a function of temperature (Bubnik et al., 1995; Van der Poel et al., 1998).

It was assumed that no significant deviation occurred in the change of the thermal expansivity of the mixed solution as a consequence of the pressure effect.

The empirical correction factor ξ was used in order to correctly fit the experimental results:

$$\xi = \frac{Cp_{water}(T, 0.1\ MPa)}{Cp_{water}(T, p)^{0.75}} \qquad \text{Eqn.13}$$

where the upper part of the ratio is represented by the *Cp* of water calculated at the actual temperature and at the initial value of pressure while the lower part denotes the *Cp* of water for the actual temperature and pressure.

In comparison with pure water, no significant deviation for the thermal expansion coefficient for mixed solutions was assumed in response to changes in pressure.

2.4.1.1.2 Mathematical routine

High pressure treatments were performed in the Multivessel Model U111 by using only one pressure chamber.

In order to check the experimental set-up, the thermal increase of distilled water was plotted against the pressure evolution (see Fig.2.4.1.1.2), and later compared with data from literature (NIST) which, based on theoretical considerations, give the temperature value as function of pressure. The NIST formulation consists of a fundamental equation for the Helmholtz energy per unit mass (kg), as a function of temperature and density.

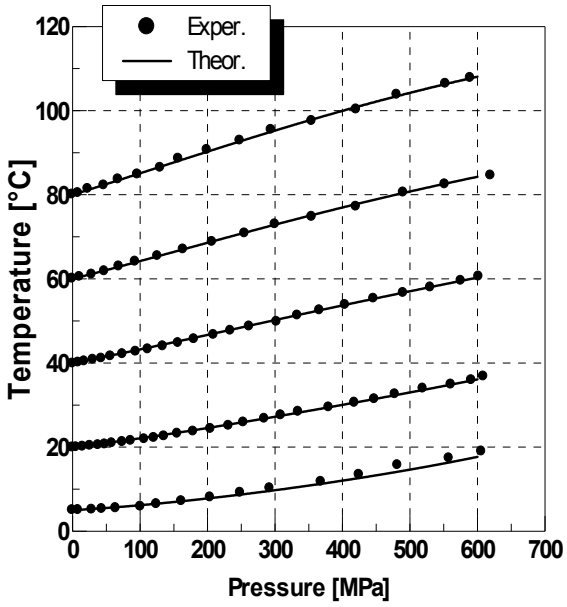


Fig.2.4.1.1.2 Comparison among experimental (dotted line) and theoretical (continuous line) data extrapolated by NIST, for distilled water at different starting temperature up to 600 MPa.

As shown in Fig.2.4.1.1.2, no significant deviations were found among the experimental and the theoretical results, which proves the suitability of experimental conditions.

The main model equation (14) was then used as a function of the thermo-physical properties of water, which were themselves function of pressure and temperature:

$$\Delta T = \int_{po}^{p1} \frac{\beta}{\rho \cdot Cp} \cdot T \cdot dp \qquad \text{Eqn. 14}$$

The numerical routine was written in MathCAD (MathSoft Inc, Massachusetts, USA) by implementing the NIST formulations for the regressive calculation of the thermal expansion coefficient β, the density ρ and the specific heat *Cp.*

Integration of equation (14) was performed by the Rhomberg method (MathCad, Handbook) for 0.1 MPa pressure increments.

2.4.1.2 Di-2-ethyl-hexyl sebacate & n-hexane

High pressure treatments were performed in the micro-system unit up to a maximum pressure level of 1400 MPa.

For measurements of the adiabatic heat of compression, the heating-cooling block was non activated and the initial temperature was stabilized through the cooling medium prior to the compression.

The investigations were carried out for a wide range of pressure (0.1- 1400 MPa) and temperature (5- 120°C) for two different fluids, di-2-ethyl-hexyl sebacate and n-hexane.

In order to obtain a reproducible measuring procedure, the first acquisition tests were performed with distilled water.

Perfect agreement was observed among experimental and theoretical data (NIST).

A quite precise technique was adopted for data acquisition, in order to exclude undesirable heating or cooling effects which might be produced by the large metal part constituting the thermocouple device.

The initial temperature was stabilized prior to the compression phase, which always

proceeded in steps of 20 MPa. Making use of the rapid occurring thermal equilibration, which was due to the small system dimensions, the sample was cooled to the initial value of temperature before the subsequent pressure step.

The numerical routine was written in MathCAD by finite element method.

For each pressure-temperature condition, which was related to the value of temperature prior the compression phase, the temperature change (ΔT) was calculated and implemented in equation (14):

$$\Delta T = \int_{po}^{p1} \frac{\beta}{\rho \cdot Cp} \cdot T \cdot dp \qquad \text{Eqn. 14}$$

Data were modelled by a simple *Taylor* series polynomial, stopped to the 2nd term, which resulted in satisfying results for both the tested fluids:

$$\frac{\partial T}{\partial p} = a + b \cdot T + c \cdot p + d \cdot T^2 + e \cdot p^2 + f \cdot T \cdot p \qquad \text{Eqn. 15}$$

2.4.2 Modelling the pressure-temperature impact on the microbial inactivation

The experimental microbial reduction levels (N/N_0) were studied by fitting an *n*th-order decay reaction:

$$\frac{dN}{dt} = -k \cdot N^n \qquad \text{Eqn. 16}$$

or upon integration:

$$\left(\frac{N}{N_0}\right) = (1 + k \cdot N_0^{(n-1)} \cdot t \cdot (n-1))^{\frac{1}{1-n}} \qquad \text{Eqn. 17}$$

The second equation was used in its logarithmic form:

$$Log\frac{N}{N_0} = Log\left(1 + k \cdot N_0^{(n-1)} \cdot t \cdot \left(n-1\right)\right)^{\frac{1}{1-n}} \qquad \text{Eqn. 18}$$

The inactivation rate constant (k) was obtained from regression analysis of the

experimental kinetic data at constant pressure and temperature.

A common reaction *n*th-order which could fit the inactivation data of all pressure-temperature investigated settings, was obtained by minimizing the accumulated residual standard error.

Regression analysis was performed using TableCurve 2D-3D (SPSS Inc., Chicago, IL, USA).

The mathematical routine was written in MathCAD.

To describe the correlation between rate constant, pressure and temperature, the following equation was used (Eyring, 1935):

$$Ln(k) = k'(T) + \frac{(-\Delta V^*)}{R \cdot T} \cdot p \qquad \text{Eqn. 19}$$

Equation (19), derived from the transition state theory, introduces the activation volume ΔV^* as the characteristic parameter of the pressure dependence of the rate constant k.

In this equation, k' denotes the natural logarithm $\ln(k_0)$ at the reference state p=0.1 MPa and it is often represented by an Arrhenius-type equation (Eqn.21). To provide an overall model of the inactivation reaction rate, it is essential to find a function relating k' with the temperature level of the pressure treatment.

Combining equations (17) and (19) and solving for the pressure level, once time t, temperature T and inactivation level $\text{Log}(N/N_0)$ have been fixed, it was possible to identify all the p-T conditions resulting in the same inactivation level for different settings of the treatment time.

3. Results & discussion

3.1 Adiabatic heat of compression of fluid systems

3.1.1 Sucrose solutions and orange juice

In Fig.3.1.1.1-2 experimental and predicted results obtained by applying the mathematical model for sucrose solutions (equations (9)-(14)) at different concentrations are shown, for different values of the initial temperature.

The predictions, which were based on NIST data for water and on the mixing rules for sucrose solutions (Eqn.9-13) were extrapolated to a maximum pressure of 1400 MPa. No significant deviations were detected in the range from 0.1 to 600 MPa while good continuity was found for extrapolation of the results from 600 to 1400 MPa.

Hence, the mathematical model was applied to predict the thermal behaviour for a real product.

Orange juice (Hohes C, Eckes-Granini, Deutschland GmbH, pH 3.8) with a sugar content of 9% was pressurized up to 600 MPa starting from different initial temperatures.

The model was then applied, simulating the adiabating heat of compression in orange juice with a sucrose solution of 9% of solid content.

In Fig.3.1.1.3 the comparison among the experimental and predicted results is shown in a range of pressure from 0.1 to 600 MPa and for initial temperatures from 5 to 90°C. No evident deviations were detected.

The model produced satisfying and reproducible data in the pressure-temperature range applied for the experimental procedure.

Orange juice, simulated as a sucrose solution with 9% solid content, presented an adiabatic heat of compression which was comparable to water, In accordance with the presence of a low solid content in the solution.

An increase of the sucrose concentration resulted in less thermal increase, which is related to the lower expansibility of the solution when compared with pure water.

As a consequence of thermal gradients, a not-uniform temperature distribution occurs in the sample, which would yield a not uniform microbial inactivation.

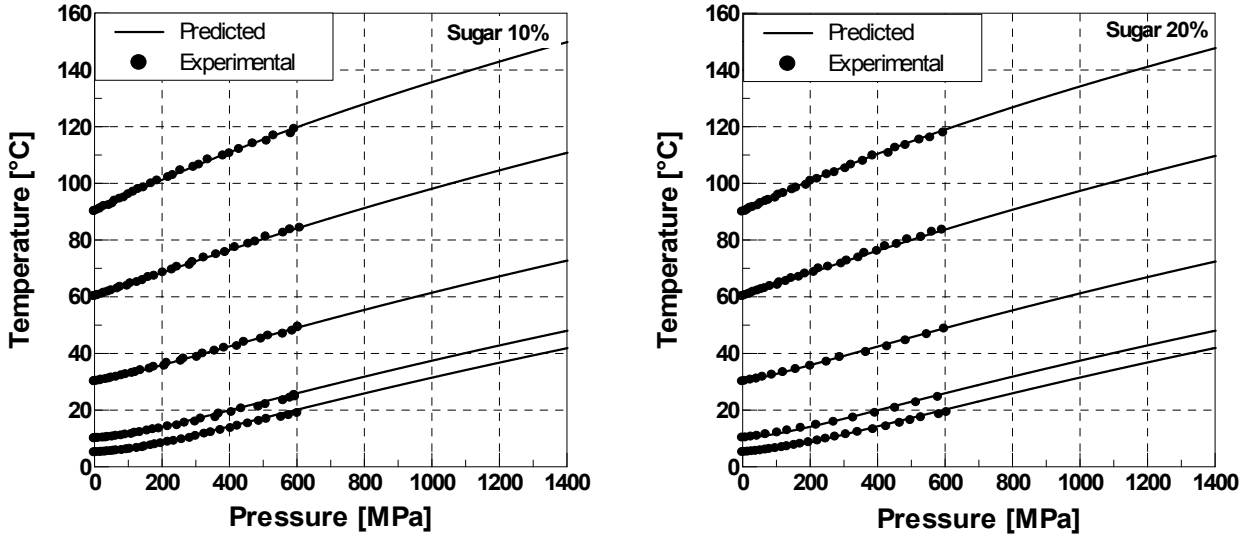


Fig.3.1.1.1 Comparison among experimental results (dotted line) and results obtained from the predictive model (continuous line) for sugar solutions at 10%-20% of solid content, in a range of pressure from 0.1 to 600 MPa at different starting temperatures. The modelled data are extrapolated up to 1400 MPa.

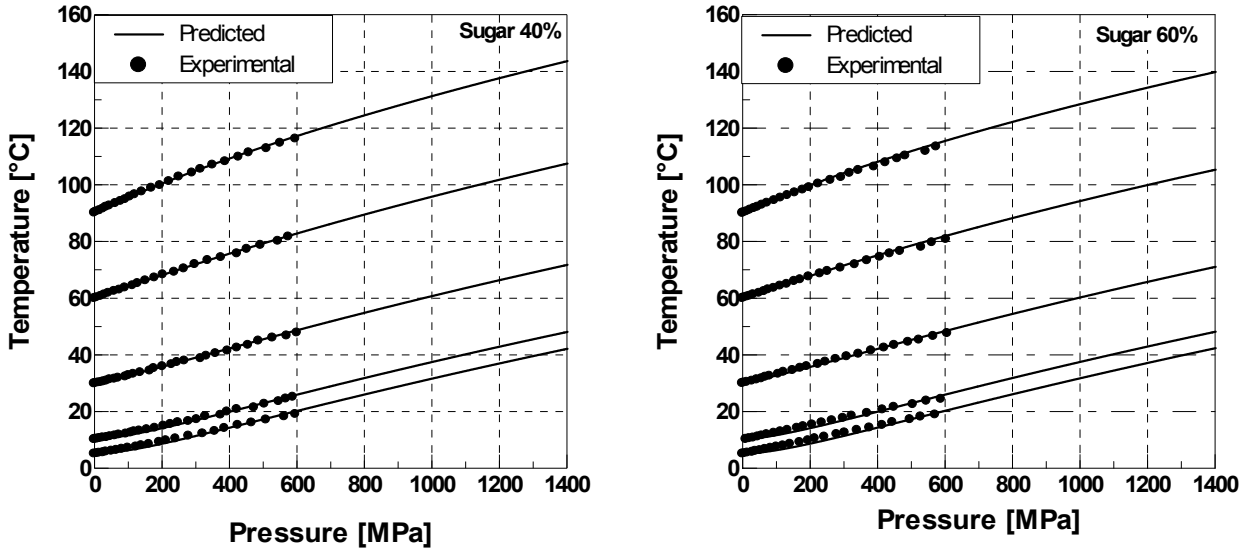


Fig.3.1.1.2 Comparison among experimental results (dotted line) and results obtained from the predictive model (continuous line) for sugar solutions at 40%-60% of solid content, in a range of pressure from 0.1 to 600 MPa at different starting temperatures. The modelled data are extrapolated up to 1400 MPa.

By the application of the model it would be possible to predict the entity of self-heating phenomenon which characterizes the product during the compression phase, hence, by considerations on the heat transfer across the high pressure system, it would be possible to evaluate and optimize the thermal profile in the product.

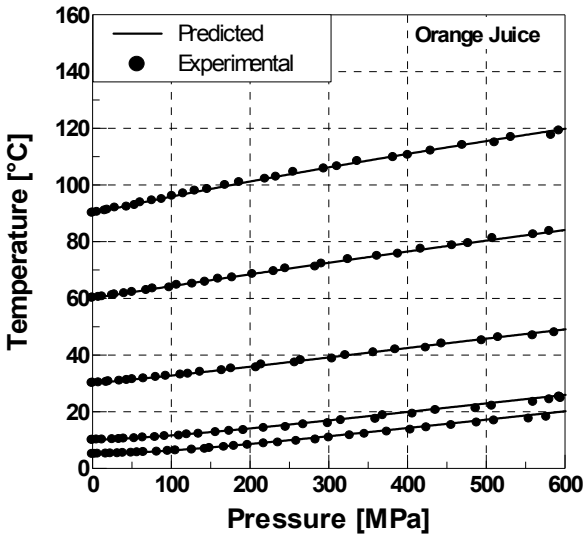


Fig.3.1.1.3 Comparison among experimental results of orange juice(dotted line) and results obtained from the predictive model (continuous line) for sugar solutions at 9% of solid content, in a range of pressure from 0.1 to 600 MPa at different starting temperatures.

3.1.2 Di-2-ethyl-hexyl sebacate and n-hexane

The adopted procedure for data acquisition (par. 2.4.1.2) provided quite satisfying results. Due to the impossibility of finding available data on the thermophysical properties of di-2-ethyl-hexyl sebacate & n-hexane as function of pressure and temperature, the model could not be based on theoretical data and no mixing rules could be adopted.

Values of the regression coefficients (a-f) for equation (15) are listed in tab.3.1.2 for both the fluids (see also annex, table 1-2). As expected, compared to water, both the fluids, sebacate acid and hexane, showed higher adiabatic heat of compression.

The increase of temperature for hexane was higher than the one produced by sebacate when the starting temperature was in the range from 5°C to approximately 80°C. Afterwards, sebacate resulted in higher adiabatic heating.

This phenomenon could be explained taking into account the different behaviour of

the viscosity of the two fluids under pressure and temperature changes.

Regression Coefficients	di-2-ethyl-hexyl sebacate	n-Hexane
a [MPa^{-1} °C]	0.0775689	0.104085
b [MPa^{-1}]	-7.0866*10^-5	-0.00011202
c [MPa^{-2} °C]	0.00011502	0.000199861
d [MPa^{-1} $°C^{-1}$]	1.82827*10^-8	3.48421*10^-8
e [MPa^{-3} °C]	-2.3313*10^-7	-1.4807*10^-6
f [MPa^{-2}]	-1.9837*10^-8	3.27904*10^-8

Tab.3.1.2 Regression coefficients for equation (15) for sebacate and hexane fluids.

Fig.3.1.2.1-2 show the adiabatic heat of compression of di-2-ethyl-hexyl sebacate and n-hexane.

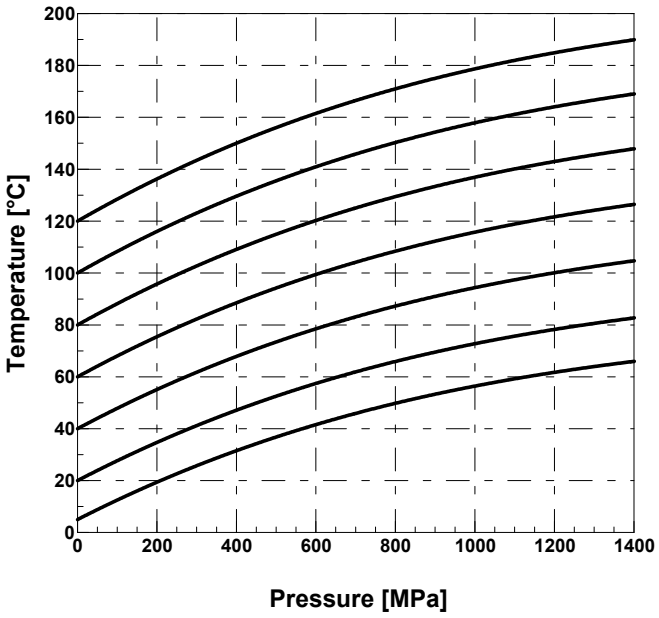


Fig.3.1.2.1 Adiabatic heat of compression of di-2-ethyl-hexyl sebacate up to 1400 MPa, starting from different initial temperatures.

In particular, hexane- less viscous compared to sebacate- would present a lower value of the cubic coefficient of thermal expansion at extreme pressure and temperature conditions and consequently higher volume reduction.

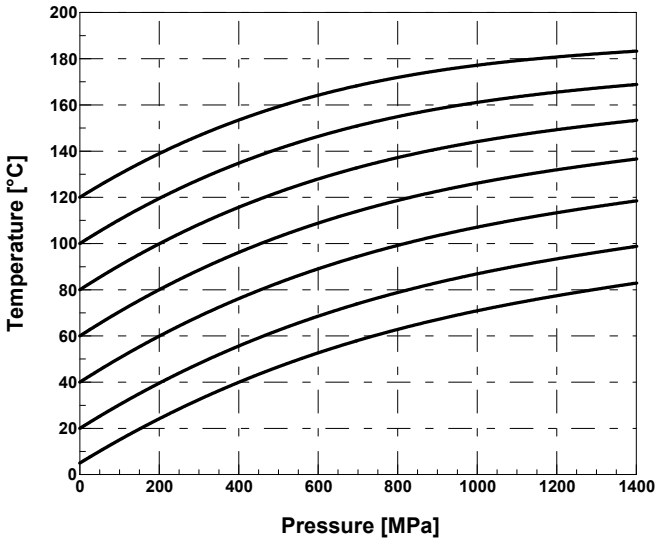


Fig.3.1.2.2 Adiabatic heat of compression of n-hexane up to 1400 MPa, starting from different initial temperatures.

The following graph shows the comparison among the adiabatic heat of compression of water, sucrose solution with 40% of solid content, sebacate, n-hexane and orange juice.

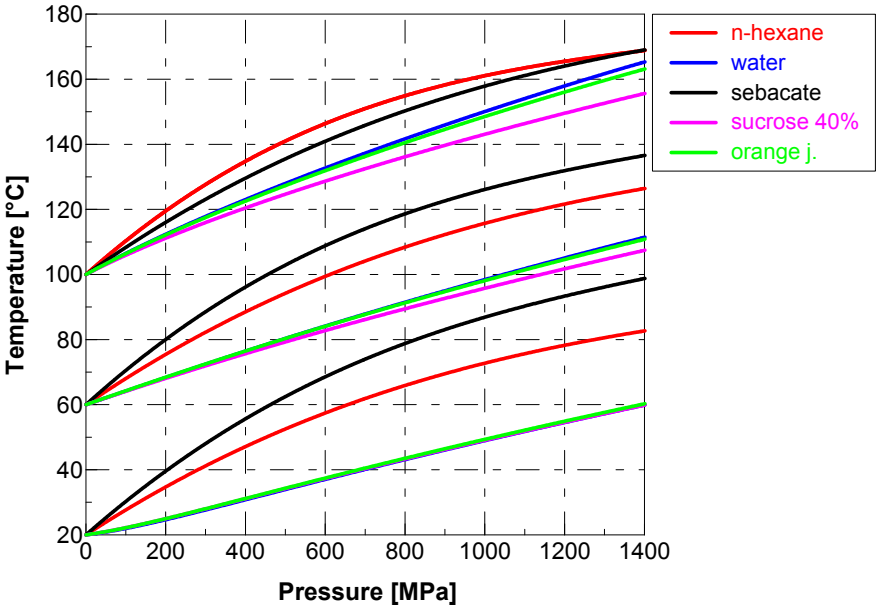


Fig.3.1.2.3 Adiabatic heat of compression of water, sucrose solution with 40% of solid content, orange juice, sebacate and n-hexane

3.2 Microbial inactivation & modelling

3.2.1 *Lactobacillus rhamnosus* GG 53103 in orange juice

Inactivation kinetics of *Lactobacillus rhamnosus GG* (*LGG*) in orange juice were determined in close-to-adiabatic conditions due to the application of very high compression and decompression rates.

In Fig. 3.2.1 the semi-logarithmic reduction of the survivors of *LGG* in orange juice is presented for several pressure levels.

It can be noticed that very high inactivation rates can be achieved by applying pressure levels higher than 400 MPa. For instance, at 600 MPa a reduction of approximately 6 log-cycles occurred within 10 seconds of treatment time while on the other hand, an inactivation of only 2 log-cycles was found at 500 MPa within the same holding time.

On the base of results showed in Fig.3.2.1.1, a modified version of the most probable number method, previously described, was applied.

It was hypothesized that, as a consequence of adhesive forces, a protective effect of fibres, which are present in orange juice, could result in an over-estimation of microbial survivors.

In contrast to the standard MPN method, sterility was found even in the sample with the highest cell concentration ($5*10^8$ CFU/ml) when exposed to 600 MPa for 15 s.

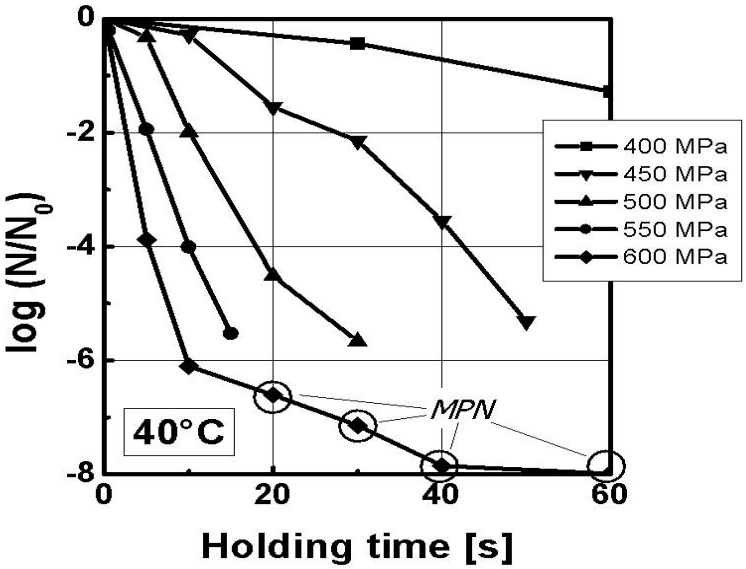


Fig.3.2.1.1 Inactivation kinetics of *LGG* in orange juice at 40°C and different pressure levels, evaluated through standard plate counting method. The kinetic at 600MPa for pressure holding times higher than 10 seconds was estimated by standard MPN method.

This result indicated that plate counting and standard MPN method resulted in an overestimation of the survivor count as a consequence of the non-uniform distribution of bacteria in serial dilution steps. In addition, a substantial damage of the bacterial membrane and of the cellular metabolism was presumed.

Hence, for orange juice, a tailing behaviour in the inactivation kinetics of *LGG* could be ruled out and the semi-logarithmic linear shape of the curves could sufficiently be described by simple first order kinetics.

Assuming first order kinetics, the rate constant k was obtained regressively by using equation (16).

The activation volume (equation (20)) was properly expressed as a linear function of pressure:

$$(-\Delta V^{*}) = A_1 + A_2 \cdot p \qquad \text{Eqn. 20}$$

From regression analysis, the polynomial parameters were determined as follows:

A_0= 15.21 ± 3.79; E_a= -79622.1 ± 14670 kJkg^{-1}; A_1= -103.49 ± 36.83 kJ kg^{-1}MPa^{-1}; A_2= 0.0581 ± 0.039 kJkg^{-1}MPa^{-2}.

Combining equations (17) and (19) and solving for the pressure level, once time t, temperature T and inactivation level Log(N/N_0) have been fixed, it was possible to identify all the p-T conditions resulting in the same inactivation level for different settings of the treatment time.

Fig.3.2.1.2 indicates the required pressure-time conditions for a 7log reduction of *LGG* in orange juice at different treatment temperatures.

The effect of a pressure cycle on the inactivation rate is strongly increased at higher pressure levels. For instance, the treatment time required to achieve a 7 log-cycles inactivation could be reduced by 7 times when applying 500 MPa instead of 400 MPa at 40°C. Higher pressure levels (i.e. 600 MPa and more) proofed to be highly efficient in eliminating lactic acid bacteria in juice within treatment times in the range of seconds.

As a consequence of high compression and decompression rates it was possible to

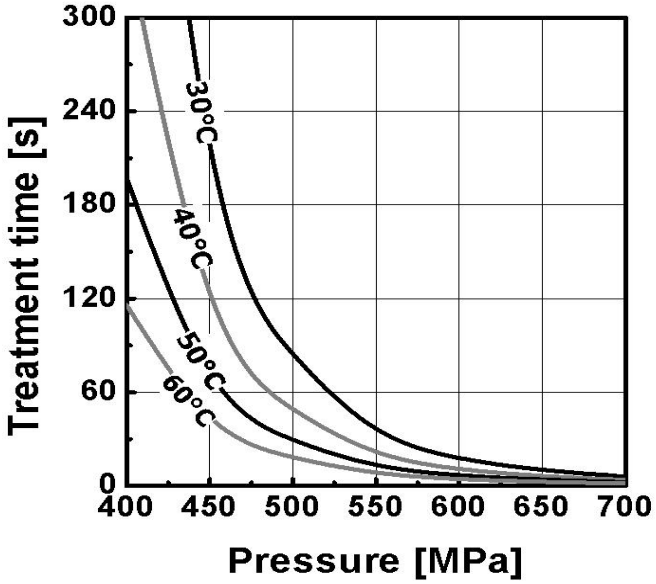


Fig.3.2.1.2 Pressure-time conditions for a 7log reduction of *LGG* in orange juice at different treatment temperatures.

figure out the minimum holding time required to obtain a satisfying reduction of the initial microbial population in orange juice.

In addition, a triangle test was performed to investigate the difference in organoleptic properties of fresh squeezed orange juice before and after pressure treatment.

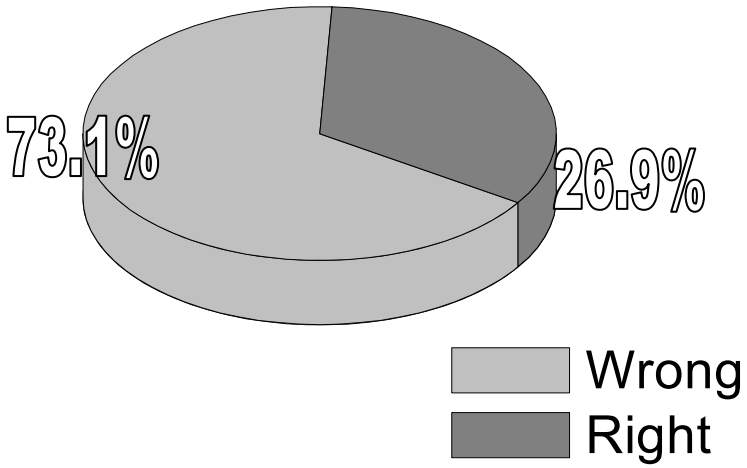


Fig.3.2.1.3 Triangle test on treated and untreated fresh squeezed orange juice stored at 4°C for 8days after pressure treatment at 600 MPa and 30 seconds. 79% of 29 panel testers preferred the treated juice.

The juice was pressure-treated at 600 MPa for 30 seconds and stored at 5°C together with the untreated samples.

The results obtained from an organoleptic test after one day of storage showed no significant difference between treated and untreated juice, as well as no distinction occurred in the preferences. In contrast, after 8 days of storage, a significant difference between the two juices was evident, since the 79% from the 29 panel testers evaluated the pressure-treated juice (Fig.3.2.1.3).

3.2.2 *Alicyclobacillus acidoterrestris* DSM 2498 in orange juice

In Figures 3.2.2.1-2 the thermal and the combined pressure-temperature inactivation of the spores, at a starting temperature of 80, 85, 90 and 95°C is presented.

Thermal kinetics followed substantially a linear behaviour, while combined treatments seemed to produce “quasi-linear” kinetics.

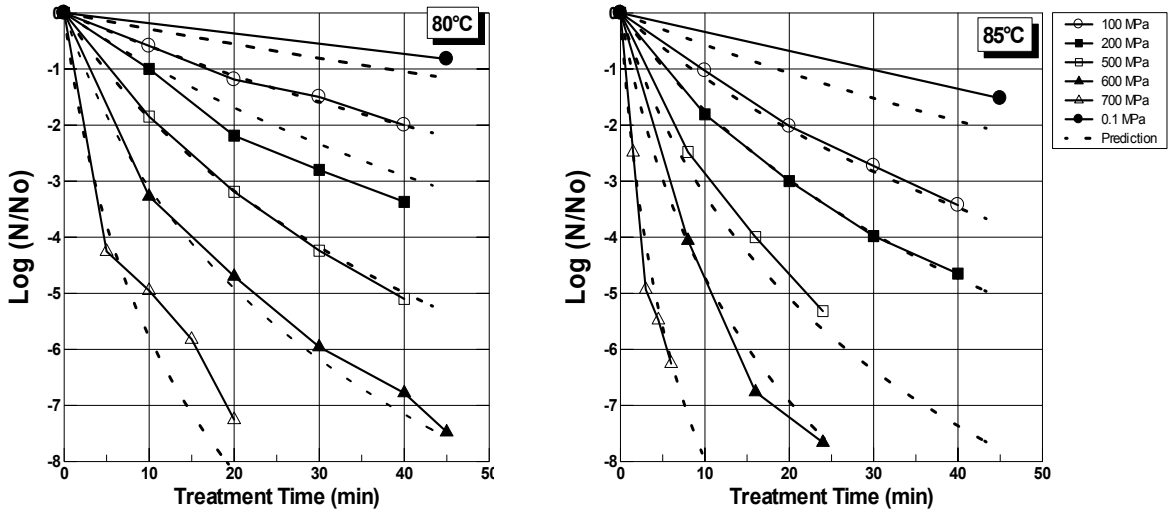


Fig.3.2.2.1 Temperature and combined temperature-pressure inactivation of spores. Starting temperature 80°C-85°C. Experimental data (solid lines) and predicted data from the model equations (17)- (19) (dotted lines).

For thermal kinetics, only the first inactivation point was considered in the graphs, in order to have a simpler view of the whole inactivation process. The sole thermal treatments were applied for 60, 165, 300 and 385 minutes at starting temperatures of 95, 90, 85 and 80 °C, respectively.

The solid lines represent the experimental data, while the dashed lines show the result of the regression analysis using a reaction order of n=1.1 (see annex, table 6).

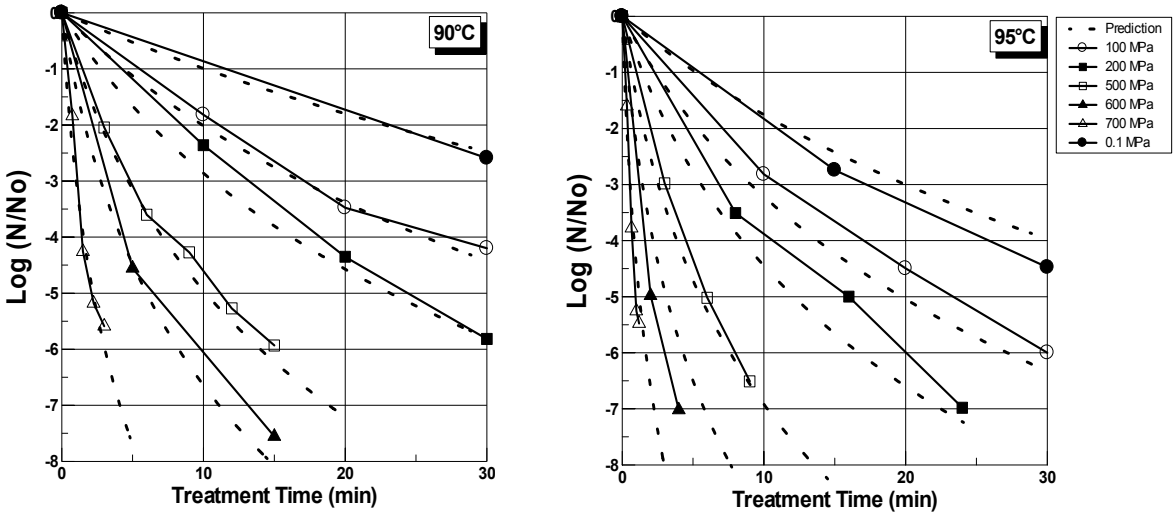


Fig.3.2.2.2 Temperature and combined temperature-pressure inactivation of spores. Starting temperature 90°C-95°C. Experimental data (solid lines) and predicted data from the model equations (17)- (19) (dotted lines).

In accordance with results from other authors (Lee et al., 2002), high temperature treatments alone did not result in a significant reduction of *A. acidoterrestris* spores, while a combination of high pressure and heat strongly reduced the numbers of survivors.

The reaction order has been derived from the accumulated standard error of all experimental inactivation kinetics investigated. The standard error function passes through a minimum at a reaction order of n=1.1 (see inset of Fig. 3.2.2.3 or annex, table 6).

To describe the correlation between rate constant, pressure and temperature, equation (19) was used:

$$Ln(k) = k' + \frac{(-\Delta V^*)}{R \cdot T} \cdot p \qquad \text{Eqn. 19}$$

An Arrhenius-type equation (21) was used for evaluating *K'*:

$$k' = A_0 + \frac{E_a}{R \cdot T} \qquad \text{Eqn. 21}$$

Equation (19) is applicable in isothermal situations. R denotes the universal gas constant (R=8.314 $Jmol^{-1}K^{-1}$).

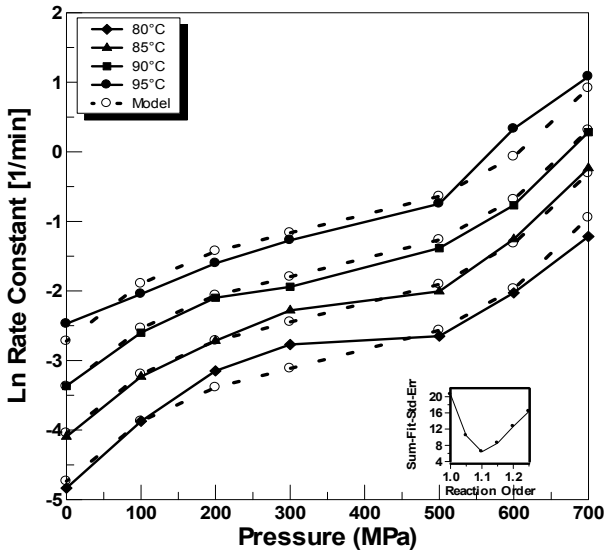


Fig.3.2.2.3 Rate constants of 1.1 order inactivation reaction plotted logarithmically versus pressure. Solid lines show the experimental data. Dotted lines are from the model fitting the original data by equation (19). The inset shows the overall standard error from the kinetic analysis.

For the activation volume (ΔV^*) a second-order polynomial equation for pressure was applied to yield a satisfying fit of the experimental data:

$$(-\Delta V^*) = A_1 + A_2 \cdot p + A_3 \cdot p^2 \qquad \text{Eqn. 22}$$

From regression analysis, the polynomial parameters were determined as follows:

A_0= 44.7 $\pm$ 2.1; E_a= -145070 $\pm$ 6340 kJkg^{-1}; A_1= -32.3 $\pm$ 3.31 kJ kg^{-1}MPa^{-1}; A_2= 0.079 $\pm$ 0.012 kJkg^{-1}MPa^{-2} ; A_3= -7.9*10^{-5} $\pm$ 1.1*10^{-5} kJkg^{-1}MPa^{-3}.

Combining equations (17) and (19) and solving for the pressure level, once time t, temperature T and inactivation level Log(N/N$_0$) have been fixed, it was possible to identify all the p-T conditions resulting in the same inactivation level for different settings of the treatment time (see Fig. 3.2.2.4).

As anticipated in equation (17), when increasing the initial concentration of spores a lower pressure level was necessary to achieve the same inactivation ratio (N/N$_0$) at the same treatment temperature.

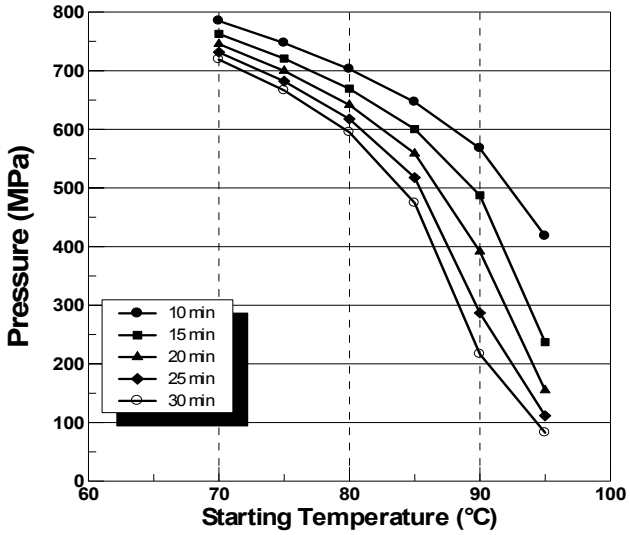


Fig.3.2.2.4 Pressure and temperature combinations for 6 log-cycles spore reduction at different treatment times (from 10 to 30 minutes). Data are calculated combining equations (17) and (19) and solving for pressure, after having fixed the starting temperature, treatment time and log reduction.

A linear behaviour of thermal inactivation was found while slight deviations from linearity were found for combined pressure and temperature treatments. As described in the figures 3.2.2.1-2, a more linear trend was found at higher pressure level and higher treatment temperature. Using the final model, it was possible to reproduce the inactivation behaviour of spores of *Alicyclobacillus acidoterrestris.* Temperature-pressure conditions could be calculated required to achieve identical inactivation level. As shown in Fig.3.2.2.4, a longer treatment time is needed to reach the same level of inactivation using lower pressures or temperatures. In particular while a 10 minutes treatment time is needed when using 600 MPa and 90°C initial temperature, starting from the same temperature but using a lower pressure, such as 300 MPa, a 30 minutes treatment time is needed.

It seems useful to notice that by coupling pressure and temperature it is possible to reduce the temperature level and the holding time of the inactivation process. Comparing the thermal effect of 80°C starting temperature and the combined effect of 80°C and 700 MPa is possible to see a substantial difference in the inactivation level and the required treatment time: 45 minutes to reach less than 1 log-cycle inactivation at 80°C and 20 minutes to inactivate more than 7 log-cycles at 80°C and 700 MPa.

In this way is possible to find the optimum combination among the operative conditions in order to reduce the strong quality damage due to the thermal treatments and the high pressure level needed for spore inactivation in food products.

Since pressurization is a “quasi-adiabatic” transformation, a temperature profile is present in the vessel and in the product itself.

An increase of temperature is consequently applied during the pressure build-up phase only. Once the set pressure is reached, the temperature of the product decreases to its starting value because of the equilibration with the temperature of the pressure medium and the temperature of the vessel.

Calculating the increase of temperature due to the adiabatic heating, it is possible to start the process with a lower temperature of the product, resulting in more benefits for the product quality and in a lower cost of the whole sterilization process.

3.2.3 *Bacillus stearothermophilus* ATCC 7953 in buffer solutions

Preliminary investigations on the thermal resistance of *B. stearothermophilus* spores, in pH 7.0 phosphate buffer, showed that the microbial reduction could be expressed with sufficient accuracy as a linear function of the processing temperature.

Indeed, an Arrhenius-type equation was used to fit the experimental rate constant data (equation 23).

$$Ln(k) = A_0 + \frac{E_a}{R \cdot T} \qquad \text{Eqn. 23}$$

The coefficients of regression were estimated as follows:

A_0= 88.45 $\pm$ 3.9; E_a= -310815 $\pm$ 12588 kJkg^{-1}.

Fig.3.2.3.1 shows the rate constant as a function of the thermal intensity of the treatment, for a temperature range from 90 to 130°C.

Fig.3.2.3.2 shows the temperature-time conditions to achieve 8 log-cycles inactivation in phosphate buffer pH 7.0.

Low temperature levels resulted in quite large treatment times while a treatment performed at 130°C required approximately 400 seconds.

Fig.3.2.3.2 shows the inactivation kinetics of B*. stearothermophilus* in phosphate buffer pH 7.0 by combined pressure-temperature treatments.

A wide range of pressure condition was investigated.

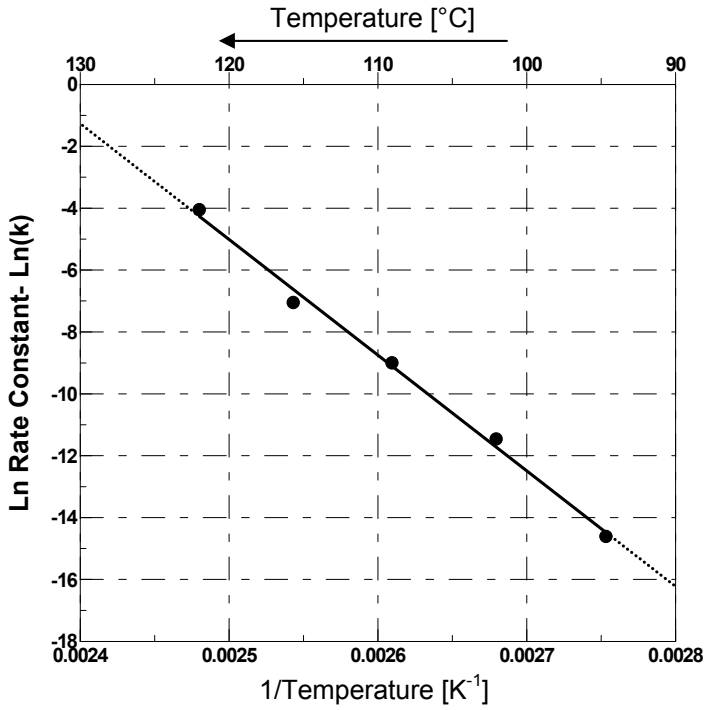


Fig.3.2.3.1 Natural logarithm of the rate constant for thermal treatments plotted versus the inverse of temperature at ambient pressure.

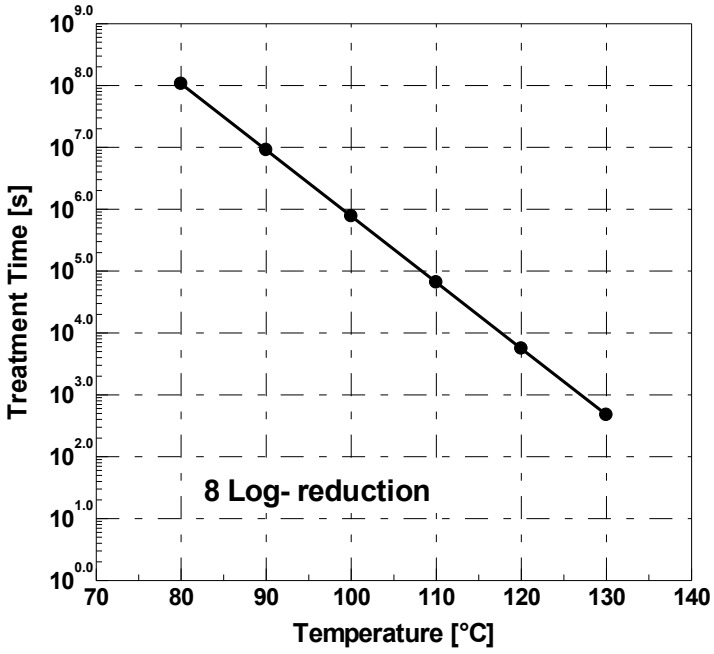


Fig.3.2.3.2 Temperature-time conditions to achieve 8 Log-cycles inactivation of *B. stearothermophilus* spores by sole thermal treatments in pH 7.0 phosphate buffer at ambient pressure.

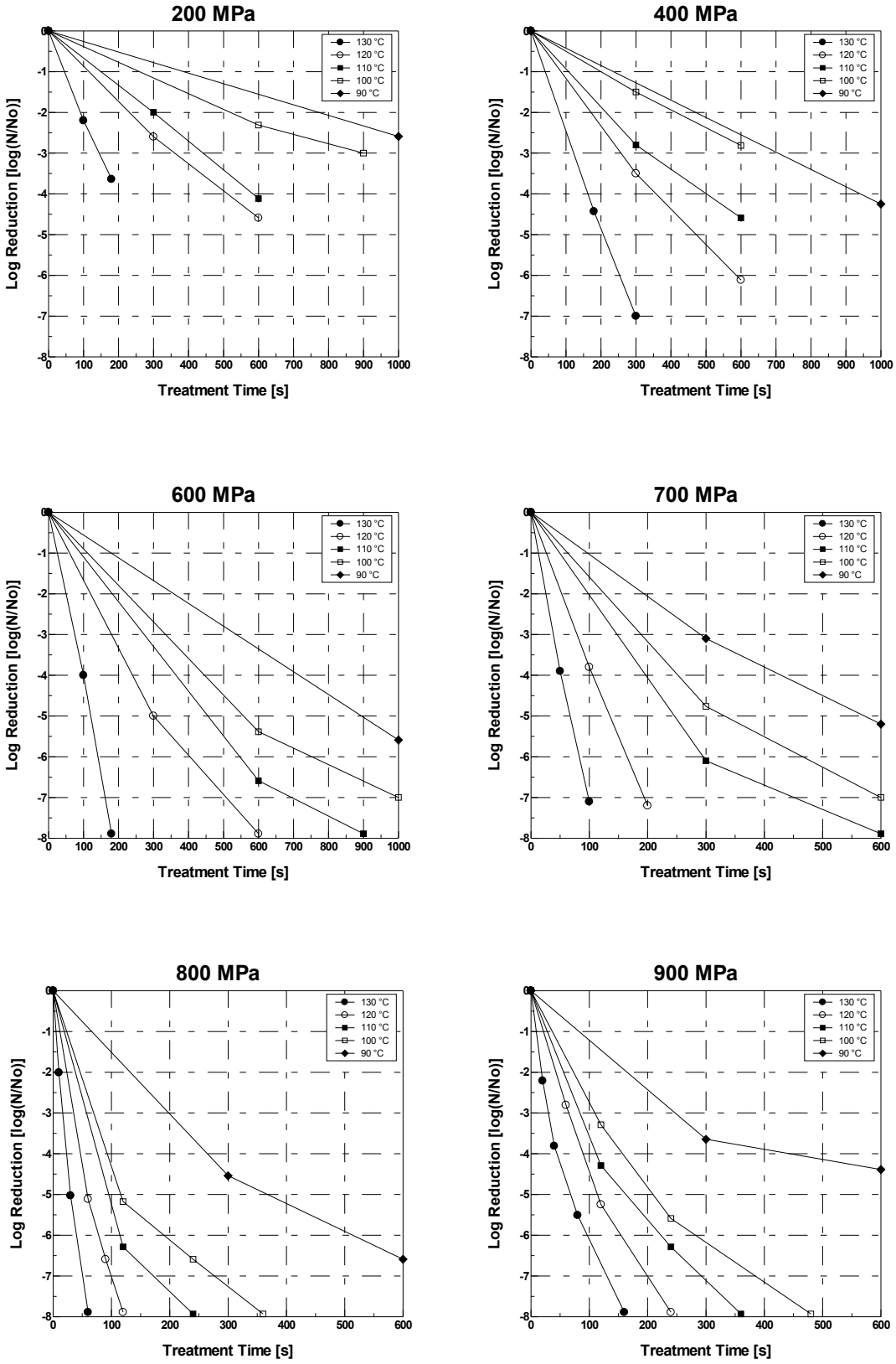


Fig.3.2.3.3 Inactivation kinetics of *B. stearothermophilus* spores by combined pressure-thermal treatments in pH 7.0 phosphate buffer (to be continued).

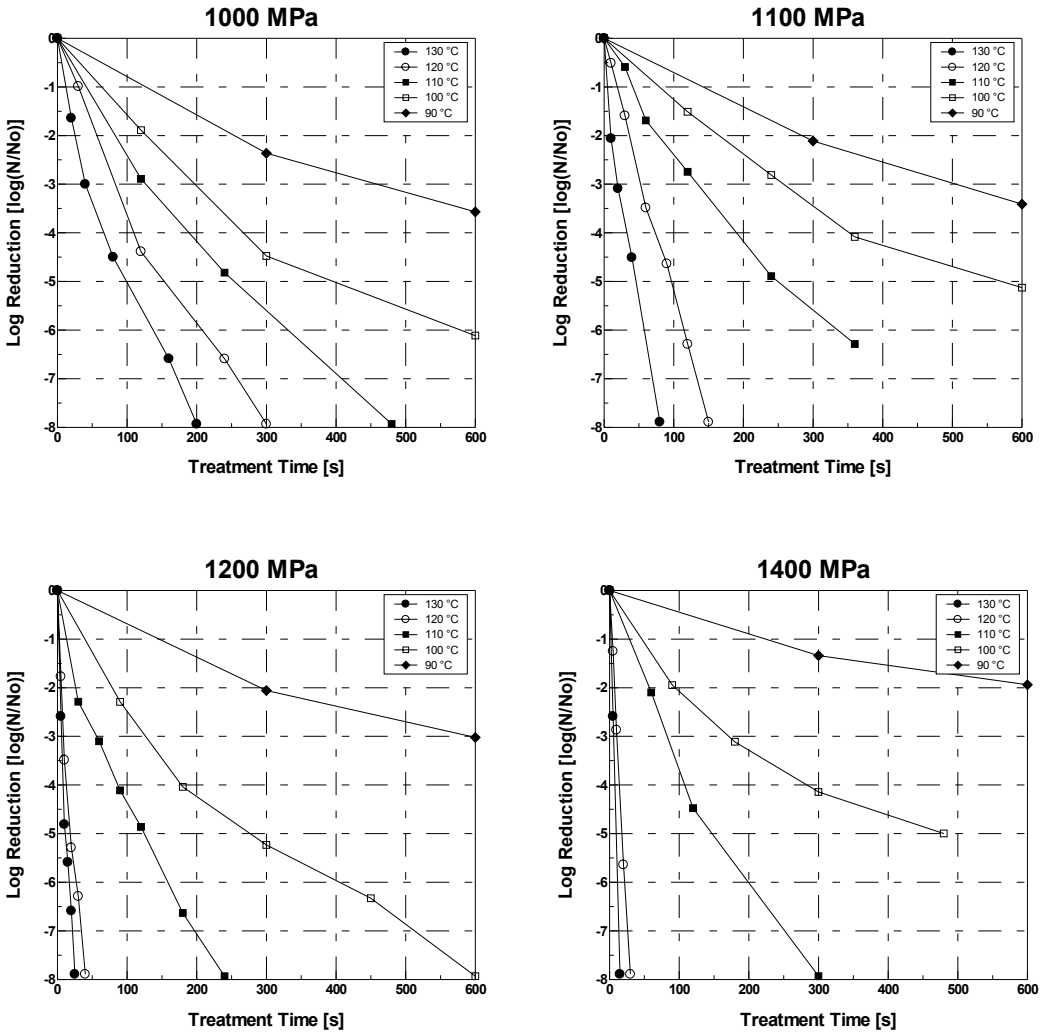


Fig.3.2.3.3 Inactivation kinetics of *B. stearothermophilus* spores by combined pressure-thermal treatments in pH 7.0 phosphate buffer.

An increase of temperature resulted in higher inactivation rates for each considered pressure level. When plotting the effect of different pressure treatments at fixed temperature, an unexpected behaviour was observed.

In order to present a clear representation of the results, only two temperature conditions are shown in the following figure, 120°C and 90°C.

As it is possible to note from the graphs, at the two temperature levels no continuity in pressure increase was found. Similar situation was characterizing the other temperature levels.

In particular, the highest pressure level was not always resulting in the highest inactivation while, depending on the p-T combination, a sort of pressure stabilization

was revealed.

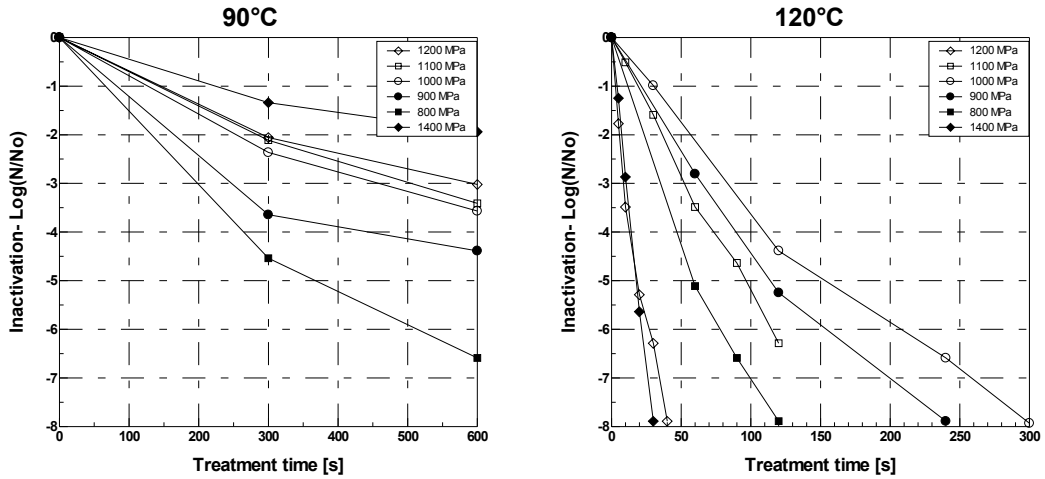


Fig.3.2.3.4 Inactivation kinetics of *B. stearothermophilus* spores by combined pressure-thermal treatments in pH 7.0 phosphate buffer at 90 and 120°C.

By considerations on the rate constant dependence on pressure and temperature changes, the inactivation kinetics were fitted with a 1.1 order reaction kinetic (see annex, table 7).

The following figure shows the natural logarithm of the rate constant as function of pressure and treatment temperature.

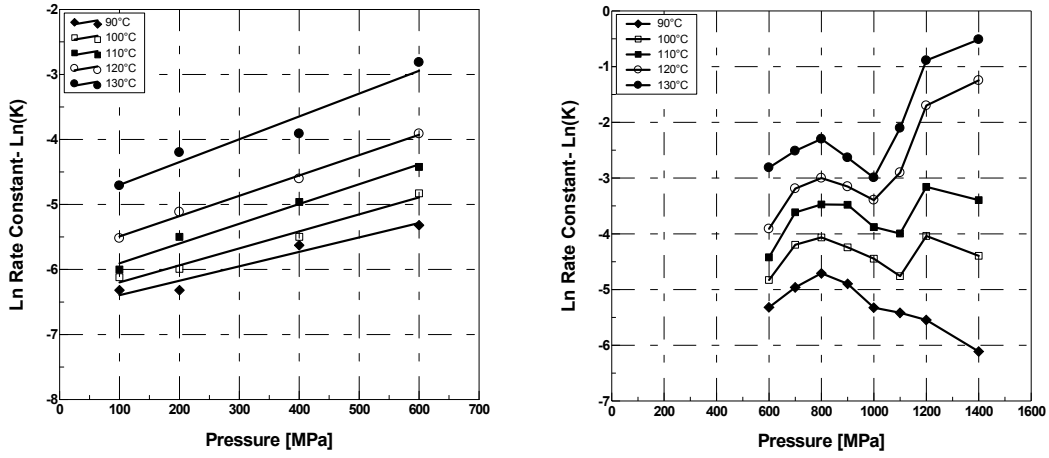


Fig.3.2.3.5 Natural logarithm of the rate constant for combined pressure-thermal treatments plotted versus pressure in the pressure range from 100 to 1400 MPa for phosphate buffer pH 7.0.

The higher temperature levels were characterized by a minimum in the inactivation in

correspondence of 1000 MPa, while at the lower temperatures a shift of the minimum value to higher pressure was observed.

In the low pressure range, form 100 to 600 MPa, a linear behaviour of the rate constant was found, for the whole temperature range (fig. 3.2.3.5), stating that the results were not affected by a wrong experimental procedure.

In order to understand the unexpected phenomenon, the buffering properties of the treated solutions were considered.

Due to the instability of phosphate buffer at high pressure-temperature conditions (Marshall et al, 1981), it was hypothesized that an increase of the acidity of the medium, related to a reduction of the pH, could influence the spore resistance.

Similar experimental conditions were tested in a pH 7.0 and 6.0 Aces buffer solutions at 800 to 1400 MPa and in a temperature range from 100 to 120°C (Fig.3.2.3.6).

Fig.3.2.3.6 shows the inactivation kinetics of *B. stearothermophilus* in Aces buffer pH 7.0 and 6.0. All the inactivation kinetics were fitted with a 1.1 order reaction (see annex, table 8-9).

Fig.3.2.3.7 shows the dependence of the rate constant with pressure and temperature in the range of interest for pH 7.0 and 6.0.

For both the pH levels, at higher temperatures the inactivation increased progressively with pressure, while higher acidity produced higher inactivation.

At lower temperatures, pressure-stabilization occurred.

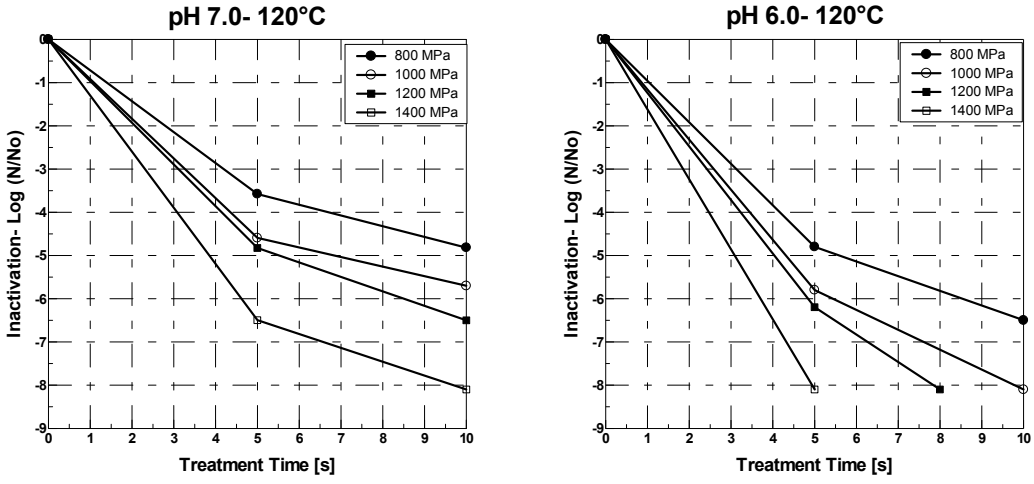


Fig.3.2.3.6 Inactivation kinetics of *B. stearothermophilus* spores by combined pressure-thermal treatments in pH 7.0 and pH 6.0 Aces buffer at 800 to 1400 MPa and 100 to 120°C (to be continued).

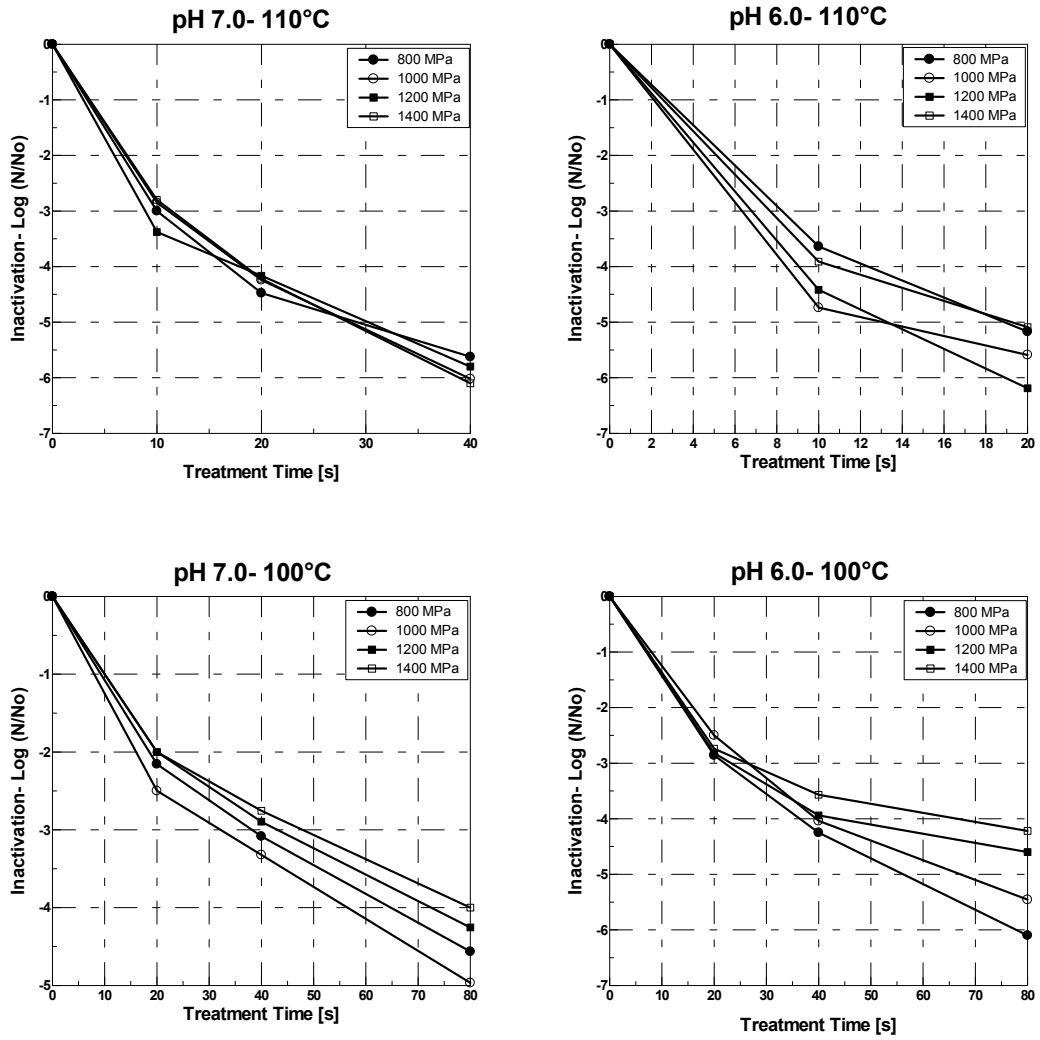


Fig.3.2.3.6 Inactivation kinetics of *B. stearothermophilus* spores by combined pressure-thermal treatments in pH 7.0 and pH 6.0 Aces buffer at 800 to 1400 MPa and 100 to 120°C.

The inactivation in a pH 6.0 Aces buffer did not improve when pressure was increased at maximum value. At the lowest temperature, the highest pressure level resulted in lower inactivations rates.

Data were modelled by a simple *Taylor* series polynomial, limited to the 2nd order terms, which produced a satisfying fitting (see annex, table, 3-4):

$$Ln(K) = a + b \cdot T + c \cdot p + d \cdot T^2 + e \cdot p^2 + f \cdot T \cdot p \qquad \text{Eqn.24}$$

Equation (24) was inserted into the modelling equation (18):

$$Log\frac{N}{N_0} = Log\left(1 + k \cdot N_0^{(n-1)} \cdot t \cdot (n-1)\right)^{\frac{1}{1-n}} \qquad \text{Eqn. 18}$$

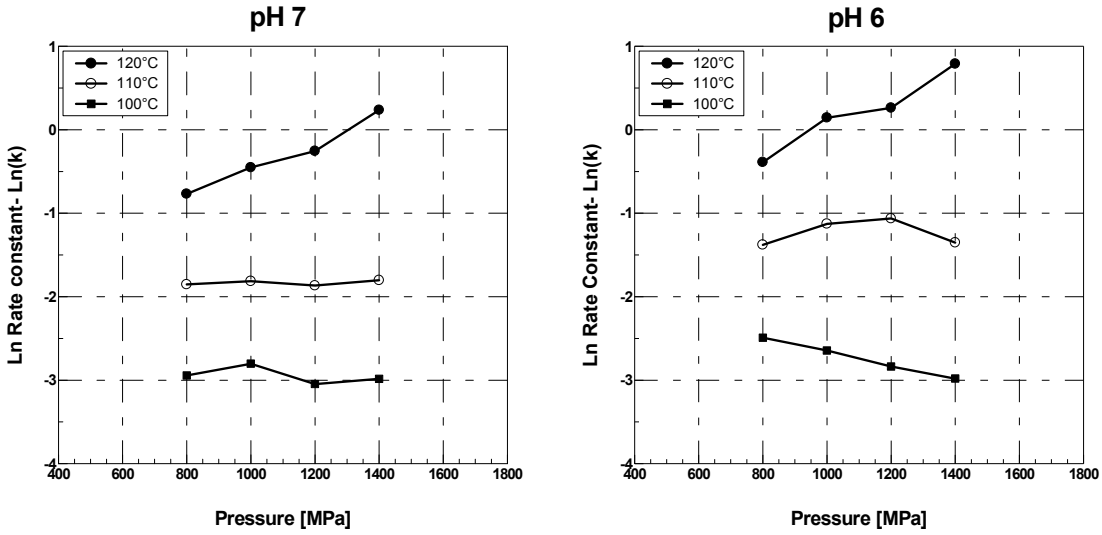


Fig.3.2.3.7 Natural logarithm of the rate constant for combined pressure-thermal treatments plotted versus pressure in the pressure range from 800 to 1400 MPa for aces buffer pH 7.0 and 6.0.

Results from the model fitting are represented in Fig.3.2.3.8 for pressure-temperature conditions required to achieve 8 Log-cycles inactivation of *B. stearothermophilus* spores in 20 seconds in pH 7.0 and 6.0 Aces buffer.

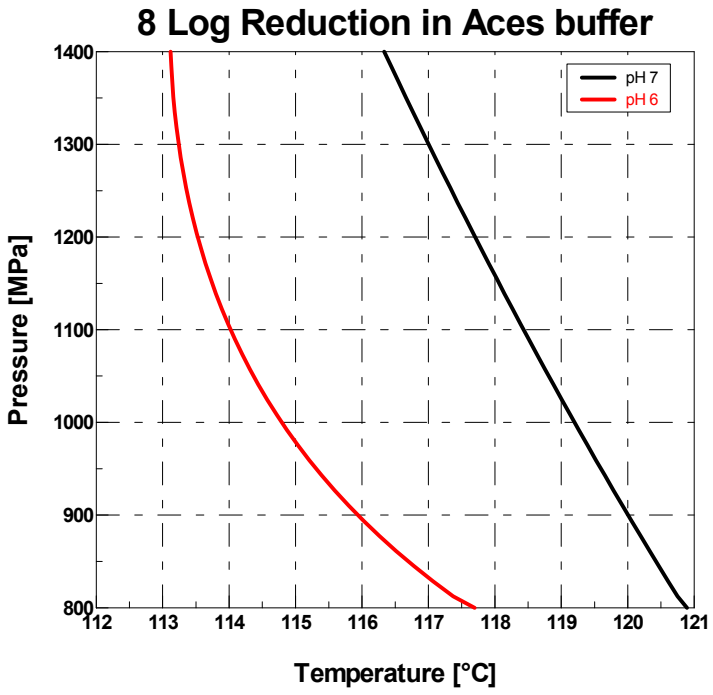


Fig.3.2.3.8 Pressure-temperature conditions to achieve 8 Log reduction of *B. stearothermophilus* spores in 20 seconds in aces buffer pH 7.0 and 6.0.

It is possible to note how the different pH value influences the rate constant behaviour. In particular, while for pH 7.0 perfect linearity was found among the pressure-temperature conditions, at pH 6.0 a threshold temperature level was observed, below which an increase of pressure was not producing an improvement in the inactivation.

Furthermore, data for the inactivation in pH 7.0 phosphate buffer were analysed by a fitting *Taylor* series polynomial truncated to the 3rd term.

$$Ln(K) = a + b \cdot T + c \cdot p + \ldots + g \cdot T^3 + h \cdot p^3 + i \cdot T \cdot p^2 + l \cdot p \cdot T^2 \qquad \text{Eqn.25}$$

Equation (25), which produced an acceptable fitting of the experimental data (see annex, table 5), was then introduced in the modelling equation (18).

Fig.3.2.3.9 shows the pressure-time conditions required to achieve 8 Log-cycles inactivation of *B. stearothermophilus* spores within different holding times in phosphate buffer pH 7.0.

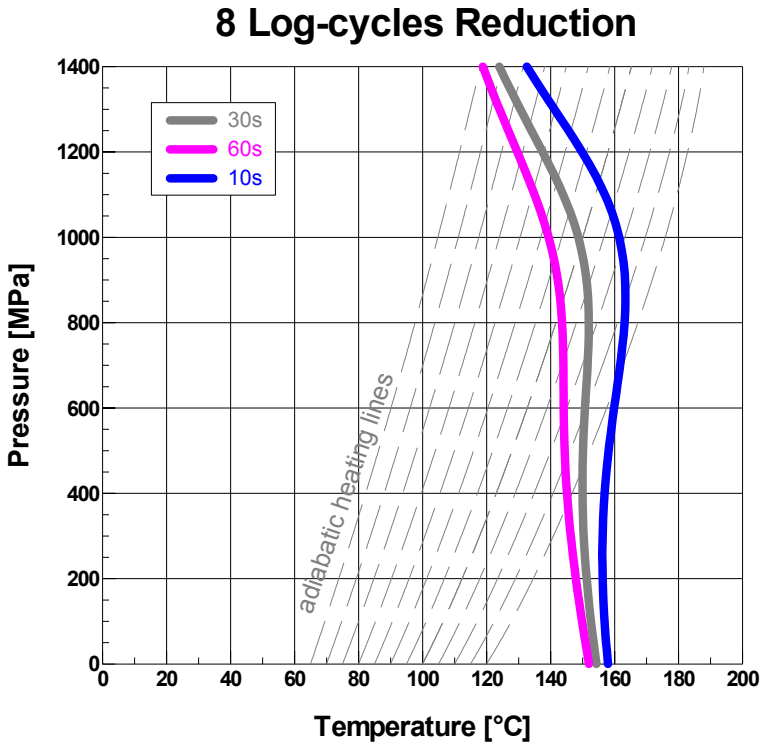


Fig.3.2.3.9 Pressure-temperature conditions to achieve 8 Log reduction of *B. stearothermophilus* spores in phosphate buffer pH 7.0 within 3 holding times. The grey lines represent the adiabatic heat of compression of water.

In Fig.3.2.3.9 also the adiabatic heating lines of water are reported, by which is possible to set the initial value of temperature required to reach the desired processing temperature as function of the pressure level.

As it is possible to note, a threshold temperature level is present, below which an increase of the pressure level is not improving the inactivation rate.

The elliptical shape which can be observed in Fig.3.2.3.9 could be the result of a composed effect of different pH levels since, as previously mentioned, the pressure-temperature instability of phosphate buffer could result in a lower pH level at extreme processing conditions.

Since a stabilizing effect was observed for the three different buffer solutions at medium and low temperature levels, it could be assumed that the impact of a different acidity level is not the main source of the phenomenon.

The elliptical shape, which is characteristic for enzymes activity inactivations, suggests that under a certain range of pressure-temperature conditions, the enzymes which catalyze the hydrolytic reaction leading to the spore destruction, are inactivated by pressure and that an ultra-high pressure treatment could only be applied in assisting a thermal treatment. In order to focus on the mechanism of inactivation, simple tests were performed to evaluate if a different inactivation mechanism could occur at different pressure-temperature conditions when a same inactivation level was obtained.

Spores were suspended in pH 7.0 Aces buffer and treated at 100, 110 and 120°C at 800, 1000 and 1400 MPa. In order to achieve the same inactivation level of 4 Log-cycles, different treatment times were applied. After treatment, the suspension was diluted in standard-nutrient broth, in pH 3.0 phosphate buffer and in pH 3.0 phosphate buffer with glucose (0.02 Mol).

Immediately after pressure treatment, 1 ml out of the broth suspension was plated on nutrient agar. All the samples were incubated at 55°C for 24 hours.

The plate count resulted in a 4 Log reduction, stating that inactivation results previously obtained could be reproduced applying the right treatment conditions. After 24 hours, 1 ml out of the two other media was plated on nutrient agar while the broth suspension was analyzed by optical density.

Spores re-suspended in pH 3.0 buffer, with and without glucose, were not able to

grow while spores suspended in nutrient broth resulted in almost the same optical density after 24 hours, ranging from 0.85 to 0.99.

Hence, it was assumed that no difference occurs in the mechanism of inactivation when the same reduction level is obtained within different pressure-temperature conditions and fixed pH value.

In the following figure the hypothesized mechanism of inactivation of spores is schematically represented.

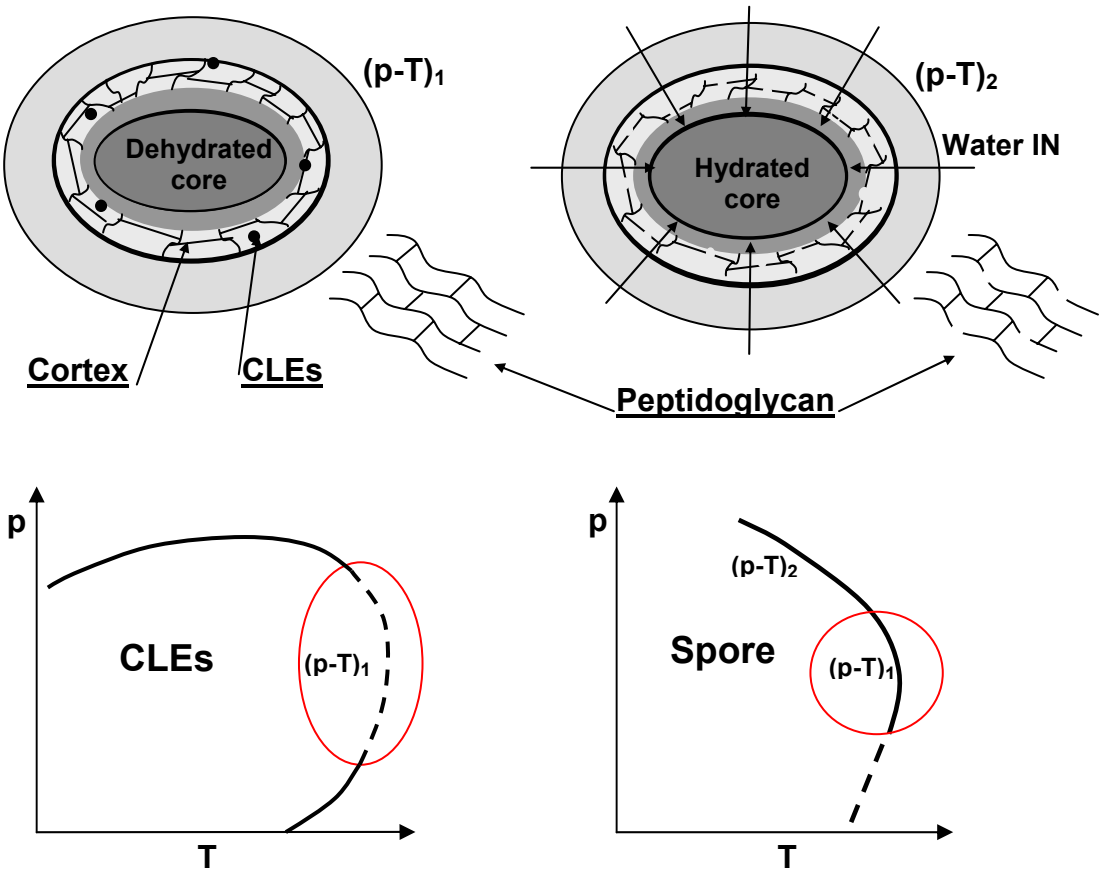


Fig.3.2.3.10 Hypothesized mechanism of spore inactivation. It is assumed that at $(p\text{-}T)_1$ the lytic-enzymes which are responsible for the cortex breakdown are inactivated by the pressure-temperature intensity of the treatment, while at $(p\text{-}T)_2$, higher pressure levels require lower temperature to achieve the same degree of inactivation.

The high temperature-pressure resistance of spores is mostly attributed to the high degree of dehydration of the core and to the presence of cortex-lytic enzymes (CLEs)

which degrade the spore cortex allowing germination.

It can be hypothesized that the key-enzymes, e. g. germination protease, which are responsible for cortex degradation and which are thought to be present in the peptidoglycan, may be inactivated by a specific combination of pressure and temperature, $(p\text{-}T)_1$, causing stability of the spore.

As can be observed in Fig.3.2.3.9, in the low pressure range (from 300 to 800 MPa) the iso-kinetic curves are characterized by a pressure independent behaviour, with the meaning that a threshold pressure level is required before that an increase of pressure can result in the reduction of the processing temperature.

This could be explained if it is assumed that the main role of pressure is to squeeze water inside the spore cortex and that depending on specific pressure-temperature conditions ($(p\text{-}T)_2$ in Fig 3.2.3.10) the spore core can be re-hydrated initiating a germination process which later leads to the inactivation of the spore.

It can be assumed that a pressure level of about 800 MPa is the minimum value which allows water entering the cortex and that, depending on the processing temperature, the core can be re-hydrated and subsequently inactivated.

At this pressure, low temperatures do not produce high inactivation.

4 Process optimization & technical aspects

4.1 Numerical modelling on the heat transfer during HHP treatments

In addition to the heat of compression calculated by equation (14) valid for adiabatic situations, the superposition of a heat flow across the boundary has to be taken into account in real high pressure systems.

The developing transient temperature field strongly depends on geometry and boundary heat transfer, and on the thermophysical and transport properties of the compressed materials.

A finite difference code based on heat conduction in radial coordinates was written which includes dT/dp as a time dependent heat source or sink during the compression or de-compression phase, respectively.

Since the use of Eqn.14 requires β, ρ and Cp for the actual temperature and pressure, these values have to be calculated at each spatial node and for every time step of the numerical routine, taking into account the different parts constituting the system.

To account for this behaviour the microbial inactivation has been implemented into the finite difference scheme, yielding the degree of inactivation for any radial position.

The time dependent inactivation was mathematically described coupling equation (17) and (19):

$$\left(\frac{N}{N_0}\right) = (1 + k \cdot N_0^{(n-1)} \cdot t \cdot (n-1))^{\frac{1}{1-n}} \qquad \text{Eqn. 17}$$

$$Ln(k) = k' + \frac{(-\Delta V^*)}{R \cdot T} \cdot p \qquad \text{Eqn. 19}$$

In order to demonstrate the effect of transient temperature conditions, two different microorganisms have been chosen.

Previous experimental studies on microbial inactivation showed that reaction orders n=1 and n=1.1 well reproduced the inactivation kinetics of *Lactobacillus rhamnosus* and *Alicyclobacillus acidoterrestris* in orange juice, respectively (Ardia et al., 2003).

The pressure-temperature dependence of the rate constant of both the microbial species, previously estimated, was related to the temperature distribution during the pressure treatment.

A PET container with a diameter of 80 mm, a length of 100 mm and a thickness of 0.5 mm was chosen for the simulations (see Fig.4.1.1).

Pure water was assumed as the pressure transmitting medium.

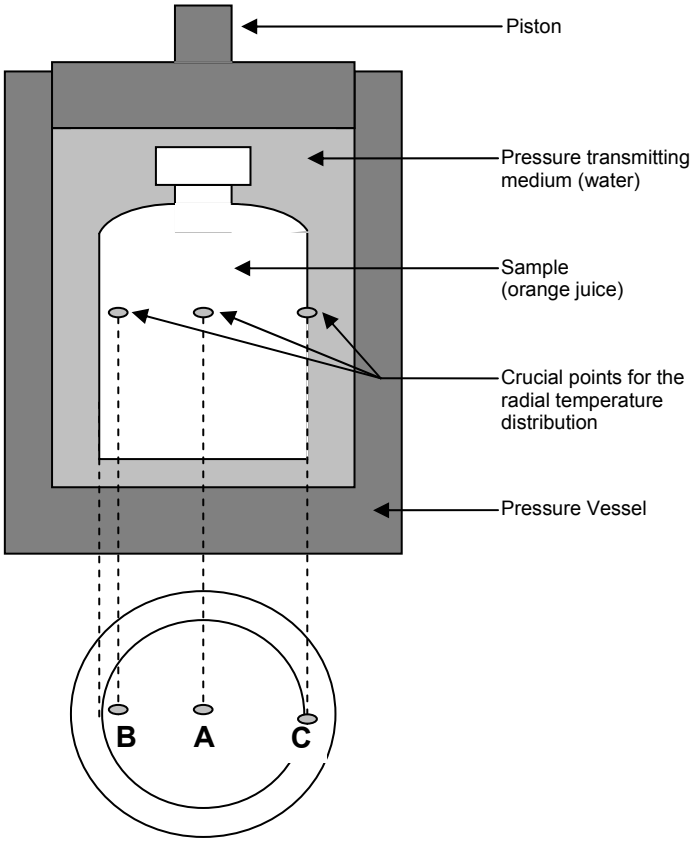


Fig.4.1.1 Schematic view of the geometry of the product sample used for the numerical simulation and the three points where the adiabatic heating has been predicted: A) centre of the sample; B) inner layer of the sample container; C) sample container (PET).

Heat transfer phenomena, conduction- described by the *Fourier* low- and convection- described by the *Nusselt* equation- have been taken into account by implementing in the finite difference code the QuickField software (Tera analysis Ltd., Denmark) for heat transfer simulations.

The pressure-temperature dependence of the thermal conductivity of pure water was derived by NIST database and implemented in the finite element code.

Once that the thermal distribution has been evaluated in the system during the whole pressure treatment, the microbial inactivation was estimated as a function of punctual pressure and temperature values.

Fig.4.1.2 shows the prediction of the temperature distribution and the inactivation kinetics of *Lactobacillus rhamnosus* (a) and *Alicyclobacillus acidoterrestris* spores (b) in orange juice, at two different locations in the sample (see Fig.4.1.1).

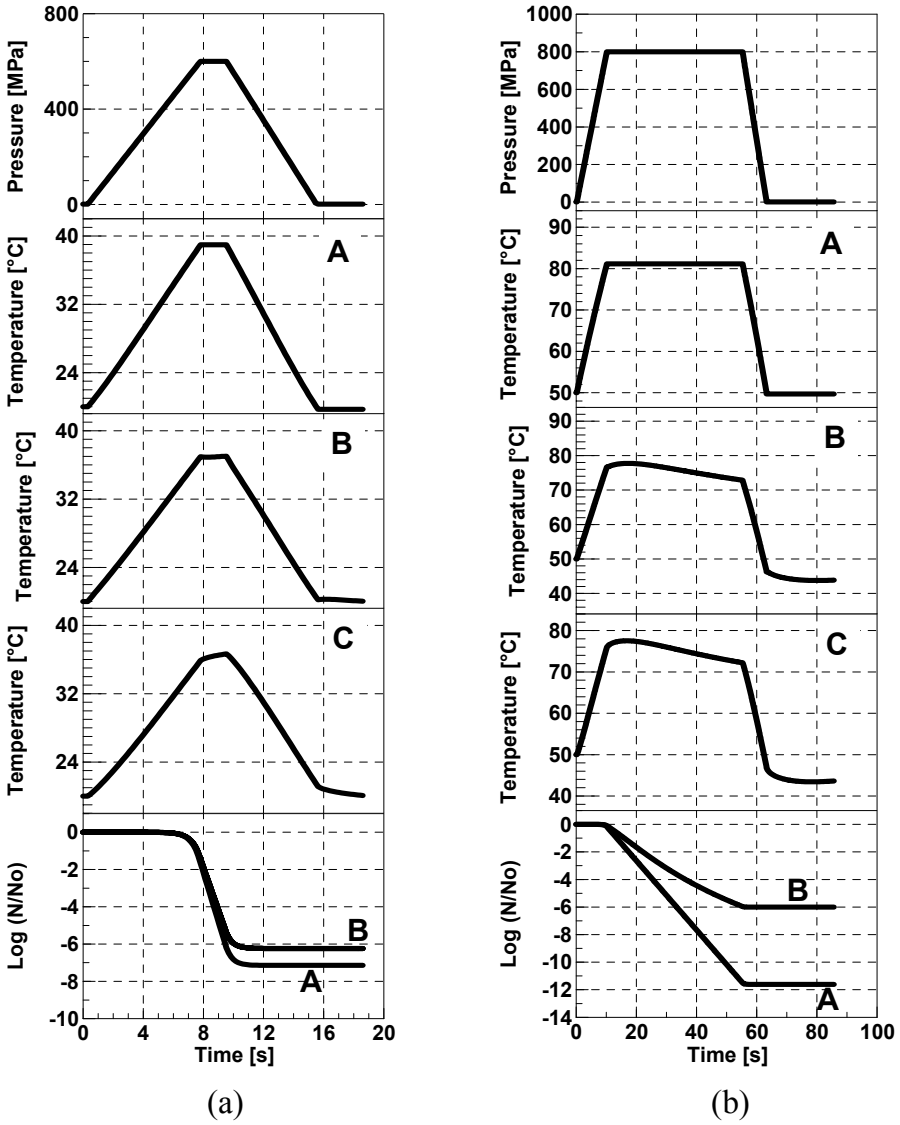


Fig.4.1.2 Simulation of adiabating heat and inactivation kinetics of *Lactobacillus rhamnosus* (a) and *Alicyclobacillus acidoterrestris* spores (b) in the crucial points of the sample, the centre (A), the inner layer of the product sample (B) and the PET container (C).

The simulation of *Lactobacillus rhamnosus* was performed in a range of pressure

from 0.1 to 600 MPa, starting from room temperature (20°C), while for *Alicyclobacillus acidoterrestris* spores a higher operative pressure of 800 MPa, and a higher value of the starting temperature, 50°C, has been chosen because of the stronger pressure and temperature resistance.

Simulating a pressure treatment on orange juice up to 600 MPa and starting from room temperature, a difference of 2°C between the centre (A) and the bulk of the sample (B), which produced a difference of almost 1 Log-cycle in the inactivation of *Lactobacillus rhamnosus* was predicted.

Similar results were detected for *Alicyclobacillus acidoterrestris*.

A pressure treatment up to 800 MPa, starting from an initial temperature of 50°C, produced a temperature increase of almost 30-32°C in the centre of the juice.

The adiabatic heating of the product at inner side-wall of the PET bottle was characterized by lower temperatures compared to the centre, because of the heat flux to the vessel which was assumed to maintain a temperature level of 50°C.

A difference of 3-4°C among the two crucial points resulted in a strong difference in the spores inactivation: soon after the pressure release, a difference of approximately 6 Log-cycles between the centre and the inner side-wall of the bottle was detected.

By the aim of the model it was possible to optimize the pressure and temperature processing conditions which are needed for pasteurization and sterilization processes.

When similar models are performed for different “ideal” products, it would be possible to predict the adiabating heating for several food products.

Knowledge about the end-temperature as function on the processing pressure would avoid any kind of thermal over-processing phenomena and would result in safer products with better quality, since it would be possible to predict the temperature distribution in the whole product.

Of course, the gradient of temperature among the crucial points of the system has to be taken into account. As shown in Fig.4.1.2 (b), the strong difference in the inactivation between the centre and the boundary of the product is related to gradient between the high temperature reached with the adiabatic heating and the low temperature of the metallic vessel.

Several option could be considered to prevent this effect: use of an insulating layer

as the inner part of the vessel; heating of the vessel to an appropriate temperature level which can minimize the gradient; use of a pressure transmitting medium with higher thermal conductivity in combination with mild heating of the vessel.

Once that the gradient of temperature which occurs in the product is known, it could be possible to optimize the pressure and temperature conditions in order to achieve the desired inactivation level and guarantee the safety margins that are required for pasteurized and sterilized products.

4.2 High pressure unit design for ultra-high pressure treatments

It has been previously mentioned that one of the main goals of this work was the research of possible technical improvements which could lead HHP technology to become a reality in the food industry.

Small volume vessels (e.g. 3L) would allow the simulation of a semi-continuous process which would be optimal for the preservation of food beverages.

The need of quick compression and decompression rates is therefore justified by the necessity of reducing the handling times and loading and unloading operations.

High degree of automation is required to shorten the whole processing time and obtain an adequate production layout.

The utilization of an internal intensifier, integrated within the high- pressure vessel, seems to be the right solution for the generation of ultra-high pressure levels in short times.

Gas accumulators, or in alternative large high pressure pumps, are feasible to provide the required amount of pressure in the low-pressure part of the intensifier.

In order to optimize the high pressure treatment, the right temperature conditions and an appropriate pressure-transmitting medium are required.

The utilization of an insulator material at the inner surface of the high pressure vessel, would be feasible to reduce the thermal gradient which otherwise would characterize the whole system because of the physics of the pressure build-up transformation.

Teflon is suggested for its good insulating properties.

Mild pre-heating of the product, prior to the compression phase, would be feasible to generate the desired operative temperature level after compression and avoid over-

processing phenomena in the food product.

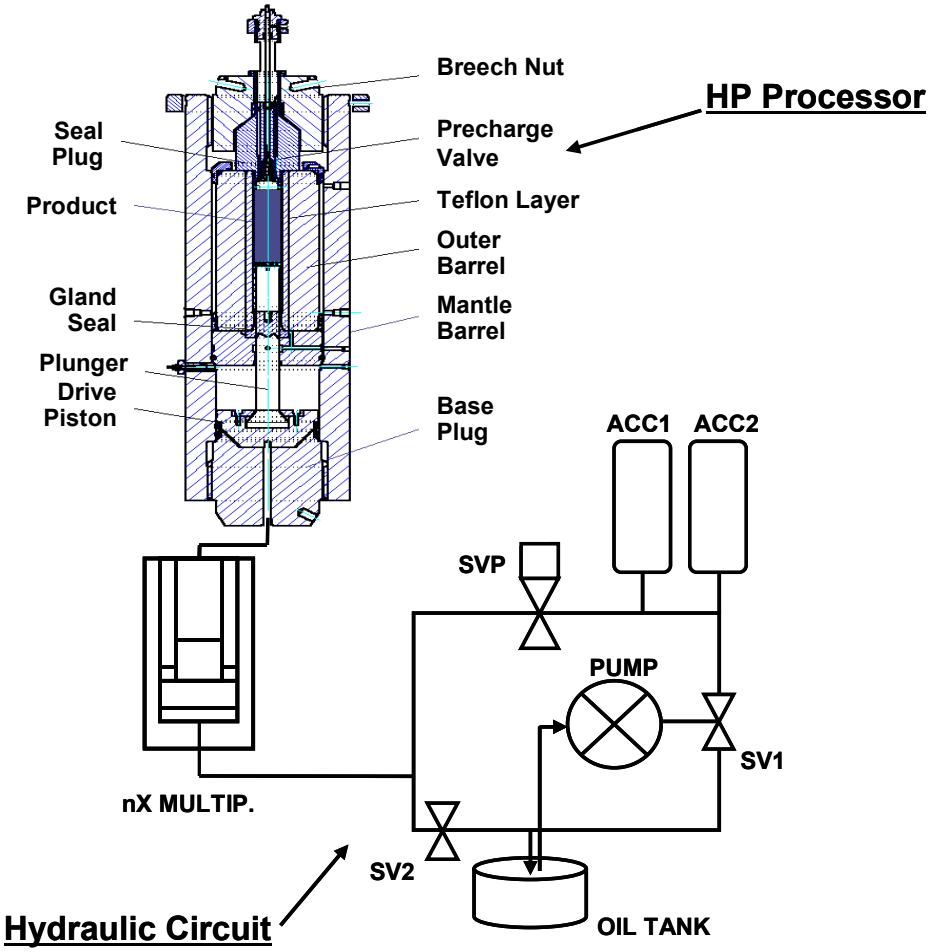


Fig.4.2.1 HHP set-up for food processing. The hydraulic circuit is constituted by a nX pressure multiplier which is loaded from gas accumulators. A second pressure intensifier is integrated within the processor (Plunger Press). The Precharge Valve keeps the Seal plug loaded to avoid extrusion phenomena and extend the durability of the seal. An inner Teflon Layer is inserted into the Barrel for reducing the thermal gradient between the food sample and the Mantle Barrel.

An appropriate pressure-transmitting medium should be used, depending on particular process which wants to be realized.

In the case of ultra-high pressure treatments (> 800 MPa) it is required a liquid medium which does not present phase-transition at the operative pressure-temperature conditions and which produces an adiabatic heat of compression which is comparable to the one generated by the food product.

In any case, the pressure-transmitting medium must be not aggressive for the packaging container and not toxic in the case it comes into contact with the food

product.

Problems related to the stability and durability of high pressure seals could be solved by the utilization of electrical actuators which could keep the seal loaded and stressed during the movements of the high pressure piston, avoiding extrusion phenomena.

Fig.4.2.1 shows a possible HHP set-up for food processing.

On the base of the micro-system set-up (par.2.1.3), high pressure treatments can be realized without the utilization of any packing system.

It is suggested that, for treatments of few seconds, tightness can be realized by face-to-face contact between two smooth metal surfaces.

In addition, it can be thought of placing a seal plug between the pressure-vessel and the upper breech nut, in the case of malfunctions of face-to-face coupling.

Such a system could guarantee rapid compression and decompression phases within short holding times and consequently safer products and high product quality.

When high degree of automation for loading and unloading operation is realized, high product layouts can be achieved, allowing HHP processing replacing conventional heat treatments in food industry.

Conclusions

Simple mixing rules considerations for water and sucrose have been used to model the temperature increase which occurs in the product during the pressure build-up.

It was possible to predict the thermal evolution for sucrose solutions at different concentrations of sugar as the first step to simulate the adiabatic heating in real products and simulate the temperature distribution all over the system.

By the use of simple mixing rules it has been shown that the NIST formulation for β, ρ and Cp of pure water can be used to accurately predict the temperature increase in response to pressure levels up to 600 MPa.

Since the NIST formulation provides data for pure water up to much higher pressure, it is suggested to extrapolate the model equations up to 1400 MPa which is still a relevant pressure level for food processing.

By the aid of the model it was possible to optimize the pressure and temperature processing conditions which are needed for pasteurization and sterilization processes.

Applying these models it is possible to predict the adiabatic heating of food products.

Knowledge about the end temperature as function on the processing pressure would avoid any kind of thermal over-processing phenomena and would result in safer products with better quality, since it would be possible to predict the temperature distribution in the whole product.

Of course, the gradient of temperature among the crucial points of the system has to be taken into account. The strong difference in the inactivation level between the centre and the boundary of the product is related to gradient between the high temperature reached within the adiabating heating and the low temperature of the metallic vessel.

Several option could be considered to prevent this effect: use of an insulating layer as the inner part of the vessel; heating of the vessel to an appropriate temperature level which can minimize the gradient; use of a pressure transmitting medium with higher thermal conductivity in combination with mild heating of the vessel.

Once that the gradient of temperature which occurs in the product is known, it could be possible to optimize the pressure and temperature conditions in order to achieve the desired inactivation level and guarantee the safety margins that are required for

pasteurized and sterilized products.

The inactivation kinetics of different microbial strains have been studied in a low pressure range and in a high pressure range.

Results obtained by the application of the standard MPN technique for enumeration of low microbial concentrations showed that the inactivation kinetics of lactic acid bacteria in orange juice can be efficiently represented by a 1st order reaction and a tailing behaviour can be ruled out. It was hypothesized that, as a consequence of adhesive forces, a protective effect of fibres, which are present in orange juice, could result in an over-estimation of microbial survivors and, consequently, standard plate count techniques resulted in an overestimation of the survivor count due to the non-uniform distribution of bacteria in serial dilution steps.

In accordance with results from other authors (Lee et al., 2002), high temperature treatments alone did not result in a significant reduction of *A. acidoterrestris* spores, while a combination of high pressure and heat strongly reduced the numbers of survivors.

By minimizing the accumulated residual standard error, a common reaction *n*th-order was obtained by which it was possible to determine the pressure-temperature conditions required to achieve identical inactivation levels within different treatment times.

It is well known that the inactivation rate of microorganisms is related to pressure and temperature. For transient situations, like those encountered in high pressure vessels, fluctuations in microbial reduction during the treatment should be anticipated. Hence, the gradient of temperature among the crucial points of the system has to be taken into account.

By modelling the inactivation kinetics of *Lactobacillus rhamnosus* and *Alicyclobacillus acidoterrestris* in orange juice, it was possible to relate the microbial inactivation to the pressure-temperature distribution at any radial position in the high pressure vessel.

This procedure allows to find the optimum combination among the operative conditions in order to reduce the strong quality damage due to the thermal treatments and the high pressure level needed for microbial inactivation in food products.

Calculating the increase of temperature due to the adiabatic heating and reducing the

thermal gradient during the whole pressure treatment, it is possible to start the process with a lower temperature of the product, resulting in more benefits for the product quality and in a lower cost of the whole preservative process.

The impact of combined pressure-temperature treatments on the inactivation of *B. stearothermophilus* spores was studied in different buffer solutions.

An increase of temperature resulted in higher inactivation rates for each considered pressure level.

When plotting the effect of different pressure treatments at fixed temperature in pH 7.0 phosphate buffer, an unexpected behaviour was observed. In the higher pressure range (> 700 MPa), the higher temperature levels were characterized by a minimum in the inactivation in correspondence of 1000 MPa, while at the lower temperatures a shift of the minimum value to higher pressure was observed.

It was hypothesized that the unexpected phenomenon could be related to the buffering properties of the treated solutions. Due to the instability of phosphate buffer at high pressure-temperature conditions (Marshall et al, 1981), it was hypothesized that an increase of the acidity of the medium, related to a reduction of the pH, could influence the spore resistance and, consequently, the elliptical shape which characterizes the iso-kinetic curves in the p-T diagram, could be the result of a composed effect of different pH levels, since the pressure-temperature instability of phosphate buffer could result in a lower pH level at extreme processing conditions. In order to clarify this behaviour, a more pressure-temperature stable buffer solution- Aces buffer- was tested at two different pH levels, 6.0 and 7.0.

At higher temperatures the inactivation increased progressively with pressure, while higher acidity produced higher inactivation. At lower temperatures, pressure-stabilization occurred and the inactivation in a pH 6.0 Aces buffer did not improve when pressure was increased at maximum value. At the lowest temperature, the highest pressure level resulted in lower inactivations rates.

In particular, a threshold temperature level was observed, below which an increase of pressure was not enhancing the microbial reduction.

Since a stabilizing effect was observed for both the three different buffer solutions at medium and low temperature levels, it was assumed that the impact of a different acidity level is not the main source of the phenomenon.

The elliptical shape, which is characteristic for enzymes activity inactivations, suggests that under a certain range of pressure-temperature conditions, the enzymes which catalyze the hydrolytic reaction leading to the spore destruction, are pressure unstable and that an ultra-high pressure treatment could only be applied in assisting a thermal treatment.

It is suggested that the main effect of pressure on the single spore cell is to squeeze water inside of spore cortex. Depending on a specific pressure-temperature combination, the enzymes which are responsible for the cortex breakdown (CLEs) and for the access of water to the spore core are inactivated, so that, maximal hydration of the core and related cell inactivation require higher temperature levels. At this stage, temperature is the main variable leading the inactivation process, while an increase of the pressure level does not produce higher inactivation.

When the specific pressure-temperature conditions- $(p\text{-}T)_1$- do not affect the CLEs stability, water has free access to the spore core which is inactivated after re-hydration. At this stage, pressure is leading the inactivation process while lower temperature conditions are required to achieve equal inactivation levels.

It can be assumed that a pressure level of about 800 MPa is the minimum value which allows water entering the cortex and that, depending on the processing temperature, the core can be re-hydrated and subsequently inactivated.

Further experiments are required to provide a proof for the hypothesized mechanism, which might be the object of future investigations.

In conclusion, a HHP process is suggested which is characterized by a strongly reduced heat intensity, producing safe foods from a microbial point of view and high production layouts, which could enhance HHP processing in replacing conventional heat treatments in the food industry.

Annex

Regression Coefficients & Standard Errors tables

1- Eqn.15. n-hexane

r^2 Coef Det	DF Adj r^2	Fit Std Error	F-value
0.9359040675	0.927163713	0.0023105776	131.4145262

Parameters	Value	Std Error
a	0.104084891	0.009490058
b	-0.00011202	1.87052e-005
c	0.000199861	8.30343e-005
d	3.48421e-008	9.5614e-009
e	-1.4807e-006	5.11554e-007
f	3.27904e-008	6.31345e-008

2- Eqn.15. Di-2-ethyl-hexyl sebacate

r^2 Coef Det	DF Adj r^2	Fit Std Error	F-value
0.9713558861	0.9684914748	0.0023288008	413.71647493

Parameters	Value	Std Error
a	0.077568887	0.002999642
b	-7.0866e-005	5.70624e-006
c	0.00011502	5.60536e-005
d	1.82827e-008	3.08539e-009
e	-2.3313e-007	3.57355e-007
f	-1.9837e-008	2.92654e-008

3- Eqn.23. *Bacillus stearothermophilus* in pH 7.0 Aces buffer

r^2 Coef Det	DF Adj r^2	Fit Std Error	F-value
0.9930140307	0.9846308676	0.1301784474	170.57287119

Parameters	Value	Std Error
a	19.31098639	9.985098488
b	-0.42214509	0.176894261
c	-0.00991392	0.003070253
d	0.002069957	0.000797177
e	2.5519e-007	9.39482e-007
f	8.94888e-005	2.0583e-005

4- Eqn.23. *Bacillus stearothermophilus* in pH 6.0 Aces buffer

r^2 Coef Det	DF Adj r^2	Fit Std Error	F-value
0.9940490744	0.9869079637	0.1343533029	200.44930316

Parameters	Value	Std Error
a	-7.66707476	10.30532311
b	0.085483581	0.182567305
c	-0.01179632	0.003168716
d	-0.00038524	0.000822743
e	-1.1233e-006	9.69611e-007
f	0.000132944	2.12431e-005

5- Eqn.25. *Bacillus stearothermophilus* in pH 7.0 phosphate buffer

r^2 Coef Det	DF Adj r^2	Fit Std Error	F-value
0.9423071385	0.9224130483	0.353479277	54.443889829

Parameters	Value	Std Error
a	-25.7833166	46.11643675
b	0.1658984	1.221452655
c	0.042900248	0.024806461
d	-0.00095546	0.010949148
e	-4.6216e-005	1.48831e-005
f	-5.3012e-005	0.000321283
g	5.98028e-006	3.29335e-005
h	7.59854e-009	4.33829e-009
i	2.17083e-007	6.44684e-008
j	-1.2232e-006	1.3377e-006

Summa of Standard Fit Error & optimal polynomial order n

6- Eqn.18. *Alicyclobacillus acidoterrestris* in orange juice

Conditions p(MPa)- T(°C)	Order n					
	o1	o1,05	o1,1	o1,15	o1,2	o1,25
0,1- 80	0,109033	0,118453	0,155245	0,199667	0,243923	0,364416
0,1- 85	0,218526	0,132519	0,267368	0,417692	0,549466	0,661834
0,1- 90	0,551033	0,26058	0,19517	0,350691	0,513778	0,646084
0,1- 95	0,52769	0,211788	0,354578	0,604117	0,795579	0,938765
100- 80	0,095971	0,070484	0,054578	0,053823	0,066325	0,084798
100- 85	0,154183	0,059548	0,052892	0,126541	0,196552	0,259053
100- 90	0,427398	0,247027	0,330516	0,493434	0,641779	0,764905
100- 95	0,670534	0,386489	0,159626	0,101975	0,227281	0,343853
200- 80	0,236014	0,148317	0,105689	0,131174	0,186908	0,24544
200- 85	0,423058	0,224504	0,0572	0,09916	0,220073	0,32227
200- 90	0,528996	0,135066	0,250122	0,500246	0,699643	0,857032
200- 95	0,686842	0,29984	0,05056	0,266554	0,455538	0,603466
300- 80	0,972473	0,561149	0,312706	0,348637	0,518267	0,684343
300- 85	0,400853	0,151283	0,138189	0,303681	0,450701	0,572475
300- 90	0,623244	0,189093	0,265252	0,509727	0,714851	0,877502
300- 95	1,087862	0,563208	0,178706	0,226379	0,445675	0,621454
500- 80	1,593164	0,732277	0,335964	0,180718	0,305152	0,470073
500- 85	0,526535	0,181217	0,128668	0,347035	0,523988	0,662708
500- 90	1,666821	0,82164	0,35802	0,195836	0,336265	0,504243
500- 95	1,020695	0,474828	0,214757	0,357102	0,560406	0,735035
600- 80	0,948126	0,478641	0,158878	0,252117	0,456356	0,619918
600- 85	1,361547	0,509758	0,261058	0,445236	0,678362	0,863472
600- 90	1,421899	0,820728	0,263504	0,11552	0,384125	0,576961
600- 95	0,986936	0,456527	0,099575	0,145251	0,315797	0,43736
700- 80	1,31829	0,908258	0,591147	0,409841	0,379194	0,438339
700- 85	0,889293	0,536784	0,335583	0,35798	0,498914	0,641663
700- 90	0,719829	0,448388	0,364958	0,448005	0,585093	0,716599
700- 95	0,367759	0,277751	0,392233	0,555219	0,70858	0,842813
SUM Fit St. Err.	20,5346	10,40614	**6,432738**	8,543361	12,65857	16,35687

7- Eqn.18. *Bacillus stearothermophilus* in pH 7.0 phosphate buffer

Conditions p(MPa)- T(C°)	Order n					
	o1	**o1,05**	**o1,1**	**o1,15**	**o1,2**	**o1,25**
800-130	0,66359	0,1079	0,36657	0,7276	1,0059	1,051
800-120	0,622501	0,1417	0,1889	0,4042	0,5513	0,6545
800-110	1,469	0,8294	0,383	0,0934	0,09867	0,2304
800-100	1,43	0,838	0,3897	0,1415	0,2346	0,3787
800-90	0,78824	0,3708	0,0547	0,17056	0,331	0,4474
900-130	1,2097	0,5887	0,1	0,3415	0,637	0,6916
900-120	0,7804	0,2049	0,3803	0,7137	0,9608	1,044
900-110	0,958807	0,39386	0,093	0,3628	0,5777	0,6852
900-100	1,057	0,4041	0,17544	0,50933	0,7664	0,8705
900-90	0,9182	0,7041	0,5217	0,3752	0,2911	0,26
1000-130	0,8817	0,4207	0,1948	0,4644	0,7404	0,7567
1000-120	0,5698	0,2259	0,4083	0,7231	0,9989	2,1229
1000-110	0,6396	0,07769	0,4147	0,7431	0,9825	1,1594
1000-100	0,7984	0,4339	0,2495	0,3715	0,5569	0,7177
1000-90	0,3673	0,2413	0,12987	0,0348	0,0447	0,11096
1100-130	0,7674	0,3722	0,4037	0,6524	0,8799	1,0643
1100-120	0,1404	0,367	0,67411	0,926	1,1326	1,3035
1100-110	0,4305	0,1537	0,2464	0,4712	0,6688	0,8349
1100-100	0,5439	0,2885	0,1699	0,25912	0,39	0,5066
1100-90	0,2579	0,1465	0,0472	0,03826	0,1107	0,1716
1200-130	0,8254	0,3663	0,2555	0,4817	0,6965	0,8654
1200-120	0,869	0,3963	0,2234	0,4806	0,7363	0,9424
1200-110	0,8015	0,3429	0,2984	0,5315	0,7456	0,9179
1200-100	0,9407	0,4417	0,2076	0,4258	0,6641	0,8547
1200-90	0,3466	0,2543	0,1704	0,0967	0,033	0,0216
1400-130	0,0273	0,5548	0,998	1,3281	1,5684	1,7452
1400-120	0,1868	0,3883	0,77429	1,077	0,3109	1,49187
1400-110	0,7403	0,2415	0,4983	0,8531	1,1219	1,3232
1400-100	0,7953	0,5225	0,2866	0,0979	0,09859	0,22407
1400-90	0,234	0,1951	0,1583	0,1239	0,0922	0,0633
SUM Fit St. Err.	21,06124	11,01455	**9,46258**	14,01997	18,02736	23,5115

8- Eqn.18. *Bacillus stearothermophilus* in pH 7.0 Aces buffer

Conditions p(MPa)- T(°C)	Order n					
	o1	**o1,05**	**o1,1**	**o1,15**	**o1,2**	**o1,25**
800-120	0,7387	0,4992	0,02998	0,2427	0,2124	1,18461
800-110	1,1776	0,799	0,01087	0,2262	0,4763	0,1965
800-100	0,6698	0,4524	0,2603	0,1118	0,9747	0,905
1000-120	1,1037	0,7567	0,018115	0,2776	0,1684	0,0814
1000-110	0,937	0,547	0,02168	0,2523	0,2484	0,4035
1000-100	0,7901	0,5404	0,3277	0,1798	0,1573	0,2486
1200-120	0,997	0,573	0,2509	0,218	0,1408	0,2112
1200-110	1,208	0,846	0,5386	0,309	0,1856	0,2043
1200-100	0,6261	0,4346	0,2623	0,1199	0,639	1,7463
1400-120	1,548	0,879	0,417	0,12	0,751	0,2082
1400-110	0,889	0,4948	0,1644	0,1057	0,3036	0,4586
1400-100	0,6504	0,4803	0,3254	0,6941	0,799	0,3992
SUM Fit St. Err.	11,3354	7,3024	**2,627245**	2,8571	4,59107	6,24741

9- Eqn.18. *Bacillus stearothermophilus* in pH 6.0 Aces buffer

Conditions p(MPa)- T(°C)	Order n					
	o1	**o1,05**	**o1,1**	**o1,15**	**o1,2**	**o1,25**
800-120	1,905	1,129	0,5286	0,1164	0,1893	0,339
800-110	0,6559	0,3997	0,1822	0,0139	0,1143	0,2125
800-100	0,923	0,527	0,1946	0,0824	0,2779	0,433
1000-120	1,0106	0,4799	0,044	0,2797	0,4292	0,5597
1000-110	1,229	0,8843	0,6093	0,4051	0,2556	0,2077
1000-100	0,8827	0,5483	0,2646	0,0916	0,1957	0,1166
1200-120	0,6803	0,1857	0,1293	0,329	0,4604	0,55
1200-110	0,8374	0,4593	0,1669	0,0456	0,1995	0,3123
1200-100	1,1988	0,9256	0,1778	0,6712	0,7079	0,488
1400-120	1,12	1,056	0,056	0,123	0,567	0,689
1400-110	0,865	0,593	0,37	0,198	0,0674	0,0325
1400-100	1,1491	0,9202	0,2078	0,7243	0,57198	0,5201
SUM Fit St. Err.	12,4568	8,108	**2,9311**	3,0802	4,03618	4,4604

References

Alpas, H., Kalchayanand, N., Bozoglu, F., Sikes, A., Dunne, C. P. and Ray, B., **1999**. Variation in Resistance to Hydrostatic Pressure among Strains of Food-Borne Pathogens. Applied and Enviromental Microbiology, 65, 9, 4248-4251.

Ananta, E., Heinz, V., Schlüter, O. and Knorr, D., **2001**. Kinetic studies on high pressure inactivation of *Bacillus stearothermophilus* spores suspended in food matrices. Innovative Food Science & Emerging Technologies, 2, 261-272.

Ando, Y., Tsuzuki, T., **1983**. Mechanism of chemical manipulation of the heat resistance of *Clostridium perfringens* spore, Journal of Applied Bacteriology, 54, 197-202.

Ardia, A., Knorr, D., Ferrari, G. and Heinz, V., **2003**. Kinetics studies on combined high pressure and temperature inactivation of *Alicyclobacillus acidoterrestris* spores in orange juice. Applied Biotechnology , Food Science and Policy, 1,3, 169-173.

Ardia, A., Knorr, D. and Heinz, V., **2004**. Adiabatic heat modelling for pressure build-up during high pressure treatment in food processing. Trans IChemE, Part C, Food and Bioproducts Processing, 82(C1), 89-95.

Bender, G. R., Marquis, R.E., **1982**. Sensitivity of various salt forms of *Bacillus megaterium* spores to the germinating action of Hydrostatic pressure. Journal of Microbiology. 28, pp. 643-649.

Bender, G. R. and Marquis, R. E., **1985**. Spore hat resistance and specific mineralization. Applied and Environmental Microbiology, 50,1414-1421.

Benito, A., Ventoura, G., Casadei, M., Robinson, T. and Mackey, B., **1999**. Variation in Resistance of Natural Isolates of *Escherichia coli O157* to High

Hydrostatic Pressure, Mild Heat, and Other Stresses. Applied and Enviromental Microbiology, 65, 4, 1564-1569.

Bergère, J. L., **1991**. Spores formation and germination of *Bacillus cereus* : the spore cycle. Bulletin of the IDF 275, 9-14.

Berlin, D. L., Herson, D. S., Hicks, D. T. and Hoover, D. G., **1999**. Response of Pathogenic *Vibrio* Species to High Hydrostatic Pressure. Applied and Enviromental Microbiology, 65, 6, 2776-2780.

Bubnik, Z., Kadlec, P., Urban, D., Bruhns, M., **1995**. Sugar Technologists Manual., Edited by *Dr. Albert Bartens KG*, Berlin, Germany, pp. 116.

Butz, P., Ries., Traugott, U., Weber, H. and Ludwig, H., **1990**. Hochdruck-inaktivierung von bacterien und bakteriensporen. Pharmazeutische Industrie, 52, 487-491.

Capellas, M., Mor-Mur, M., Gervilla, R., Yuste, J., Guamis, B., **2000**. Effect of high hydrostatic pressure combined with mild heat or nisin on inoculated bacteria and mesophiles of goat's milk fresh cheese. Food Microbiology, 17, 6, 633-641.

Cerny, G., Hennlich, W. and Poralla, K., **1984**. Spoilage of fruit juice by bacilli: isolation and characterization of the spoilage organisms. Z. Lebensm. Unters. Forsch., 179, 224-227.

Cheftel, J. C., **1992**. Effect of high hydrostatic pressure on food constituents: an overview. In: Balny, C., Hayashi, R., Heremans, K., Masson, P., editors. High Pressure and Biotechnology (Proceedings of the First European Seminar on High Pressure and Biotechnology, La Grand Motte), 224, 195-209.

Cheftel, J. C., **1995**. High pressure, microbial inactivation and food preservation, Food Sci. Technol. Int. 1:75.

Cheftel, J. C., Culioli, J., **1997**. Effects of high pressure on meat: a review. Meat Science, 46, 3, 211-236.

Chen, H., Hoover, D. G., **2003**. Modelling the combined effect of high hydrostatic pressure and mild heat on the inactivation kinetics of *Listeria monocytogenes Scott A* in whole milk. Innovative Food Science and Emerging Technologies, 4, 25-34.

Clouston, J. G. and Wills, P. A., **1969**. Initiation of germination and inactivation of *Bacillus pumilus* spores by hydrostatic pressure. Journal of Bacteriology, 97, 684-690.

Earnshaw, R. G., **1995**. Kinetics of high pressure inactivation of microorganisms. In “High Pressure Processing of Foods”, Ledward, D. A., Johnston, D. E., Earnshaw, R. G., Hasting, A. P. M., eds. Nottingham University Press, p. 37.

Eyring, H., **1935**. The activated complex in chemical reactions. Journal of Chemical physics, 3, 107-115.

Furukawa, S., Noma, S., Shimoda, M. and Hayakawa, I., **2002**. Effect of initial concentration of bacterial suspensions on their inactivation by high hydrostatic pressure. International Journal of Food Science and Technology, 37, 573-577.

Furukawa, S., Noma, S., Yoshikawa, S., Furuya, H., Shimoda, M. and Hayakawa, I., **2001**. Effect of filtration of bacterial suspensions on the inactivation ratio in hydrostatic pressure treatments. Journal of food Engineering, 50, 59-61.

Furukawa, S., Shimoda, M. and Hayakawa, I., **2003**. Mechanism of the inactivation of bacterial spores by reciprocal pressurization treatment. Journal of Applied Microbiology, 94, 836-841.

Garcia-Graells, C., Valckx, C. and Michiels, C. W., **2000**. Inactivation of

Escherichia coli and *Listeria innocua* in milk by combined treatment with high hydrostatic pressure and the lactoperoxidase system. Applied and Environmental Microbiology, 66, 10, 4173-4179.

Garthright, W. E., **1998**. Most probable number from serial dilutions. Food and Drug Administration Bacteriological Analytical Manual, 8th edition, revision A, appendix 2. Gaithersburg MD, AOAC Intl.

Gola, S., Foman, C., Carpi, G., Maggi, A., Cassara, A. and Rovere, P., **1996**. Inactivation of bacterial spores in phosphate buffer and in vegetable cream treated with high pressure. High Pressure Bioscience and Biotechnology, pp. 253-259, Sanei Schuppan, Kyoto, Japan.

Gould, G. W. and Sale, A. J. H., **1970**. Initiation of germination of bacterial spores by hydrostatic pressure. Journal of General Microbiology, 60, 335-346.

Gould, G. W., **1996**. Methods for preservation and extension of shelf life. International Journal of Food Microbiology, 33, 1, 51-64.

Haas, G. J., Prescott, H. E. et al., **1989**. Inactivation of microorganisms by carbon dioxide under pressure. Journal of Food Safety, 9, 253, 265.

Hayakawa, I., Furukawa, S., Midzunaga, A. et al., **1998**. Mechanism of inactivation of heat-tolerant spores of *Bacillus stearothermophilus IFO 12550* by rapid decompression. Journal of Food Science, 63, 3, 371-374.

Heinz, V., **1997**. Wirkung hoher hydrostatisher Drucke auf das Absterbe- und Keimunungsverhalten sporenbildender Bakterien am von *Bacillus subtilis* ATCC 9372. PHD thesys.

Heinz, V. and Knorr, D., **2001**. Effects of High Pressure on Spores. In: Ultra High Pressure Treatments of Foods, Hendrix, M. E. G. and Knorr, D. (eds.), 4, 77-113.

Kluwer Academic/ Plenum Publishers, New York.

Hayakawa, I., Kanno, T., Yoshiyama, K. and Fujio, Y., **1994**. Oscillatory compared with continuous high pressure sterilization on *Bacillus stearothermophilus* spores. Journal of Food Science, 59, 164-167.

Karatzas, A. K., Kets, E. P. W., Smid, E. J. and Bennik, M. H. J., **2001**. The combined effect of carvacrol and high hydrostatic pressure on *Listeria monocytogenes Scott A*. Journal of Applied Microbiology, 90, 3, 463.

Khadre, M.A., Yousef, A.E., **2002**. Susceptibility of human rotavirus to ozone, high pressure, and electric field. Journal of Food Protection, 65, 9, 1441-1446.

Kingsley, D. H., Hoover, D. G., Papafragkou, E., Richards, G. P., **2002**. Inactivation of *Hepatitis A* virus and a *calcivirus* by high hydrostatic pressure. Journal of Food Protection, 65, 10, 1605-1609.

Knorr, D. **1993**. Effects of high-hydrostatic pressure process on food safety and quality. Food Technology, 47, 6, 156.

Lee, J. K., Movahedi, S., Harding, S. E. and Waites, W. M., **2003**. The effect of acid shock on sporulating *Bacillus subtilis* cells. Journal of Applied microbiology, 94, 2, 184.

Lee, S.Y., Dougherty, R. H. and Kang, D.H., **2002**. Inhibitory effects of high pressure and heat on *Alicyclobacillus acidoterrestris* spores in apple juice. Applied and Environmental Microbiology, 68, 8, 4158-4161.

Lee, S.Y., Dougherty, R. H. and Kang, D.H., **2002**. Effect of high pressure combined with heat on reducing populations of *Alicyclobacillus acidoterrestris* in orange juice. In: Food Microbiology: fruit and vegetables. Section 15D-19. Annual Meeting and Food Expo. 2002 Jun 16: Anaheim, CA, USA

Lewis, M. J, **1987**. Physical Properties of Foods and Food Processing Systems. Edited by *Ellis Horwood Ltd*., Chichester, England, pp. 54, 222.

López-Pedemonte, T. J., Roig-Sagués, A. X., Trujillo, A. J., Capellas, M., Guamis, B., **2003**. Inactivation of spores of *Bacillus cereus* in cheese by high hydrostatic pressure with the addition of nisin or lysozyme. Journal of Dairy Science, 86, 3075-3081.

Marshall, W. L. and Frank, E. U., **1981**. Ion Product of Water Substance, -- 1000°C, 1-10,000 Bars – New International Formulation and its Background. Journal of Phys. Chem. Ref. Data 10, 2, 295-304.

Marquis, R. E. and Bender, G. R., **1985**. Mineralization and heat resistance of bacterial spores. Journal of Bacteriology, 161, 789-791.

Masschalck, B., Van Houdt, R., Van Haver, E. G. R. and Michiels, C. W., **2001**. Inactivation of Gram-Negative bacteria by lysozyme, denaturated lysozyme, and lysozyme-derived peptides under high hydrostatic pressure. Applied and Environmental Microbiology, 67,1, 339-344.

McIntyre, S., Ikawa, J. Y., Parkinson, N., Haglund, J. and Lee, J., **1995**. Characteristics of an acidophilic Bacillus strain isolated from shelf-stable juices. Journal of Food Protection, 58, 319-321.

Meyer, R. S., Cooper, K. L., Knorr, D., Lelieveld, H., L. M., **2000**. High pressure Sterilization of Foods, Foodtechnology, 54, 11, pp. 67-72.

Morgan, S. M., Ross, R. P., Beresford, T. and Hill, C., **2000**. Combination of hydrostatic pressure and lacticin 3147 causes increased killing of *Staphylococcus* and *Listeria*. Journal of Applied Microbiology, 88, 414-420.

Murakami, M., Tedzuka, H. and Yamazaki, K., **1998**. Thermal resistance of

Alicyclobacillus acidoterrestris spores in different buffers and pH. Food Microbiology, 15, 577-582.

Nakayama, A., Yano, Y., Kobayashi, S., Ishikawa, M. and Sakai, K., **1996**. Comparison of pressure Resistances of spores of six *Bacillus* strains with their heat resistances. Applied and Environmental Microbiology, 62, 10, 3897-3900.

Nicholson, W. L., Munakata, N., Horneck, G., Melosh, H. J. and Setlow, P., **2000**. Resistance of *Bacillus* Endospores to Extreme Terrestrial and Extraterrestrial Environments. Microbiology and Molecular Biology Reviews, 64, 3, 548-572.

NIST (National Institute of Standard and Technology) database, U.S. Commerce Department's Technology Administration.

Oh, S., Moon, M. J., **2003**. Inactivation of *Bacillus cereus* spores by high hydrostatic pressure at different temperatures. Journal of Food Protection, 66, 599-603.

Okazaki, T., Kakugawa, K., Yoneda, T. and Suzuki, K., **2000**. Inactivation behaviour of heat-resistant bacterial spores by thermal treatments combined with high hydrostatic pressure. Food Science Technology, 6, 3, 204-207.

Okazaki, T., Kakugawa, S., Yamauchi, S., Yoneda, T., Suzuki, K., **1996**. Combined effects of temperature and pressure on inactivation of heat-resistant bacteria. In Hayashi, R. and Balny, C. (Eds.), High Pressure Bioscience and Biotechnology, pp. 415-422, Elsevier Science B. V., The Netherlands.

Paidhungat, M., Setlow, B., Daniels, W. B., Hoover, D. G., Papafragkou, E. and Setlow, P., **2002**. Mechanisms of induction of germination of *Bacillus subtilis* spores by high pressure. Applied and Environmental Microbiology, 68, 6, 3172-3175.

Patterson, M.F., Queen, M., Simpson, R. and Gilmour, A., **1995**. Effect of high pressure on vegetative pathogens. In “High Pressure Processing of Foods”, Ledward, D. A., Johnston, D. E., Earnshaw, R. G., Hasting, A. P. M., eds. *Nottingham University Press*, p. 47.

Perry, R. H., **1984**. Perry’s Chemical Engineers’ Handbook, Sixth Edition. Edited by McGraw- Hill Book Co., section 4, pp. 54.

Ponce, E., Pla, R., Capellas, M., Guamis, B., Mor-Mur, M., **1998**. Inactivation of *Escherichia coli* inoculated in liquid whole egg by high hydrostatic pressure. Food Microbiology, 15, 3, 265-272.

Ponce, E., Pla, R., Sendra, E., Capellas, M., Guamis, B., Mor-Mur, M., **1999**. Destruction of *Salmonella enteritidis* inoculated in liquid whole egg by high hydrostatic pressure: comparative study in selective and non-selective media. Food Microbiology, 16, 4, 357-365.

Pontius, A. J., Rushing, J. E. and Foegeding, P. M., **1998**. Heat resistance of *Alicyclobacillus acidoterrestris* spores as affected by various pH values and organic acids. Journal of Food Protection, 61, 41-46.

Reddy, N. R., Solomon, H. M., Fingerhut, g. A., Rhodehamel, E. J., Balasubramaniam, V. M. and Palaniappan, S., **1999**. Inactivation of *Clostridium Botulinum type E* spores by high pressure processing. Journal of Food Safety, 19, 277-288.

Ritz, M., Tholozan, J. L., Federighi and Pilet, M. F., **2001**. Morphological and Physiological Characterization of *Listeria monocytogenes* Subjected to High Hydrostatic Pressure. Applied and Enviromental Microbiology, 76, 5, 2240-2247.

Roberts, C. M. and Hoover, D. G., **1996**. Sensitivity of *Bacillus coagulans* spores to combination of high hydrostatic pressure, heat, acidity and nisin. Journal of

Applied microbiology, 81, 363-368.

Sale, A. J. H., Gould, G. W. and Hamilton W. A., **1970**. Inactivation of bacterial spores by hydrostatic pressure. Journal of General Microbiology, 60, 323-334.

Sale, A. J. H., Gould, G. W. and Hamilton, W. A., **1970**. Inactivation of bacterial spores by hydrostatic pressure. Journal of General Microbiology, 60, 323.

Sale, A. J. H., Gould, G. J. W. and Hamilton, W. A., **1970**. Inactivation of bacterial spores by hydrostatic pressure. Journal of Bacteriology., 121, 664.

Setlow, B., Cowan, A. E., Setlow, P., **2003**. Germination of spores of *Bacillus subtilis* with dodecylamine. Journal of Applied Microbiology, 95, 3, 637.

Setlow, B., Loshon, C. A., Genest, P. c., Cowan, A. e., Setlow, C. and Setlow, P., **2002**. Mechanisms of killing spores of *Bacillus subtilis* by acid, alkali and ethanol. Journal of Applied Microbiology, 92, 2, 362.

Setlow, B., Melly, E. and Setlow, P., **2001**. Properties of spores of *Bacillus subtilis* blocked at an intermediate stage in spore germination. Journal of Bacteriology, 183, 4894-4899.

Shearer, A. E. H., Dunne, C. P., Sikes, A. and Hoover, D. G., **2000**. Bacterial spores inhibition and inactivation in foods by pressure, chemical preservatives, and mild heat. Journal of Food Protection, 63, 11, 1503-1510.

Sojka, B. and Ludwig, H., **1994**. Pressure-induced germination and inactivation of *Bacillus subtilis* spores. Pharmazeutische Industrie, 56, 660-663.

Sojka, B. and Ludwig, H., **1997**. Effects of rapid pressure changes on the inactivation of *Bacillus subtilis* spore. Pharmazeutische Industrie, 59, 436-438.

Splittstoesser, D. F., Churey, J. J. and Lee, C. Y., **1994**. Growth characteristics of aciduric sporeforming bacilli isolated from fruit juice. Journal of Food Protection, 57, 1080-1083.

Stevens, C. M., Daniel, R., Illing, N. and Errington, J., **1992**. Characterization of a sporulation gene *spoIVA* involved in spore coat morphogenesis in *Bacillus subtilis*. Journal of Bacteriology, 174, 586-594.

Taki, Y., Awano, T., Toba, S. and Mitsuura, N., **1991**. Sterilization of *Bacillus sp*. spores by hydrostatic pressure. In High Pressure Science for Food, Hayashi, R. (Ed), pp. 217-224, Sanei Pub. Co., Kyoto, Japan.

Tholozan, J. L., Ritz, M., Jugiau, F., Federighi, M. and Tissier, J. P., **2000**. Physiological effects of high pressure treatments on *Listeria monocytogenes* and *Salmonella typhimurium*. Journal of Applied Microbiology, 88, 202-212.

Ting, E., Balasubramanian, V. M., Taghubeer, E., **2002**. Determining Thermal Effects in High pressure Processing. Food Technology Feature, 56, 2, pp. 31-35.

Tipper, D. J. and Gauthier, J. J. **1972**. Structure of the bacterial endospore. In "Spores V", Halvorson, H.O., Hanson, R. and Campbell, L. L., eds. American Society for Microbiology, Washington, DC, pp. 3-12.

Tsuchido, T., Katsui, N., Takeuci, A., Takamo, M. and Shibasaki, I., **1985**. Destruction of the outer permeability barrier of *Escherichia coli* by heat treatment. Applied and Environmental microbiology, 50, 298-303.

Van der Poel, P.W., Schiweck, H., Schwartz, T., **1998**. Zuckertechnologie, Edited by *Dr. Albert Bartens KG*, Berlin, Germany, pp. 88.

Warth, A. D., **1978**. Molecular structure of the bacterial spore. Adv. Microbial Physiol. 17, 1-45.

Wuytack, E. Y., Boven, S. and Michiels, C. W., **1998**. Comparative study of pressure-induced germination of *Bacillus subtilis* spores at low and high pressures. Applied and Environmental Microbiology, 64, 9, 3220-3224.

Wuytack, E. Y., Soons, J., Poschet, F. and Michiels, C. W., **2000**. Comparative study of pressure- and nutrient-induced germination of *Bacillus subtilis* Spores. Applied and Environmental Microbiology, 66, 1, 257-261.

Wuytack, E. Y.and Michiels, C. W., **2001**. A study on the effects of high pressure and heat on *Bacillus subtilis* spores at low pH. International Journal of Food Microbiology, 64, 333-341.

Yamazaki, K., Teduka, H. and Shinano, H., **1996**. Isolation and identification of *Alicyclobacillus acidoterrestris* from acidic beverages. Bioscience, Biotechology and Biochemistry, 60, 543-545.

Yamazaki, K., Murakami, M., Kawai, Y., Inoue, N. and Matsuda, T., **2000**. Use of nisin for inhibition of *Alicyclobacillus acidoterrestris* in acid drinks. Food Microbiology, 17, 315-320.

Young, S. B. and Setlow, P., **2003**. Mechanisms of killing *Bacillus subtilis* spores by hypochlorite and chlorine dioxide. Journal of Applied Microbiology, 95, 1, 54.

Yuste, J., Mor-Mur, M., **1998**. Surviving bacteria from mechanically-recovered poultry meat treated with high hydrostatic pressure. In Meat consumption and culture, Diestre, A., Monfort, J. M. (Eds), Proceeding of the 44th International Congress of Meat Science and Technology, Madrid, 2, 548-549.

Yuste, J., Mor-Mur, M., Capellas, M., Guamis, B., Pla, R., **1998**. Microbiological quality of mechanically recovered poultry meat treated with high hydrostatic pressure and nisin. Food Microbiology, 15, 4, 407-414.

Yuste, J., Pla, R. and Mor-Mur, M., **2000**. *Salmonella enteritidis* and aerobic mesophiles in inoculated poultry sausages manufactured with high pressure processing. Letters in Applied Microbiology, 31, 374-377.

Yuste, J., Pla, R., Capellas, M., Sendra, E., Betran, E., Mor-Mur, M., **2001**. Oscillatory high pressure processing applied to mechanically recovered poultry meat for bacterial Inactivation. Journal of Food Science, 66, 3, 482-484.

Woo, S. Y., Lee, Y. J., Choi, I. M. and Kim, B. S., **2002**. How to reduce non-linearity in effective area caused by the O-ring seal in a high pressure balance. Metrologia, 39, 537-541.

List of publications, international congresses attendance and oral presentations

Articles:

Ardia, A., Knorr, D., Ferrari, G. and Heinz, V., **2003**. Kinetics studies on combined high pressure and temperature inactivation of *Alicyclobacillus acidoterrestris* spores in orange juice. Applied Biotechnology , Food Science and Policy, 1,3, 169-173.

Ardia, A., Knorr, D. and Heinz, V., **2004**. Adiabatic heat modelling for pressure build-up during high pressure treatment in food processing. Trans IChemE, Part C, Food and Bioproducts Processing, 82(C1), 89-95.

International congresses attendance, poster and oral presentations:

Heinz, V., Ardia, A., Buckow, R. and Knorr, D. Inactivation of spores by pressure assisted heating, 1st FEMS Congress of European Microbiologists, CANKARJEV DOM SLOVENIA, Ljubljana, Slovenia, 29 June- 3 July 2003.

Heinz, V., Ardia, A., Buckow, R. and Knorr, D. Survival of bacteria under pressures up to 1.5 GPa – new apparatus for experiments under isothermal conditions, European High Pressure Research Group Meeting 2003, 11 July, Bordeaux, France.

Ardia, A., Heinz, V. and Knorr, D. Very short treatment times for high pressure pasteurization and sterilization: a new concept. Workshop on Nonthermal Food Preservation, Wageningen International Conference Centre, Wageningen, The Netherlands, 7-10 September 2003.

Heinz, V. and Ardia, A. High pressure in food processing. Fall Meeting of the European Material Research Society, High Pressure School, Unipress, Warsaw, 13-15 September 2003.

Ardia, A., Heinz, V. and Knorr, D. Very short treatment times for high pressure processing: a new concept. ICEF9, International Congress on Engineering and Food. Montpellier, France, 7-11 March 2004.

Ardia, A., Heinz, V. and Knorr, D. Very short treatment times for high pressure processing: a new concept. GVC FA LVT, Baden-Baden, Germany, 22-23 March 2004.

Curriculum vitae

Personal information

Name:	Adriano Ardia
Date of birth:	25/01/1975
Nationality:	Italy
E-mail:	ardiaa@tiscali.it

Education and training

09/89- 07/94	High School Diploma (Salerno, Italy)
09/94- 03/01	Degree in Chemical Engineering (Salerno, Italy)
09/01- 09/02	Annual Scholarship Grant in Food Technology (Berlin, Germany)
10/02- 05/03	Full-time position at TU-Berlin (Berlin, Germany)
06/03- 05-04	Collaboration with SIG-Simonazzi S.p.A (Parma, Italy- Berlin)