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High hydrostatic pressure inactivation of *Bacillus subtilis var. niger.* spores: the influence of the pressure build-up rate on the inactivation

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CONTENT

Abstract

In the context of the development of commercially applied high pressure processes it is necessary to evaluate the impact of high pressure on the inactivation of bacterial populations. From the results kinetic models should reliably predict inactivation under dynamic conditions. High pressure processes imply an initial phase of pressure build-up, accompanied by a temperature increase due to adiabatic heating.
Since pressure application leads to the generation of heat, the question may arise whether this dynamic p-T profile will influence the spore inactivation.
The come-up time (the time needed to reach the constant treatment conditions) is an important variable that needs to be considered in the design and optimization of high hydrostatic pressure processes due to its significant contribution to microbial inactivation.

The pressure-temperature inactivation kinetics of *Bacillus subtilis var. niger* spores was first studied under isobaric-isothermal conditions in the pressure range 50–400 MPa at temperatures between 25–70°C. Isobaric-isothermal inactivation of spores appears to follow a fairly linear trend and can be described by a first order model. The kinetic parameters (k_{ref}, E_a and $\Delta V^{\neq}$) were calculated at different pressure and temperature levels.
Assuming first order kinetics for non-isobaric/non-isothermal inactivation of *Bacillus subtilis* spores during the come-up time, the integral effect of the inactivation process under dynamic conditions was quantified. To do that, the above mentioned kinetic parameters were used to calculate the inactivation constants as a function of temperature and pressure. The total come-up time was divided in time steps of one second and the inactivation constants were calculated for each seconds time interval.

The experimental pressure build-up was carried out in two different ways: (a) fast – pressure was developed with the highest possible rate and the come-up time was between 118 and 677 seconds depending on the pressure level of the treatment; (b) slow – pressure was built-up with a rate of 20 MPa/min and the come-up time was between 180 and 1337 seconds.
The inactivation effects during these come-up times after fast and slow pressure build-up processes were measured and compared with the calculated effects. The comparison reveals an additional inactivation effect on spores due to dynamics of fast pressure build-up. This is the first time that such an effect has unambiguously been shown.

Zusammenfassung

Die Entwicklung kommerziell angewandter Hochdruckprozesse macht es notwendig, die Auswirkungen von Hochdruck auf die Inaktivierung bakterieller Populationen zu untersuchen. Von den erarbeiteten Ergebnissen sollten Modelle entwickelt werden, die es erlauben die Inaktivierung unter dynamischen Prozessbedingungen zuverlässig vorauszusagen.

Diese Hochdruckprozesse bestehen aus einer Druckaufbauphase, bei der durch die adiabatische Kompression ein Temperaturanstieg zu verzeichnen ist. Es stellt sich nun die Frage, ob das dynamische Profil einen Einfluß auf die Sporeninaktivierung hat.

Die Vorlaufzeit (die Zeit bis zum Erreichen der konstanten Behandlungsbedingungen - Temperatur und Druck) stellt eine wichtige Variable bei der Mikroorganismeninaktivierung dar. Sie muß bei der Optimierung hydrostatischer Druckprozesse und der technischen Ausstattung berücksichtigt werden.

Die Inaktivierungskinetik von *Bacillus subtilis var.niger* Sporen wurde zuerst unter isobar-isothermen Bedingungen im Druckbereich zwischen 50 und 400 MPa und bei Temperaturen zwischen 25 und 70°C untersucht. Zu Anfang folgt die isobar-isotherme Sporen-Inaktivierung näherungsweise einem linearen Verlauf und kann durch ein Modell erster Ordnung beschrieben werden. Die kinetischen Parameter (k_{ref}, E_a und ΔV) zu den unterschiedlichen Drücken und Temperaturen konnten errechnet werden.

In der Annahme, daß für nicht-isobare/nicht-isotherme Inaktivierung der *Bacillus subtilis* Sporen während der Vorlaufzeit ebenfalls eine Kinetik erster Ordnung vorliegt, wurde der integrale Effekt des Inaktivierungsprozesses unter dynamischen Bedingungen quantitativ bestimmt. Für diese Berechnungen, wurden die oben erwähnten kinetischen Parameter verwendet und so konnten die Inaktivierungskonstanten in Abhängigkeit von Temperatur und Druck erhalten werden. Die Vorlaufzeit wurde dazu in Sekunden-Intervalle eingeteilt, für die jeweils die Inaktivierungskonstanten nach dem oben beschriebenen Verfahren errechnet wurden.

Experimentell wurde der Druck auf zwei unterschiedliche Weisen aufgebaut: (a) *schnell* mit höchstmöglicher Geschwindigkeit und einer Vorlaufzeit zwischen 118 und 677 Sekunden (abhängig vom erreichten Enddruck); (b) *langsam* mit einer Rate von 20 MPa/min und einer Vorlaufzeit zwischen 180 und 1337 Sekunden. Damit war es möglich, die Inaktivierungseffekte während der Vorlaufzeit bei schnellem und langsamem Druckaufbau zu messen und mit den theoretisch errechneten Werten zu vergleichen. Aus den Ergebnissen ist

zu entnehmen, daß sich bei schnellem Druckaufbau ein zusätzlicher Inaktivierungseffekt ergibt, wahrscheinlich reagieren die Sporen auf die Dynamik des schnellen Druckaufbaus. Durch diese Arbeit konnten solche Effekte erstmalig eindeutig nachgewiesen werden.

Part I General considerations

The destruction of microorganisms has permanently concerned the scientific communities.

Traditionally, the most-used preservation technologies for the microbiological control have been heat treatment, the addition of chemical preservatives and the control of storage temperature of products. All these techniques (excepting autoclave sterilization) slow or prevent the growth of organisms without inactivating them. In the last years, there are many developments that may change this situation. There is much interest in alternative, non-thermal methods that may be applied commercially for the inactivation of microorganisms. One of these novel processes that is already used is high hydrostatic pressure (HHP). High pressure processing (HPP) is gaining popularity in the preservation field since it offers some advantage comparing with traditional preservation methods.

High pressure processing, a process wherein a product is compressed hydrostatically, is known to yield sterile products. The potential microbiological effects of pressure processing was not recognized by the food industry until around 1985. High pressure processing has recently received a great deal of attention in the food, pharmaceutical and biotechnological industries. Many disadvantages of traditional thermal processes regarding microbiological control are overcome by HPP. The advantages are: lethal effect on vegetative forms of bacteria, moulds and yeast; inactivation of viruses used for vaccines production.

HPP represents an interesting and promising alternative for microbiological control of products, but a lot of research remains to be done in terms of understanding the critical limits of the process.

Part II Literature review

Chapter 1 Minimal processing methods

1.1. Introduction

This chapter is a literature review of several processes worldwide used for microbiological control. The food industry, and meanwhile also the pharmaceutical industry, continues to expand the use of current methods and to search for new technology which will ensure microbiological safety in products while at the same time providing the consumer with products of high quality which appear to have experienced little or no processing.

These technologies include thermal (sterilization, pasteurization, blanching, etc) and non-thermal (irradiation, high intensity electrical field, pulsed light, UV light, high pressure processing, ozone, etc) methods.

The term " minimal processing " covers a wide range of technologies and methods for preserving food or pharmaceutical products. Very short shelf-life products require preservation methods that will prolong their shelf-life and improve their quality. Such methods are found amongst the group of technologies and methods known as minimal processing.

The majority of techniques that are currently employed to combat the microorganisms that cause products poisoning or spoilage act by inhibiting or completely preventing their growth rather than by inactivating them (reduction in temperature – chilling, freezing; reduction in water activity – conserving, drying; reduction in pH – addition of acids, lactic or acetic fermentation; modified atmosphere packaging – vacuum, N_2). Traditional techniques continue to be important but newer technique such as use of carbon dioxide-enriched "modified atmosphere" packaging or high pressure processing greatly improve the preservation effect (Gould,1998).

There is much interest in alternative, non-thermal techniques that may be applied commercially for the inactivation of microorganisms. These include the use of enzymes (such as lysozyme which is already used in large amounts in cheese preservation (Scott et al., 1987), lactoperoxidase, lactoferrin, lactoferricin), small peptides (histatins and magainins) and microbially derived polypeptide bacteriocins. These methods act by inactivation rather than inhibition. Processes that are already applied include gamma irradiation, high hydrostatic pressure, high voltage electric discharges (electroporation), sonication. These new and improved techniques for the inactivation of spoilage and poisoning microorganisms are being

increasingly exploited. Mild preservation techniques can "encourage" microorganisms to undergo stress reactions that increase their resistance, and sometimes their pathogenicity (Gould, 1998). In Table 1.1 different new minimal processing technologies are presented.

Process	Mechanism
New thermal processing methods	
Ohmic heating	Optimized heating regime reduces levels of undesired microorganisms while minimizing thermally induced quality losses.
Radio frequency heating	
Microwave heating	
Sous-vide technology	
Non-thermal processing methods	
High pressure treatment	Microorganisms inactivated under high pressure;
Gamma irradiation	Ability of microorganisms to reproduce eliminated;
High electric field pulses	Microbial cell rupture due to uneven distribution of electrical change across cell.

Table 1.1 Minimal processing methods (Ohlsson, 1994)

1.2. Novel thermal processes

Conventional heating is the method mostly used in the processing and preservation of food/pharmaceutical products to reduce or eliminate microbial and biochemical activity. With conventional heating processes it is usual for energy to be supplied in the form of steam or hot water. Heat transfer relies on conduction from the heating medium to the product or by direct condensation of steam. Unfortunately, foods generally have low thermal conductivity so that heat transfer is limited for particles of any size much greater than 2 mm; core temperatures will lag behind that of the liquid, requiring overcooking of both the product surfaces and the liquid (Schreier et al.,1993).

To overcome this limitation, novel thermal technologies have been developed such as electroheat processes which comprise ohmic, microwave and radio frequency (RF) heating systems, or ultrasounds.

For any of these heat-based processes, the magnitude of time/temperature will determine the preservative effect.

1.2.1.Electroheat technologies

Electroheat technologies are now becoming increasingly significant in the thermal processing of food products. In addition to microbial inactivation by conventional methods of heating ohmic, microwave and radio frequency heating are also considered to be heat-based processes that can inactivate microorganisms by thermal effects. By all these technologies the energy source is electricity which is applied either directly as in ohmic heating or indirectly in the form of electromagnetic energy as in microwave and radio frequency systems.
The term ”volumetric heating” can be used to describe the principle of these three processes.

The basic principle of ohmic heating, also called resistance heating or Joule heating is the passage of an electrical alternating current (50-60Hz) through an electrically conducting product. The longer the food stays in the electrical field, or ohmic column, the greater the amount of heat generated. Special electrodes are used to avoid adverse electro-chemical reactions. The major benefit of the process is that heating takes place volumetrically and the product does not experience a large temperature gradient within itself as it heats.
The applicability of ohmic heating is dependent on the electrical conductivity of the product. Most food products and pharmaceutical solutions contain a moderate percentage of water with dissolved ionic salts and hence the conductivity is sufficient for the ohmic effect to be applied. The industrial applications are: aseptic processing, pre-heating and hot-filling, pasteurization (APV Baker Ltd).

The principle of microwave and radio frequency technologies is that if a product is placed in an electric field alternating at microwave or radio frequencies then the energy will be reflected, transmitted or absorbed. If the energy is absorbed heat is generated throughout the mass of the product by the rapid reversal – or polarization – of individual molecules. Two types of polarization mechanisms are significant at radio and microwave frequencies: orientation polarization (is the rapid movement of molecules already permanently polarized due to their chemical structure) and space charge polarization (is the accumulation of charge within the product due to the movement of charged particles such as (Na^+) or chloride (Cl^-) ions under the influence of the applied electromagnetic field). The second mechanism is not notable at microwave frequencies but is significant at radio frequencies. The physical properties of individual products are important in determining which mechanism will be significant and subsequently whether radio or microwave heating is most appropriate.

Tempering is by far the largest application for microwave heating in the food industry and pasteurization of meat products for radio frequency heating.
APV Baker Ltd., West Sussex, England is currently the only manufacturer able to offer a complete electroheat portfolio: ohmic, microwave and radio frequency heating systems.

1.2.2. Sonication

Ultrasound technology entails the transmission of mechanical waves through materials at frequencies above 20 kHz. The use of ultrasound may be divided broadly in two areas. The first area involves low amplitude (higher frequency) propagation and is commonly referred to as “low power” or “high frequency ultrasound”. Typically, low amplitude waves (between 2-10 MHz) are used in medical scanning, chemical analysis and the study of relaxation phenomena. The second area involves high energy (low frequency) waves known as “power ultrasounds” (between 20-100kHz) which is used for cleaning, plastic welding and more recently to effect chemical reactivity.

Presently, the most applications of sonication could be found in pharmaceutical and chemical industry where ultrasound technology is used for emulsification, accelerating freezing and cleaning, crystallization processes, but is also used in food industry. High frequencies are used for non-destructive testing, i.e. evaluation of the internal quality and latent defects of whole fruits and vegetable (Mizrach et al. 1994). Because viscous liquids and solids impede the propagation of ultrasound waves this technique (low frequency) is potentially most useful for sterilization of liquids such as milk and juices. Some recent investigations have focused on the possible uses of ultrasound for bacterial inactivation (Liliard 1994; Lee 1989).

Data about inactivation of food microorganisms are scarce and most applications of ultrasounds involve its use in combination with other preservation methods. The bactericidal effect of ultrasound on vegetative bacteria is generally attributed to intracellular cavitation (Hughes and Nyborg, 1962). When high power ultrasound propagates into a liquid the micro-bubbles which are commonly present in it or that may form will oscillate according to the pressure wave. High acoustic pressure will determine their growth and violent collapse which is accompanied by a sudden increase of the temperature and the pressure in the surrounding area. These shocks disrupt cellular, structural and functional components up to the point of cell lysis. The mechanism of spore inactivation by ultrasound is not clear. Cavitation must play a role but it is an auxiliary one since ultrasound alone has no effect on spores (Garcia et al., 1989).

In conclusion, ultrasound technology has the potential for future use as a preservation process in combination with other preservation technologies.

1.3. Non-thermal processes

Non-thermal processing techniques are regarded with special interest by the food industry and presently also by the pharmaceutical industry.
Processes that are under evaluation or development are: ionising radiation treatment, high hydrostatic pressure treatment, high voltage electric field pulses, high intensity ultrasounds, the application of supercritical carbon dioxide or the use of magnetic fields.
Also various combinations of the above mentioned technologies with thermal processes are being currently evaluated.
Some of these processes that are already applied are reviewed.

1.3.1. Ionizing radiation

Ionizing radiation is a versatile form of processing energy used already in a wide range of food and non-food applications. Since the 1940's irradiation has been used as a method to supply safe food to US army combat troops (Barbarosa et al., 1998).
Irradiation of food refers to the process by which food is exposed to enough radiation energy to cause ionization. Ionization can lead to the death of microorganisms due to the genetic damage which prevents cellular replication. The energy employed in food irradiation technology is denominated ionizing irradiation. The dose is generally measured in Grays (Gy) or kiloGrays (kGy) or in Rads (100 Rads =1 Gray). Products exposed to radiation levels between 30 and 40 kGy are considered to be commercially sterile and this level of irradiation is called radapperation. Exposure to a radiation level between 2.5 and 10 kGy will rid most products of all vegetative pathogens, is comparable with pasteurization and is called radicidation. Exposure levels of between 0.75 and 2.5 kGy will rid most products of spoilage organisms. This process is called radurization.

The ionizing radiation is in the form of gamma rays from a radioactive source or from an "electron beam" generated electronically. Electron beam technology does not require the use of radioactive material, and can be "turned on and off". The gamma rays are similar to ultraviolet light or microwaves but of much shorter wavelength and greater energy. Gamma

rays pass energy through food in the same way as microwaves but in this case the food remains cool.

For the treatment of foods, FDA (Food and Drug Administration) has approved the use of gamma rays from decaying isotopes of cobalt-60 or cesium-137, x-rays with maximum energy of five million electron volts (MeV), and electrons with a maximum energy of 10 MeV on pork, beef, poultry, and lamb meat, spices, seasonings, fruits, vegetables and grains.
The term ionizing comes from the fact that certain rays produce electrically charged particles called ions when these rays strike a material (Urbain, 1986). Although each electron behaves individually electrons can be used in large numbers (called electron beams) to irradiate food (Barbarosa-Canova et al., 1997).
Lethality of irradiation depends on the microorganism (number, type and age of the organism), condition of the treated item and environmental factors. Addition or removal of salt or water, time/temperature of the treatment, presence or absence of oxygen are factors that will influence the antimicrobial effect of irradiation.
Radiation treatment at doses of 1.5 to 7 kGy – depending on condition of irradiation and of the food – can effectively eliminate potentially pathogenic non-sporeforming bacteria from suspected food products without affecting nutritional and technical quality. These bacteria include both long-time recognized hazards, such *as Salmonella spp*. and *Staphylococcus aureus*, as well as emerging or "new" pathogens such as *Campylobacter spp., Listeria monocytogenes* or *Escherichia coli* O157:H7 (Farkas, 1998).
In general:

- spores are more resistant to radiation than vegetative cells;
- Gram positive bacteria are more resistant to radiation than Gram negative bacteria;
- yeasts are more resistant than molds;
- high protein foods and dry foods offer increased protection to microorganisms against radiation.

Applications in Food Industry

Irradiation cannot be used with all foods. It causes undesirable flavor changes in dairy products, for example, and it causes tissue softening in some fruits such as peaches and nectarines.

Irradiation is most useful in four areas:

Preservation. Radiation pasteurization refers to the destruction of pathogenic, non-sporeforming foodborne bacteria. In radiation pasteurization medium dose treatments (1-10 kGy) reduce microbial populations including pathogens in food. The resulting products are closer to the fresh state in texture, flavor, and color. Using irradiation to preserve foods requires no additional liquid, nor does it cause the loss of natural juices.

Sterilization. Radiation sterilization is used for radiation processes that will render the food commercially sterile or for foods that are both, sterile and shelf stable. In this last case, sterilization must ensure the elimination of the most resistant pathogen, endospores of *Clostridium botulinum*. In order to achieve this higher doses (42-71 kGy depending on the product) than the ones currently permitted for foods (up to 10 kGY, except for spices) are needed. Only frozen meats consumed by NASA astronauts have been permitted by FDA to be sterilized through irradiation. They are, however, in the market in other countries.

Control sprouting, ripening, and insect damage. In this role, irradiation offers an alternative to chemicals for use with potatoes, tropical and citrus fruits, grains, spices, and seasonings. However, since no residue is left in the food, irradiation does not protect against reinfestation like insect sprays and fumigants do.

Control foodborne illness. Irradiation can be used to effectively eliminate those pathogens that cause foodborne illness such as *Salmonella*.

Since 1986 all irradiated products must carry the international symbol called a radura, which resembles a stylized flower.

Irradiation is considered an additive in the U.S. and as such it needs to be approved by the FDA office of pre-market approval for each new application and labeled. Presently over 30 countries have approved applications to irradiate approximately 40 different foods. The safety and nutritional adequacy of irradiated food have been well established, the technology is ready to use technically and the need to improve the microbial safety of food became a major driving force.

Applications in medicine and medical and pharmaceutical science

Using irradiated blood in transfusions is recognized as the most effective way of preventing Graft Versus Host Disease (GVHD). GVHD most commonly occurs following bone marrow transplants; GVHD occurs when blood from the donor makes antibodies that try to destroy the cells of the recipient. GVHD has become a major concern in current blood transfusion practices, since it is nearly always fatal. Since treatment of transfusion-associated GVHD is almost always ineffective efforts have been made to minimize the risk of

contracting it. These efforts have focused on reducing or inactivating transfused donor lymphocytes, the cells that make antibodies. Inactivation of transfused lymphocytes by gamma irradiation of blood remains the most efficient method for preventing transfusion-associated side effects.

It is also used to sterilize about 50 percent of all medical disposable materials such as bandages, sutures, and surgical drapes. More recently, consumer products such as cosmetics, baby nipples, teething rings, and so on are being sterilized using irradiation.

In Table 1.2 are presented the advantages and disadvantages of using irradiation for microbial destruction.

Microbial destruction by irradiation	
Advantages	Disadvantages
Effective against all types of microorganisms	May be more expensive than other available technologies
May be less destructive to some heat sensitive products	May cause changes in the product and must be approved for food additive use on that product
Gamma rays can penetrate dense material	Electron beam will not penetrate dense products
Will extend shelf life of products	Public acceptation is not good

Table 1.2 Advantages and disadvantages of irradiation

1.3.2. High intensity pulsed electric fields

The effect of high intensity pulsed electric field (PEF) processing on inactivation of microorganisms was explored and these studies led recently to the development of prototype and industrial scale devices (Barbarosa-Canovas, 1997).

PEF processing involves the application of pulses of high voltage (typically 20-80 kV/ cm) to the product placed between two electrodes. It may be applied in the form of square wave, bipolar, or oscillatory pulses at ambient or slightly above ambient temperature for less than 1 s. Use of PEF can reduce energy usage compared to thermal processes.

The application of electrical fields to biological cells in a medium (for example water) causes build up of electrical charges at the cell membrane (Schoenbach et al., 1997). Membrane disruption occurs when the induced membrane potential exceeds a critical value of 1V. Several theories have been proposed to explain microbial inactivation by PEF. Among

them, the most studied are electrical breakdown and electroporation or disruption of cell membranes (Zhang et al., 1995; Castro, 1993).

When microorganisms are subjected to electrical pulses cell membranes are ruptured due to an uneven distribution of electrical charges on either side of the cell membranes. Ruptures of the cell walls causes inactivation of microorganisms. The method is more efficient the larger the cell (e.g. for yeast cells).

This technology has been applied mainly to extend the shelf life of food products and the most interesting future application is likely to be for fruit products. Application of PEF is restricted to those products that can withstand high electric fields, have low electrical conductivity, and do not contain or form bubbles.

Research that provide conclusive data on the PEF microbial inactivation are still not available. However, numerous critical process factors exist and carefully designed studies need to be performed to better understand how these factors affect the microorganisms.

Pulsed electric field technology is industrially being applied in Germany (Elcrak system) for fat recovery from fish and slaughter house offal's. The advantages and disadvantages of using this method are presented in Table 1.3.

Microbial destruction by high intensity pulsed electric field	
Advantages	Disadvantages
Destroys pathogenic and spoilage organisms	Suitable only for liquid products
Energy costs are lower compared with thermal processes.	High dosage and longer periods of time needed for spores
Vitamins and enzymes are not destroyed; no product changes	Process must be designed for each specific product

Table 1.3 Advantages and disadvantages of high intensity pulsed electric field

1.3.3. High intensity pulsed light

High intensity pulsed light involves the application of rapid, intense, magnified flashes of light from inert gas lamps. The pulsed light is produced using technologies that multiply power by accumulating electrical energy in an energy storage capacitor over long periods of time (fractions of a second) and releasing this energy over short periods of time (millionths or thousandths of a second). The pulsed light contains wavelengths from 200 nm of ultraviolet through 1 mm in the near infrared at about 20.000 times the intensity of sunlight at sea level.

Most of the pulsed light is in the visible range. Ionization of small molecules does not occur because the pulsed light wavelengths are too long. The intensity of the pulsed light treatment is measured as fluence or incident light energy per unit area, in Joule/cm^2.

Pulsed light appears to eliminate populations of vegetative bacteria, bacterial endospores, fungal conidiospores with equal effectiveness. It is also suitable for treatment of contaminated water. In addition to destruction of microorganisms the use of pulsed light deactivates those enzymes which may lead to product deterioration.

Information supplied by Pure Pulse Technologies, San Diego, California for their pulsed light technology to the Food and Drug Administration in the form of a food additive petition has resulted in the acceptance of pulsed light as a source of irradiation in the production, processing and handling of food (FDA, 21 CFR Part 179.41).

Table 1.4 presents the advantages and disadvantages of using high intensity pulsed light for microbial destruction.

Microbial destruction by high intensity pulsed light	
Advantages	Disadvantages
Effective against all types of microorganisms	Can only be used for surface treatment.
Can be used to treat products through transparent packaging materials	Initial investment in equipment may be high
Can be used to treat processing water	Employees must be shielded from light source
Inhibits enzymatic activity	

Table1.4 Advantages and disadvantages of high intensity pulsed light

Chapter 2 High hydrostatic pressure

2.1. Introduction

After irradiation, high hydrostatic pressure processing (HHP) is probably the most utilizable of what are now termed the „new“ non-thermal food processing methods. HHP has been known for over a century as a physical application with potential as a food preservation method but the state of equipment technology and, just as important, consumers were not ready to support the application earlier.

High hydrostatic pressure has recently been developed for food processing in Japan, especially as a mean of preservation for different types of food (fruit juice, jam, dairy products), but the effect of high pressure on food and microorganisms was known at the beginning of XXth century. Before the turn of the century, Hite first experimented with high hydrostatic pressure on food and beverages. It was obvious that the technology could be used for food preservation and particularly for fruit juice.

In 1903 Chlopin and Tamman observed various inhibitory effects of pressure on bacteria, i.e. loss of mobility, decrease in ability to multiply, various changes in metabolism, and decrease of virulence. At the same time Bridgman observed structural changes in food components : complete coagulation of egg-albumen in 30 min at 700 MPa and at room temperature. Bridgman was also involved in studies concerning the structure of water under pressure. He underlined, in particular, a very interesting property in relation to freezing and thawing of food, i.e. water was not frozen at -20°C under a pressure of 200 MPa.

From the 1950`s modification of the internal structure of living organisms under pressure was studied. The first model were deep-sea bacteria.

It was concluded that the barotolerant property of deep-sea bacteria was due to genetic adaptation selection of metabolic reactions. These bacteria are less pressure sensitive than those which live at atmospheric pressure.

After the 1980`s the pressure induced denaturation of various proteins and enzymes was investigated. Many of these were denatured or inactivated by pressures of 200 MPa to 800 MPa applied for short periods.

The last decade has seen a tremendous increase in basic research, development of commercial equipment and the continuing market introduction of many new products.

2.2. Physical and chemical aspects of high hydrostatic pressure

2.2.1. Dimension and nomenclature

A number of different measures of pressure are used throughout the scientifical world. The International System (SI) unit of pressure is the Pascal (Pa) or Newton per square meter ($N \cdot m^{-2}$). Some units of pressure are listed in Table 2.1

atm	Pa	bar	kg / cm^2	psi
1.000	$1.013 \cdot 10^5$	1.013	1.034	$1.470 \cdot 10^1$

Table 2.1 Pressure units in use

Because the pressure level of HHP is much higher than several Pascal, the standard prefix mega- (10^6) is often multiplied to the Pascal and yields the unit of mega-Pascal (MPa). Thus, a pressure of 100 MPa is equivalent to 1 kbar, 986.9 atmospheres or 14504 lb. $\cdot$ in^{-2} (psi).
These pressures are higher than those naturally occurring on the earth surface but are used routinely in industrial processes.

2.2.2. Generation of high hydrostatic pressure

Pressure is a physical parameter which has influenced the evolution of both micro- and macroorganisms. Normal atmospheric pressure is approximately 100 kPa, and a 100 meters depth of water may be equated to approximately 1 MPa. In nature hydrostatic pressure increases by one atmosphere for every ten meter depth of water. The high pressure science uses the pressure range of 100 - 1000 MPa which is almost the same level to ten times higher than the pressure of the deepest sea. Special equipment is needed to generate such high pressure.
A typical HHP system consists of a high pressure vessel of cylindrical design and its closure, a low pressure pump, an intensifier which uses liquid from the low pressure pump to generate high pressure process fluid for system compression, and necessary system controls and instrumentation. The most important part is the pressure vessel which is in many cases a forged monolithic cylindrical vessel constructed in low alloy steel of high tensile strength. The wall thickness of the vessel determines the maximum working pressure. Depending on the internal diameter of the vessel, the use of vessels is typically limited to maximum working pressure of 400-600 MPa (FDA/CFSAN, 2000). Pressure vessels are available as laboratory units with volumes of 0.1 to 2 liters while batch production pressure vessels can be supplied with volumes of several hundred liters.

HHP can be generated either by direct compression or by indirect compression.
In the case of direct, piston type compression the pressure medium in the high pressure vessel is directly pressurized by a piston driven at its larger diameter end by a low pressure pump.
The indirect compression method uses a high pressure intensifier which pumps the pressure medium from the reservoir into the closed and de-aerated high pressure vessel, until the desired pressure is reached. Most of industrial isostatic pressing systems use the indirect pressurization method (Deplace & Mertens, 1992).
The required work to increase the pressure to the desired value can be estimated if we know the compressibility of pressure medium. Assuming the pressure (P) increases following the equilibrium path the element of work (W) to increase the pressure (or decrease the volume(V)) of a system can be written :

$$dW = -p \cdot dV \qquad \text{equation 2.1}$$

The variation of V with P at constant T defines the compressibility ß of a substance :

$$\beta = -\frac{1}{V} \cdot \left(\frac{\partial V}{\partial P} \right)_T \qquad \text{equation 2.2}$$

Introducing equation 2.2 in equation 2.1 yields :

$$W = \int pV\beta dp \qquad \text{equation 2.3}$$

The temperature within the pressure vessel does not remain constant during pressure build-up. The temperature within the pressure system will increase as the pressure increases. Equation 2.4 can be used to predict this adiabatic heat generation during pressure build-up.

$$\left(\frac{\partial \mathrm{T}}{\partial \mathrm{P}} \right)_{\text{adiabat}} = \frac{\alpha \cdot \mathrm{T}}{\rho \cdot \mathrm{c_p}} \qquad \text{equation 2.4}$$

where α is the thermal expansivity, ρ is the density, c_p is the specific thermal capacity of the substance.

2.2.3. Mechanism of high hydrostatic pressure

There are two main principles involved in high pressure processing :

- *The Isostatic rule* which states that the transmittance of pressure is uniform and instantaneous (independent of size and geometry of the samples. The hydrostatic pressure process is volume independent and pressure gradients do not exist.
- *The Le Chatelier principle* states that the application of pressure to a system in equilibrium will favor a reduction in volume to minimize the effect of pressure. Thus, reactions which result in a decrease in total volume (negative activation volume) are enhanced by pressure. Conversely, reactions resulting in an increased total volume (positive activation volume) are slowed down by pressure.

Closely associated with Le Chatelier principle is *the principle of microscopic ordering.* The principle states that, at constant temperature, an increase in pressure increases the degree of ordering of the molecules of a substance.

Another interesting rule concerns the small energy needed to compress a solid or liquid to 500 MPa as compared to heating to 100°C. The energy for compressing is relatively small.

The HHP affects non-covalent bonds (hydrogen, ionic, and hydrophobic bonds) which mean that low molecular weight components are not affected whereas high molecular weight components are sensitive.

2.2.4. Pressure and temperature as thermodynamic variables

Pressure and temperature dependence of the chemical equilibrium

Chemical reactions move towards a dynamic equilibrium (with the negative free enthalpy change) in which both reactants and products are present. This equilibrium may be shifted by pressure and temperature as follow:

At equilibrium the free enthalpy can be expressed as

$$\Delta G^0 = -RT \ln K \qquad \text{equation 2.5}$$

and

$$\ln K = -\frac{\Delta G^0}{RT} \qquad \text{equation 2.6}$$

where ΔG^0 is the free standard enthalpy, R the gas constant, T the absolute temperature and K is the equilibrium constant.

The basic concept to interpret the effects of pressure on chemical reactions is the Principle of Le Chatelier. The variation of ln K with pressure is given by

$$\left(\frac{\partial \ln K}{\partial p}\right)_T = -\frac{1}{RT}\frac{\partial(\Delta G^\circ)}{\partial p} \qquad \text{equation 2.7}$$

and $$\frac{\partial(\Delta G^{\circ})}{\partial p} = \Delta V$$ equation 2.8

Then, on combining the last two equations, yields:

$$\left(\frac{\partial \ln K}{\partial p}\right)_T = -\frac{\Delta V}{RT}$$ equation 2.9

Where ΔV is the reaction volume, i.e. the difference between the partial molar volumes of the products and reactants.

The last equation shows that if ΔV is positive then lnK decreases with increasing pressure, implying a shift back towards reactants. If ΔV is negative the opposite is true.

The variation of ln K with temperature is therefore given by

$$\left(\frac{\partial \ln K}{\partial T}\right) = -\frac{1}{R}\frac{\partial\left(\frac{\Delta G^{\circ}}{T}\right)_p}{\partial T}$$ equation 2.10

and $$\frac{\partial\left(\frac{\Delta G^{\circ}}{T}\right)_p}{\partial T} = -\frac{\Delta H}{T^2}$$ equation 2.11

Combining the last two equations it arrives at van't Hoff isochore:

$$\left(\frac{\partial \ln K}{\partial T}\right)_p = \frac{\Delta H}{RT^2}$$ equation 2.12

The van't Hoff isochore shows that the slope dlnK/dT is negative for a reaction that is exothermic (ΔH<0) and therefore lnK decreases as the temperature rises. In this case the equilibrium shifts towards reactants. The opposite occurs in the case of endothermic reactions (ΔH >0).

Pressure and temperature dependence of the rate constant

Pressure and temperature changes affect not only the equilibrium situation of a chemical reaction, but also the rate of the equilibrium installation. The pressure dependence of the rate constant *k* is described by the activation volume ($\Delta V^{\neq}$), which indicates the variations in volume on the way to the transient condition.

$$\left(\frac{\partial \ln k}{\partial p}\right)_T = -\frac{\Delta V^{\neq}}{RT}$$ equation 2.13

The temperature dependence of the *k* is described by the activation energy (E_a).

After Arrhenius only those particles can react whose energy exceeds a certain limit value. This limit value is the activation energy. With rising temperature grows the portion of the particles with sufficient energy, the reaction rate becomes higher.

$$\left(\frac{\partial \ln k}{\partial 1 / T} \right)_p = - \frac{E_a}{R} \qquad \text{equation 2.14}$$

The effects of pressure and temperature on biochemical reactions are antagonistic: by lowering the temperature the velocity of a reaction decreases (positive activation energy) whereas by increasing the pressure the velocity of a reaction increases or decreases depending on the sign of the activation volume.

Starting from the Principle of Le Chatelier, it can roughly be estimated how pressure will affect any process if the net change in volume that accompanies the process is known (Table 2.2). Pressure tends to dissociate electrostatic interactions because ionization in aqueous systems is accompanied by a decrease in volume. Also pressure disrupts hydrophobic interactions because this process results in volume contraction. On the contrary, the breakage of hydrogen or covalent bonds is restricted because that should result in volume expansion.

Bond type	Example	ΔV (ml/mol)	Effect of pressure
Ionic	$H_2O \longrightarrow H^+ + OH^-$	-21	Disrupts electrostatic interactions
Hydrophobic	$CH_{4\ hexane} \longrightarrow CH_{4\ water}$	-23	Disrupts hydrophobic interactions
Covalent	C – C	+12	Inhibit bong breakage
Hydrogen bond formation	poly(L-lysine) (helix formation)	+1	Enhances hydrogen bonding
Protein denaturation	Myoglobin (pH 5, 20°C)	-98	Enhances denaturation

Table 2.2 Volume changes associated with biochemically important bond breakage at 25°C (from Gross and Jaenicke, 1994)

2.3. Effects of high hydrostatic pressure on protein systems

The fundamental structural units in proteins are aminoacids, linked together by peptide bonds. This sequence of amino acids is called primary structure of the protein. Secondary structure is

based on the formation of regular structural elements such as α helix and β sheet. The tertiary structure refers to the totally folded, three dimensional structure of protein. Now, the protein has more complex interactions between elements of secondary structures and their side chains. Fully folded native proteins frequently interact with other polypeptide chains and yield the quaternary structure of protein (Zubay, 1988).

A protein in its native state is stabilized by:

- covalent bonds including disulfide bridges;
- electrostatic interactions (ion pairs, polar groups);
- hydrogen bridges;
- hydrophobic interactions.

The native structure of proteins is easily affected by environmental conditions such as solvent composition, temperature and pressure. Changes in solvent composition (presence of sugars, salts or extreme pH conditions) may have a strong influence on the stability of the structure.

The term « denaturation of protein » indicates a complex phenomenon in which the higher structure of protein is ruptured by environmental changes, while the primary structure is kept without damage (Mason, 1992). The effect of pressure on proteins may be reversible (observed below 200 MPa) or irreversible (observed above 300 MPa). Because pressure effects on proteins often differ from those of temperature, pH, and chemical denaturants, pressure is now regarded as an important variable for examining protein structure-function relationships.

Proteins are flexible structures and compress when exposed to high pressure.

Generally, most of the proteins denature when exposed to pressure above 400 MPa. It is shown that ß sheet structures are more stable against pressure than α helical ones. ß sheet structures are nearly incompressible. Compact proteins show little compressibility and high stability. Oligomeric proteins dissociate to subunits, with $\Delta V^{\neq}$ being negative, at pressure below 300 MPa.

2.3.1.Mechanism of denaturation

There are a number of "mechanism" theories:

1. Intramolecular interactions:

- *Hydration*. Expanded cavities become filled with water, leading to hydration of the interior and denaturation (Mozhaev et al., 1988).

Water molecules exert a profound influence on protein conformation through interactions with amino acids. Upon application of relatively low pressures (< 200 MPa), increased conformational fluctuations provide pathways for water to penetrate into the interior of the native protein. These fluctuations are enhanced by pressure due to increased water exchange between the protein interior and bulk solvent (Tanaka et al., 2000)

As a result of water penetration pressure likely leads to conformational transitions resulting in unfolding (proteins can adopt the conformation of a molten globule).

At very high pressure (>500 MPa) protein aggregation and loss of secondary structure can occur due to hydration.

Beside protein unfolding and aggregation increased hydration of the polypeptide can cause changes in protein compressibility and flexibility. Increased hydration leads to decreases in compressibility due to electrostriction of solvent around charged and polar groups and loss of void volume (Royer et al.,1995).

- *Hydrogen bonds*. The lengths of existing hydrogen bonds within proteins have been observed to shorten under pressure (Linowski et al., 1976). The shortening of hydrogen bonds in addition to the collapse of internal cavities, can contribute to the compression of proteins under pressure (Gekko et al., 1986)

- *Van der Waals forces*. These forces can contribute to protein destabilization under pressure. Pressure below 300 MPa promotes dissociation of oligomers resulting in replacement of the weakest non-covalent interactions between amino acids residues with amino acid-water interactions and changes in peptide chain conformations. These protein-water interactions are promoted by pressure because they produce stronger and shorter bonds that decrease the overall volume (Silva, 1993).

- *Ionic interactions*. Volume change under pressure favors dissociation of ionic interaction, tertiary structure is weakened.

The role of ion pairs (sometime termed salt bridges) in proteins is to stabilize the tertiary and quaternary structure. Dissociation of ion pairs leads to electrostriction, which is the

contraction of solvents. Because pressure favors electrostriction, pressure can promote disruption of protein salt bridges and induce protein denaturation (Heremans, 1980).

2. Intermolecular interactions

- *Protein-protein.* In many cases relatively low pressure (100- 200 MPa) has been found to promote dissociation of oligomeric proteins. This dissociation is accompanied by negative and relatively large $\Delta V^{\neq}$ values (-50 to -200 ml/mol) (Silva et al., 1993).
- *Enzyme-substrate.* Binding of a substrate to the active site of an enzyme is typically accompanied by a small positive or negative volume change at atmospheric pressure (Michels et al., 1996).

2.3.2. Thermodynamics of denaturation

A fundamental difference between pressure and temperature induced protein denaturation is that no change in covalent bonding has been observed in the pressure induced protein denaturation (Masson, 1992). Further, the structure of pressure denatured proteins differs significantly from that of heat denatured proteins. The pressure denatured proteins are relatively compact and retain elements of secondary structure while the heat denatured proteins have the extended, nearly random coil configurations (Ghosh et al., 2001).

Several studies have dealt with the changes in structure and/or function of proteins under high pressure (Heremans, 1982 ; Weber and Drickamer, 1983 ; Balny et al., 1989). It is generally assumed that in protein macromolecules the formation of hydrogen bonds, the rupture of hydrophobic interactions, and the separation of ion pairs are accompanied by a decrease in volume and therefore enhanced by pressure.

The biologically active structure of a protein is only stable within restricted conditions of temperature, pressure and solvent composition. Outside this range, unfolding or denaturation takes place.

Denaturation of proteins may be regarded as two-state transition:

Native (Folded, Active) ⟷ Denatured (Unfolded, Inactive)

Starting from this assumption Hawley calculated the Gibbs free energy difference of the denatured and the native state of the protein and obtained a second order curve. This curve

turned to be elliptical in case of proteins. In frame of this theory temperature, pressure and cold denaturations are only three very special ways of unfolding.

The temperature-pressure dependence of protein denaturation is shown in Fig.2.1.

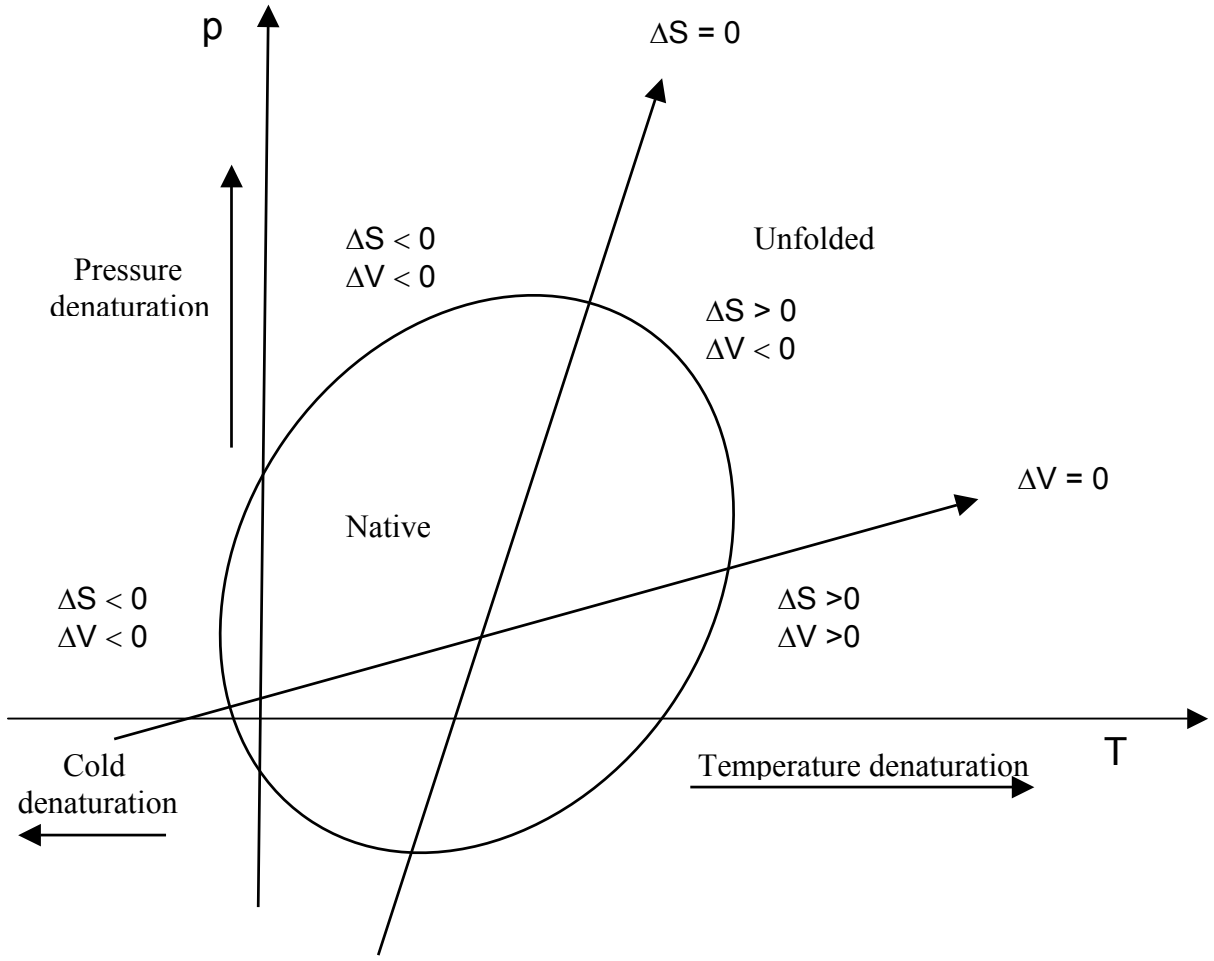

Fig.2.1 The elliptical temperature-pressure stability phase diagram characteristic for proteins (Suzuki and Hawley)

This temperature - pressure dependence was first demonstrated by Suzuki in 1960. As is shown in Fig.2.1, the denaturation temperature rises initially with increasing pressure. At maximal transition temperature the sign of $\Delta V^{\neq}$ changes; from this point on the protein denatures at lower temperatures at the given pressure. At maximal transition pressure the sign of ΔS changes; from this point on the protein denatures at lower pressure at the given temperature.

So, at high temperatures pressure stabilizes the protein against temperature denaturation. At low temperature, increasing temperature stabilizes the protein against pressure denaturation. This typical elliptical diagram has also been observed for the inactivation of enzymes, microorganisms and viruses.

The thermodynamic aspects of pressure effects on biomolecules have been reviewed by Heremans (1992) and Tauscher (1995).

2.4. Effects of high hydrostatic pressure on enzymes and enzymatic reactions

Enzymes are proteins of biocatalytic function which accelerate chemical metabolic reactions. They may be affected by high pressure in a variety of ways depending on other parameters of processing and also of the type of enzyme (Cheftel, 1995). The mechanisms of enzyme inactivation are probably similar to those of protein denaturation. Jaenicke (1991) reported that pressure increases reaction rate that involve enzymes. Thus the catalytic behavior of enzymes is affected by the application of pressure and this is probably due to volume changes resulting from changes in protein conformation.

Pressure may influence enzymes in many ways:

- *Change the substrate specificity* : a macromolecular substrate may become more sensitive to enzymatic depolymerization once it has been unfolded through pressurization.
- *Increasing or inhibition of enzyme activity*: an enzymatic reaction may be enhanced or inhibited by pressure, depending on the positive or negative value of the reaction volume. Enhanced enzyme activity may be due from enzyme release from cellular compartments and closer contact with substrates.
- *Partial or complete enzyme inactivation*: pressurization at room temperature may bring reversible or irreversible enzyme inactivation depending on the enzyme, pressure, temperature and treatment time (Hara et al., 1990).

Browning enzymes have proven to be especially troublesome in HHP. Polyphenoloxidase was treated at pressure levels necessary to achieve complete inactivation of microorganisms (600 – 800 MPa). At these levels polyphenoloxidase was not inactivated (Asaka and Hayashi, 1991).

Basak and Ramaswamy (1996) performed a kinetic study on the pectin methyl esterase during HHP of orange juice and evaluated the effect of pH and soluble solids concentration on pectin methyl esterase inactivation. They subjected pasteurized and non-pasteurized orange juice to pressures of 100 – 400 MPa for 0 – 720 min and reported that pectin methyl esterase inactivation was dependent on the pressure level, holding-time, pH and total soluble solids. They found firstly pressure inactivation to be dependent only on the pressure level and secondly inactivation to be dependent on the holding-time at each pressure level.

2.5. Effects of high hydrostatic pressure on microorganisms

To design appropriate processing conditions for food / pharmaceutical materials, it is essential to know the precise tolerance level of different microbial species to HHP and the mechanism by which that tolerance level can be minimized. The knowledge of critical factors that affect the baroresistance of microorganisms is a priority for the development of more effective and accurate high pressure processors.

In spite of a number of basic studies on the effects of high pressure on ultra-structure and the metabolism of given bacteria, little is known concerning the exact mechanisms of bacterial destruction by high hydrostatic pressure.

2.5.1. Inactivation mechanism

High hydrostatic pressure brings about a number of changes in the morphology, cell membrane or biochemical reactions of microorganism, and all these processes are related to the inactivation of microorganisms. Various morphological changes are observed with increasing pressure: compression of gas vacuoles, cell lengthening, separation of the cell membrane from the cell wall, contraction of the cell membrane, modification of the nucleus and the intracellular organelles, release of intracellular material into the extracellular space (Cheftel 1992). In 1990, Ludwig et al. showed that the cells of inactivated bacteria are not mechanically destroyed by the action of pressure.

The cell membrane is considered to be the major target for the pressure-induced inactivation of microorganism, and it is generally accepted that the leakage of intracellular constituents through the permeabilized cell membrane is the most direct reason of cell death by high pressure treatment.

If applied pressure was not enough to induce a total permeabilization of the cell, the permeabilization took place at the outer membrane in the case of Gram negative bacteria, and the permeabilized membrane was rapidly restored after pressure release (Hauben et al.,1996).

They observed that most of the proteins in the outer membrane had disappeared after HHP, whereas the cytoplasmic membrane bounded proteins were still retained after HHP. The fluidity of cell membrane plays an important role in susceptibility of microorganism to pressure treatments. Microorganism with less fluid membranes were most sensitive to HHP

(Macdonald, 1992). The conformation of nucleic acids appears to be relatively stable up to 10 kbar. DNA replication and transcription, and translation into protein appear to be inhibited at much lower pressure (*E. coli*). Microbial death may result from ATPase inhibition (Smelt et al., 1994) or from the crystallisation of membrane phospholipids with consecutive irreversible changes in cell permeability and ion exchanges.

The inactivation mechanism of bacterial spores is different from that of vegetative micro-organism. The intrinsic resistance of spores has been explained by the lack of solvation derived effects in the relatively dry structure (Sale et al., 1970). It was assumed that pressure caused inactivation of spores by first initiating germination and then inactivating germinated forms (Sale et al., 1970). Biochemically, binding of germinants to it's receptor is believed to promote the germination process followed by the efflux of Ca^{2+} and other ions and the influx of water into the spore, which result in the activation of spore specific cortex lytic enzyme (Wuytack et al., 1998). Furthermore, there is good evidence that moderate increases in temperature enhance the germinative effect of pressure (Ludwig et al.,1989; Butz et al., 1990). Leakage of DPA from pressurized spores provides good evidence of the opportunity for combined pressure/temperature combinations for spore destruction (Earnshaw et al., 1995).

2.5.2. Critical parameters for microbial inactivation by high hydrostatic pressure

The conditions under which high pressures are applied significantly influence the level of inactivation as well as the overall effect on the characteristics of the product (food or pharmaceutical products).

The critical process factors in HPP include**:** *type of microorganism; pressure level; time of pressurization, time to reach treatment pressure (come-up time); decompression time; treatment temperature (including adiabatic heating); initial product temperature, product pH, and water activity (a_w); packaging material integrity; and concurrent processing aids.*

Interestingly, because HPP acts instantaneously and uniformly through a mass of product package size and shape are not factors in process determination.

- ***Type of microorganism***

The resistance of microorganisms to high pressure is very variable. Hoover et al. (1989) reported that most bacteria are baroduric, i.e., they are capable of enduring high pressures but grow well at atmospheric pressures.

In most cases the effect of high pressure on Gram-positive bacteria is less pronounced that on Gram-negative species. Vegetative cells in the growth phase are most sensitive. Yeast and moulds are very sensitive, while viruses have a high resistance to pressure. By the far the most pressure resistant microbial forms are the endospores of Gram positive bacteria. Unless elevated temperature (45°C to 75°C) is added as a simultaneous treatment to HHP, spore numbers will scarcely be reduced by pressure in the 500 – 700 MPa range.

The spores inactivation was intensively studied Ludwig and co-workers (*B. subtilis, B. stearothermophilus, B. thuringiensis, Clostridium stricklandii*).

- ***Pressure level and time of pressurization***

Increasing the pressure level or time of pressurisation will usually increase the number of bacteria destroyed (with the exception of spores).

- ***Treatment temperatures***

The treatment temperature above or below room temperature tend to increase the inactivation rate of microorgamisms (Ludwig et al., 1992; Knorr and Heinz, 1999). There are several reports of enhanced lethal effects when pressurizing at -20°C compared with 20°C. It has been postulated that the enhanced lethal effects may be related to the fact that different proteins can be denatured at low temperatures compared with ambient temperature.

Process temperatures in the range of 90 – 110°C in conjunction with pressures of 500 – 700 MPa have been used to inactivate spore – forming bacteria such as *Clostridium botulinum*.

A preheat treatment followed by pressurization is generally more effective to inactivate spores than heating during pressurization.

- ***Time to reach treatment pressure (come-up time), decompression time; treatment temperature (including adiabatic heating)***

The effects of come-up time on the viability of microorganisms may be significant. Obviously, long come-up times will add appreciably to the total process time and will affect inactivation kinetics of microorganisms.

It is believed that rapid decompression invokes the physical breakdown of spore coat that result in physical disruption and death (Hayakawa et al., 1998). Further information is presently quite limited.

Temperature increases due to compression is a very important factor that influence the microbial inactivation. Temperature during processing may not be uniform because of compression–heating differences between the product and the pressure media, and heat loss or

heat gain between the sample, media and the pressure vessel. Temperature increase with pressure and decrease with pressure release.

This temperature transient for water is about 2-3°C for every 100 MPa. It depends on the composition of the product. For oils and fats the compression heating value can be as high as 9°C/100 MPa (Rasanayagam et al., 2001). There stands a chance that the pressure-medium fluid upon compression will heat the sample. This lead to errors in the inactivation study. Balasubramaniam and Buchanan (2002) have investigated the adiabatic heating characteristics of different pressure transmitting fluids during high pressure processing and they found that the adiabatic temperature raise was greatest (27°C above the target process temperature) in the case of 75:25 glycol : water mix. The temperature raise was in the order of 25°C and 22°C for 50:50 and 25:75 glycol : water mixes, respectively. The difference in temperature could be attributed to the difference in the viscosities and thermal heating characteristics of the fluids. Microbial studies indicated that none of the three glycol based fluids had a significant inactivation rate at 50°C. However at 70°C, the inactivation of spores was higher (up to 7 log) for a 25:75 fluid than for a 75:25 and 50:50 fluid (up to 5 log).

- ***Initial product temperature***

At lower initial temperatures, heat sensitive products can be processed without heat damage. The initial temperature can be lowered as the pressure is increased so that the same sterilizing end temperature due to instant adiabatic heating is achieved.

- ***pH and water activity***

The pH plays a very important role in determining the extent to which pressure affects the micro-organisms under study. The change in pH was thought to affect membrane ATPase and intracellular functions of the spores thereby destabilizing the microorganisms (Macdonald, 1992). When pH is lowered most micro-organisms (whether spores or vegetative forms) become more susceptible to inactivation by HHP with injured cells less able to recover. This is why acid foods such as fruit products usually work well with HPP (Hoover, 1996). The pH of aqueous solutions decreases with increasing pressure because of electrostriction although the extend of the change is extremely difficult to measure during pressure treatment.

The water activity (a_w) of cells also affects the pressure resistance of microorganisms. A reduction of water activity exerts protective effect for microorganisms against pressure treatments. Palou et al. (1997) reported the complete inhibition of *Zygosaccharomyces bailii* at $a_w > 0.98$, and an increase in the surviving fraction with a decrease in a_w. The lowest a_w at which most spoilage organisms grow is about 0.90, while the lower limit for yeast and mould is about 0.61. The level of a_w has a direct effect on the extend of sporulation, germination of

spores and toxin production. Beyond microbiological safety a_w also influences the level of chemical and enzymatic activity – browning, lipid oxidation, protein denaturation, etc.

- ***Packaging material***

The type of packaging used also plays a very important role in HHP. Two basic requirements that the packaging materials need to possess are: the ability to withstand the magnitude of pressure under operating conditions and good heat sealability. Currently, several different types of packaging are in use like plastic stomacher bags, polyester tubes, polyethylene pouches, nylon cast polypropylene pouches, and various others flexible systems.

2.5.3. Inactivation kinetics

The inactivation of microorganisms has been assumed to follow first-order kinetics; however, significant deviations from linearity have been observed frequently in the literature.
In most cases, the inactivation of microorganisms may be described as a first order reaction or a combination of two first order reactions (biphasic inactivation kinetic) (Ludwig and Merkulow, 2001).
A biphasic pressure inactivation is frequently encountered for both vegetative bacteria and endospores. Such an inactivation curve indicates the residence of a small pressure-resistant subpopulation. In this case two rates can be calculated (Fig. 2.2).

Often deviations from the first order inactivation are obtained, i.e. the inactivation curves contained shoulder or tail or both of them. Due to the more or less pronounced tailing and shoulder-formation variation in reaction order was observed even within the same bacterial strain. A description of these curves is possible by a first order inactivation of metastable intermediate state that is reached after a certain time of resistance and is assumed to be distributed due to the diversity in the bacterial population (Heinz and Knorr, 1996).
Mathematical models based on cumulative Weibull-distribution (Heinz and Knorr, 1996), Gompertz equation (Patterson and Kilpatrick, 1998) or quadratic polynomial expression (Reyns et al., 2000) were used to predict inactivation of microorganisms with non-linear behaviour.

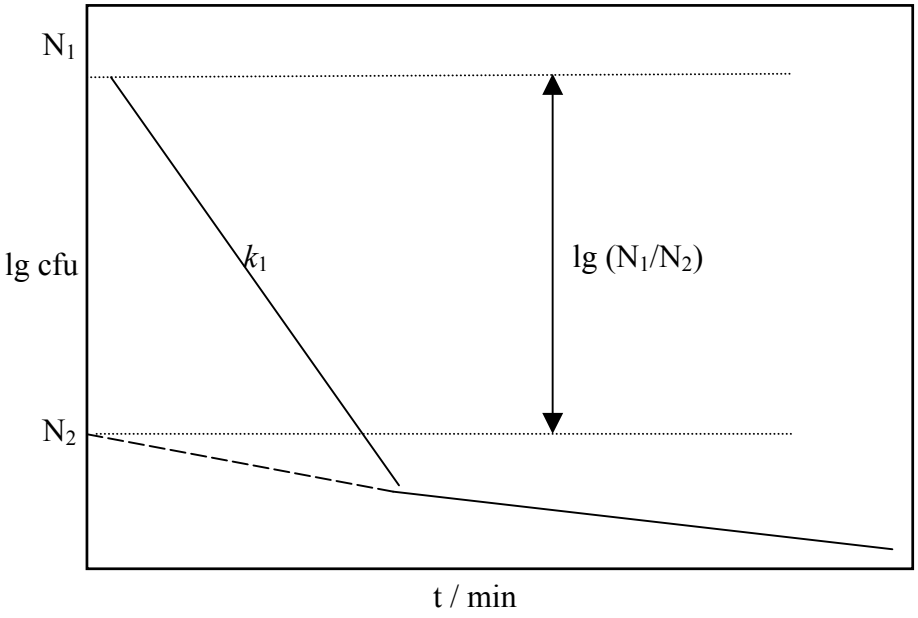

Fig. 2.2 Biphasic inactivation of microorganisms

2.6. High pressure pulsed application

The use of pressure-pulsing or oscillatory pressure treatments has been shown to be generally more effective than equivalent single pulses or continuous pressurization of equal times. Hayakawa et al., (1994) achieved a complete sterilization of *Bacillus stearothermophilus* after 6 cycles of oscillatory pressurization at 70°C and 600 MPa, but continuous pressurization was ineffective. Sojka and Ludwig (1997) showed that rapid pressure changes are an effective method to achieve inactivation of *Bacillus subtilis* at relatively moderate temperatures. They have shown that spore killing accelerates when the frequency of pressure oscillation is increased and that this effect is not due to temperature rise during adiabatic compression. Palou et al. (1998), found that cyclic applications improved inactivation of the *B. nivea* ascospores.
It was assumed that the greater rate of yeast inactivation due to oscillatory pressurization was due to greater injury to the cellular membrane from rapid changes in intracellular/extracellular differences at the membrane interface.

2.7. High pressure processing in food industry

Applications of high hydrostatic pressure in food sterilization and processing have been a central issue of research and development in Japan for more than a decade. In contrast, food technology in Europe is only beginning to enter this field (Balny et al.,1992). In 1990 Meiji-ya Food Company introduced three kinds of jam, using high pressure treatment without application of heat. The products are natural in color and taste. These jams were the first pressure-processed foods in human history.

Since 1990 a variety of pressure treated fruit juices, toppings, jams and tenderized meats have been sold in Japan.

In 1995 pressure-treated freshly squeezed orange juice was launched in France.

In 1997 sliced cooked ham became available in Spain, and an U.S. company launched guacamole, followed in 1999 by .pressure-processed fresh oysters. In 2000 a range of salsas were commercialized in USA. Recently, Japanese unrefined rice wine appeared on the market. Various commercial HHP treated products have entered the global marketplace.

Studies have been shown that HHP could be applied to increase safety of meat products (Carlez et. al., 1992), cheese ripening (Yokoyama et. al., 1991), milk and dairy products sterilization (Trujillo, 2002; Needs, 2001), fruits (Otero et al., 2000) and vegetables (Indrawati et al., 2000).

Work still needs to be directed towards a reliable method to predict the HHP process.

Concluding, the benefits of high hydrostatic pressure processing in food industry are:

- effective and fast inactivation of microorganisms and enzyme;
- retention of original, fresh-like product flavor, taste, color and texture;
- no nutrient degradation;
- addition of preservatives can be avoided or minimized;
- uniform application of pressure, no dead zones or gradients;
- waste free technology.

2.8. High pressure biotechnology in medicine and pharmaceutical science

High pressure biotechnology is an emerging technique initially applied for food processing and more recently in pharmaceutical and medical science. The effects of high pressure could have potential applications in medical fields as follow (Masson, 2001):

- *Effects of pressure on constituents of living matter*

In the pressure range of biotechnological interest pressure has in general no effect on covalent bonds. Therefore natural compounds such as flavors, aromas, dyes, and pharmacologically active molecules are not destroyed by high pressure treatment at room temperature. Several pro-vitamins and vitamins (thiamin, riboflavin, folic acid, retinal, tocopherol) have been found to resist to pressures as high as 6 – 8 kbar.

- *Chemical modifications of proteins and other macromolecules*

Enzyme modification of proteins and polysaccharides can be achieved under pressure**.**

- *Enzyme-catalyzed chemical reactions*

The enantio-selective synthesis of esters such as ibuprofen esterification by lipases, pharmacological peptides by thermolysin seem to be possible under pressure. These reactions are performed in bioreactors at pressures lower than 200 MPa, i.e. pressures that do not alter the stability and functionality of enzymes.

Pressure processing was found to be efficient to reduce the allergenic activity of food (Kato, 1990).

- *Pressure-assisted freezing and thawing and storage at sub-zero temperature without freezing*

The effect of high pressure on the phase transition of water is a possible alternative for the preservation of biopharmaceuticals, blood derivatives, cells and organs for transplantation.

The freezing induction of a product cooled at temperature from -10°C to -20°C under a pressure of 100 MPa to 400 MPa is quite instantaneous when pressure is released. This technique could probably allow fragile biopharmaceutical products to be frozen without damage.

The property of water under pressure allow also bioproducts to be stored at subzero temperatures without freezing. The process has less dramatic effects than freezing and thawing processes.

- *Inactivation of biological agents*

Inactivation of numerous viruses such as herpes viruses (Nakagami et al.,1995).

- *Development of vaccines.*

The enhanced immunogenity of some pressure killed bacteria and viruses could be applied for making new vaccines.

- *Inactivation of microorganisms*

Rigaldie et al. (2002) studied the effect of high pressure on fragile pharmaceutical molecules and its efficacy on viability of various strains of microorganisms described in Pharmacopoeia. They demonstrate the safety of HHP treatment on several sensitive biomolecules (insulin, monoclonal antibodies anti-influenza A, etc). At the pressure-temperature range they studied, a total inactivation of *Pseudomonas aeruginosa* and *Candida albicans* was achieved without altering the sensitive therapeutic molecules. These results show that HHP treatment could represent a low cost mild alternative sterilization process.

Chapter 3 Bacterial spores

3.1. Introduction

In the latter half of the 19th century Tyndall, Cohn, and Koch independently discovered that certain species of bacteria spend at least part of their lives as dormant cellular structures now known as endospores

Under condition of a limitation in the supply of C, N, or P certain gram-positive rods form highly resistant, dehydrated forms, called spores or endospores (so called because the spore is formed within the cell). Examples of sporeforming bacteria are rather widespread within the low Gram positive C subdivision of the Gram positive bacteria and represent inhabitants of diverse habitats such as aerobic heterotrophs (*Bacillus and Sporosarcina spp*.), halophiles (*Sporosarcina halophila)*, microaerophilic lactate ferments (*Sporolactobacillus spp.*), anaerobes (*Clostridium* and *Anaerobacter spp.*), sulfate reducers (*Desulfotomaculum spp.*) Despite the diversity exhibited by sporeforming bacterial species the bestknown sporeformers are commonly rod-shaped soil inhabitants belonging to the *Bacillus* and *Clostridium* species.

Bacterial spores are the toughest form of life known. In the dormant state spores undergo no detectable metabolism and they can withstand a wide range of assaults such as heat, chemical disinfectants, acids, radiation, desiccation, hydrostatic pressure that would destroy a vegetative cell.

3.2. Endospore formation and structure

Spores are unusually dehydrated, impervious, highly refractile cells and they do not take ordinary stains (Gram's, methylene blue).To stain spores specifically special spore-staining procedures must be used.

During endospore formation a vegetative cell is converted to a non-growing, heat-resistant structure – the endospore. The differences between the endospore and the vegetative cell are profound. The first stage in sporulation is the formation of an area of increased refractility, **the forespore**, at one end of a cell. The refractility gradually increases and the mature spore is completed in 6-8 h. The mature spore is freed by autolysis of the sporangial wall. The stages in endospore formation are shown in Figure 3.1.

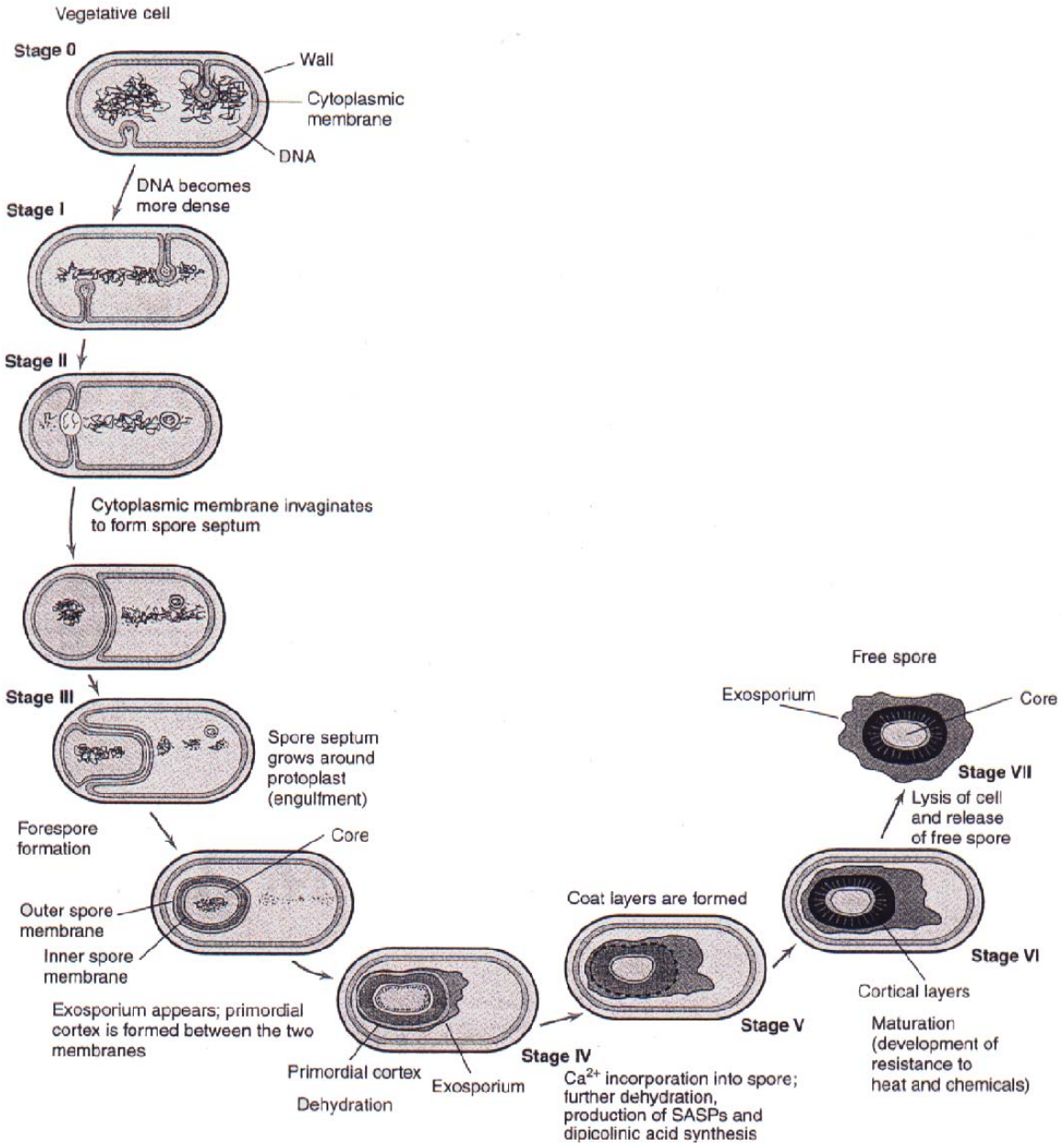

Fig 3.1 Stages of spore formation (Brock)

Basically, a spore is a structure that contains the absolute minimum of genetic informations. The outermost layer is the **exosporium**, a thin, delicate covering made of proteins. The exosporium is a lipid–protein membrane with 20% carbohydrate. It is not essential for survival, and its function is unknown. Within this are the **spore coats** composed of layers of protein. The coats are made of a keratinlike protein, rich in S-S, which constitutes as much as 80% of the total protein of a spore. The protein coat is responsible for the resistance of spores to chemicals. Below the spore coat is the **cortex** which consist of loosely cross-linked

peptidoglycan and inside the cortex is the **core** or **spore protoplast** which contains the usual cell wall (core wall), cytoplasmic membrane, cytoplasm, nucleoid, and so on. The core of a mature endospore contains only 10-30% of the water content of the vegetative cell and thus the consistency of the core cytoplasm is that of a gel. Dehydration of the core increases the heat resistance of the endospore and also confers resistance to chemicals such as hydrogen peroxide. The core contains high level of core-specific proteins called small acid-soluble spore proteins (SASPs). These proteins have two functions: carbon and energy source for the outgrowth of a new vegetative cell from the endospore (process called germination) and SASPs bind to DNA in the core and protect it from potential damage from UV radiation, desiccation and dry heat.

A striking feature of spores is their huge content of Ca^{+2}. Normally the Ca^{+2} is accompanied by a roughly equivalent amount of dipicolinic acid which can chelate Ca^{+2}. The calcium-dipicolinic acid complex is almost unique to bacterial spores and may constitute as much as 10% of their weight. This substance is located in the core.

In addition to high levels of dipicolinic acid *Bacillus subtilis* spore core contains a large amount of sulpholactic acid.

3.3 Activation, germination and outgrowth processes

Dormancy is the central function of sporulation and express the stage or condition of a living organism that is characterized by a lack of metabolism and developmental processes. Despite their metabolic inactivity spores are still capable of continually monitoring the nutritional status of their surroundings and they respond rapidly to the presence of appropriate nutrients by germinating. In the sequence of events that lead from a dormant spore to the vegetative cell three conceptually distinct processes have been postulated to occur. These processes are *activation, germination* and *outgrowth*. These three processes are fundamentally different from each other.

The process of "conditioning the spore to germination" has been called activation. Though some bacterial spores will germinate spontaneously in a favorable medium others (especially if freshly formed) remain dormant unless they are activated by some traumatic agents such as heat, low pH, or high hydrostatic pressure. Heat treatment is the most used method of activation; the other methods differ from heat activation in being less efficient.

The activation process is in most cases reversible, does not involve metabolism and consist apparently in changes in the configuration of macromolecules.

When activated spores are exposed to the appropriate germination environment an irreversible process called germination occurs which involves complete loss of typical spore properties. Germination is the conversion of a resistant and dormant spore into a sensitive and metabolically-active form.

Germination occurs in the presence of inhibitors of macromolecular synthesis and thus some spore components are broken down and excreted into the medium; germination is a degradation process during which spore metabolism is initiated.

During germination spores lose their characteristic resistance to heat, desiccation, pressure, ultraviolet and ionizing radiations, chemicals. If all spores in a material could be caused to germinate, the material could be sterilized by a relatively mild treatment.

After germination is completed outgrowth process occurs which changes a germinated spore into a vegetative cell. This is a process of biological growth and differentiation and takes place only in a growth medium. During the outgrowth process some new kinds of protein not present in the spore stage have been found as well as new structures typical of the vegetative stage only (Davis, 1980).

Part III Process

Chapter 4 Material and Method

4.1. Microorganism and growth media

Bacillus subtilis var.niger ATCC 9372 purchased as lyophilized pellet from DSM (Deutsche Sammlung Mikroorganismen, Braunschweig, Germany) was used throughout this study. All growth media were set in water which had before been demineralized and released from organic components using a Millipore device.

For cultivation of *Bacillus subtilis* 25 g Standard I Nutrient Broth were solved per liter water.

For sporulation of *Bacillus subtilis* the following medium was prepared:

25 g Standard I Nutrient Broth
15 g Agar-Agar
500 mg $MnCl_2 \times 4H_2O$
500 mg $MgCl_2 \times 6H_2O$ / per liter water.

The prepared media were sterilized 20 min at 121°C. After cooling to ca. 60°C about 15 ml of the liquid agar was poured in Petri dishes. After complete solidifying of the agar the plates with open cover became in approx. 30 min at ambient temperature dried and were stored well packed at 5°C up to further use.

4.2. Preparation of *Bacillus subtilis* spore suspension

A small piece of *Bacillus subtilis* lyophilized pellet was incubated overnight in Standard I Nutrient Broth at 37°C under strong aeration. To induce sporulation the overnight culture was platted on Petri dishes with nutrient agar supplemented with 10 mg/ml $MnSO_4$ and 500 mg/ml $MgSO_4$. After 7 days of incubation at 37°C, spores were harvested by flooding the surface of the culture with sterile NaCl 0.9% solution and then gentle scraping the surface with a sterile wide spatula. The spore suspension was shaken with glass beads (2 mm diameter).

After filtering through sterile cotton wool the spores were washed three times by centrifugation at 5500 rpm for 45 min at 4 °C and finally resuspended in sterile NaCl 0.9% solution containing 0.2% Tween 80. Tween 80 was added to minimize clumping of spores and consequently to improve the accuracy of the spore counts. The spore suspension was adjusted to 10^7 -10^8 spores/ml and kept at 4°C for up to 1 month. Platting of this spore suspension before and after heat treatment (80°C for 15 min) did not result in significantly different counts. This indicated that the spore suspension consisted exclusively of spores.

4. 3 High hydrostatic pressure treatment

4.3.1. High pressure equipment

All experiments were carried out with a high pressure system (Fig. 4.1) purchased from Resato International B.V., Roden, Holland. This equipment allows pressurization up to 1000 MPa. The unit consists of (I) four pressure vessels with a capacity of 25 cm^3, 16 mm diameter and 134 mm length surrounded by a thermostated mantle connected to a Haake F8 cryostat. One vessel is provided with an internal thermocouple in order to monitor the temperature during the experiments. The vessels are handled by air operated needle valves. The necessary compressed air input is supplied by a compressor Herkules HP 1,5 Lt.24. These valves can be used to single out one vessel or to bleed the pressure in a part of the system. This make the system suitable for performing kinetic experiments; (II) a high pressure intensifier unit (Resato, P 200-815-S) responsible for building up the pressure in the system and a pre-fill hydrostatic pump. The pressure is transmitted from the intensifier to the vessels by the pressure fluid (Resato, polyglycol ISO-viscosity class VG 15) through high pressure stainless steel tubing. By means of pressure and temperature transducers the pressure and temperature were recorded every second during the entire treatment.

This equipment is also provided with a hand operated spindle pump (Resato, SP-100-3) used for pressure micro-adjustments. The equipment used to perform the experiments for this study is presented in fig. 4.1.

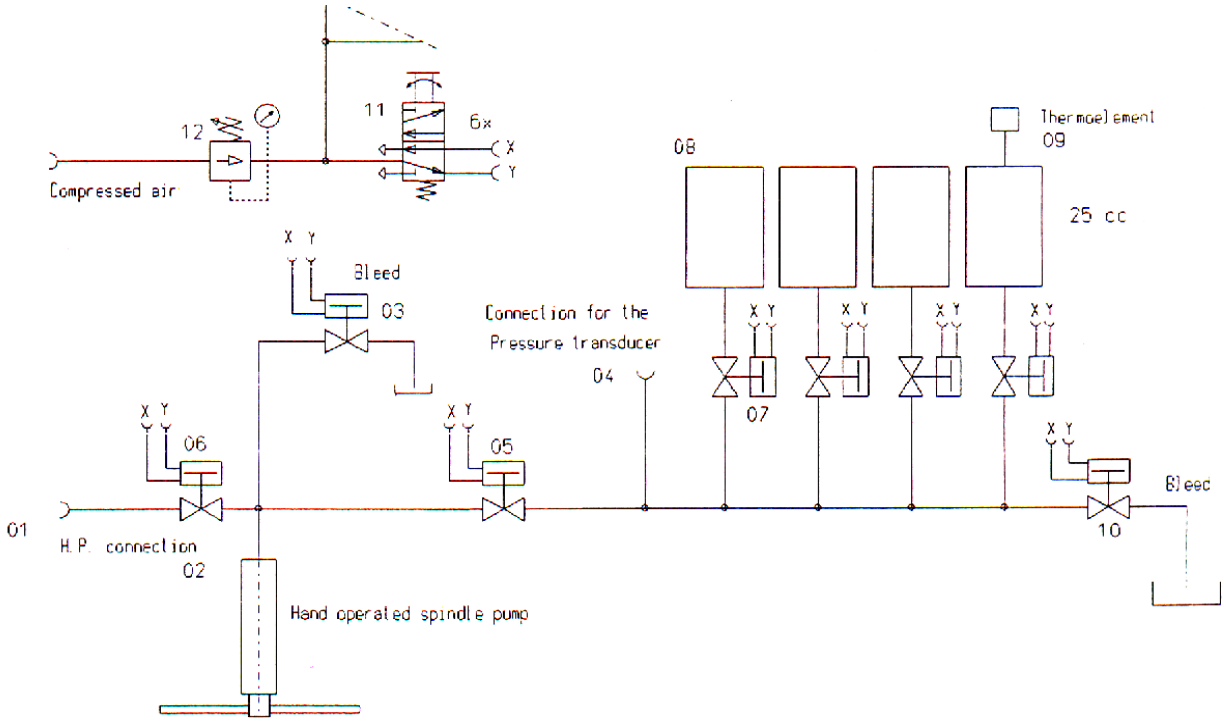

Fig 4.1 Resato high pressure equipment

4.3.2. Sample preparations and high pressure treatment

The spore suspension was enclosed in polyethylene tube pieces (Laboflex PE 8.0 NA 10.0) produced by Kronlanb Company, Sinsheim, Germany. The length of the pieces were selected in such a way that a volume could be taken up of 1.5 - 2 ml. The ends were sealed with silicone plugs (Migge Company, Heidelberg, Germany). The tube pieces were stored in 70% isopropanol and dried before use under the sterile bench. The silicone plugs were submitted to autoclave sterilization.

Subsequently the ends of the sample containers were sealed with Parafilm^{TM} and welded into HD - PE foil (Rische & Herfurth GmbH, Hamburg) in order to prevent an opening of the containers during the experiments.

After the loading of high pressure vessels with sample tubes the pressure was built-up in two ways:

- fast – pressure was developed with the highest possible rate ; the come-up time was between 5-30 s (without taking in account the equilibration period) depending on the desired pressure level ;
- slow – pressure was built up using a hand operated spindle pump with a pressurization

rate of 20 MPa/min. The time needed to achieve the treatment pressure was between 2.5- 20 min depending on the required pressure.

After the desired pressure had been reached the individual vessels were isolated so that the pressure was maintained in the vessels until the valves were opened. The pressure level micro-adjustment takes then place with the hand operated spindle pump. The conditions of high pressure testing are presented in Figure 4.2.

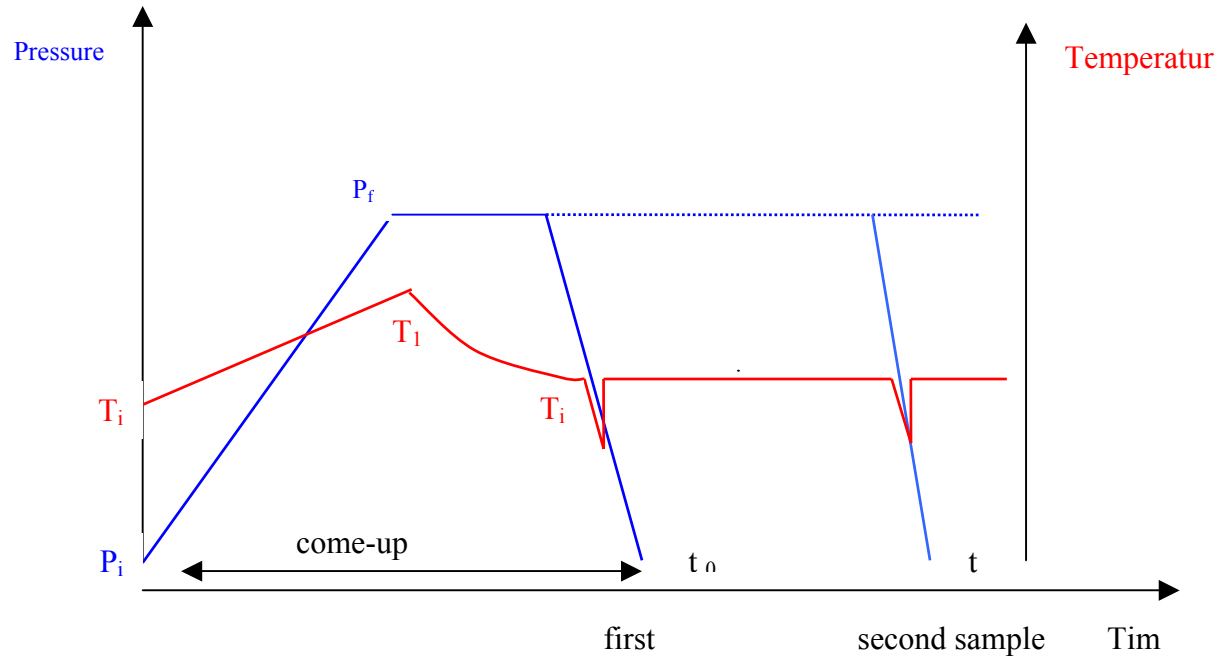

Fig.4.2 Conditions of high pressure testing;
where T_i / P_i are the starting temperature and pressure, respectively; P_f is the desired treatment pressure level; T_1 is the temperature maximum reached after pressurization; t_0 the time at which the treatment conditions are constant

The pressure build-up phase is accompanied by a temperature increase (T_i-T_1) due to adiabatic heating (between 3-25°C for fast pressure build-up and 2-3°C for slow pressure build-up) so, an equilibrating period of 3-9 min for fast pressure build-up and respectively 1-4 min for slow pressure build-up to allow temperature to evolve to its initial value (T_i), was taken into account. By starting the time course of the experiment ("zero point") after this period the inactivation of *Bacillus subtilis* during come-up times could be proper quantified. Upon decompression, the temperature falls below the initial starting temperature of the test.

Simultaneously to quantify the temperature effect on the spores inactivation, one sample (blank) was treated at ambient pressure and the corresponding experiment temperature (T_i). The blank was defined as the inactivation of a non-pressure-treated spores sample. The other vessels, each containing one spore sample, were then decompressed after pre-set time intervals (t_1, t_2 and so on).

A rapid decompression(< 2s) terminated the fast as well as slow pressure build-up treatment.

After cooling the samples were plated to determine the viable spore counts.

All experiments were carried out at least three times.

4.4. Determination of viable spore counts

The number of spores surviving the high pressure treatments (cfu/ml) was determined using the spread plate method.

Decimal dilutions series (in isotonic solution NaCl 0.9% with 0.2% Tween) of the treated spore suspensions as well as of initial spores suspension (initial germ number) were prepared and 100 µl of each dilution was plated on nutrient agar dishes in triplicates.

The spore suspension was diluted in such way before the spreading that maximally 250 colonies were counted on a plate. This was accordingly often repeated until the desired dilution stage was reached.

The agar dishes were incubated at 37°C for 24 h then the colonies formed were counted and the average number of countable colony forming units was taken as the result. The surviving spore number was logarithmic expressed as lg cfu (logarithm colony forming units).

4.5.Data analysis

First order model. On the basis of the linear curves in the plots of the logarithm of colony forming units versus time, the inactivation *of Bacillus subtilis* spores was assumed to follow a first order kinetic model.

$$-\frac{dA}{dt} = kA \qquad \text{equation 4.1}$$

where A is the spore concentration at time t (min) and *k* is the inactivation rate constant (min^{-1}).

This differential equation rearranges to

$$\left(1/[A]\right)d[A] = -kdt \qquad \text{equation4.2}$$

and can be integrated directly:

$$\int_{[A]_0}^{[A]_t}\left(\frac{d[A]}{[A]}\right) = -\int_0^t k\,dt, \quad \text{or} \quad \ln[A]_t - \ln[A]_0 = kt \qquad \text{equation 4.3}$$

The solution can be expressed in two useful forms:

$$\ln\frac{[A]_t}{[A]_0} = -kt \qquad \text{equation 4.4}$$

$$[A_t] = [A]_0 \cdot e^{-kt} \qquad \text{equation 4.5}$$

Equation 4.4 shows that if $\ln[A]_t/[A]_0$ is plotted against t, then a first order reaction will give a straight line and the value of k may be obtained from the slope. The later equation shows that in a first order reaction the concentration decreases exponentially with time with a rate determined by *k*.

The temperature dependence of *k* is given by the activation energy as expressed in the Arrhenius relationship:

$$\left(\frac{\partial \ln k}{\partial 1/T}\right)_p = -\frac{E_a}{R} \qquad \text{equation 4.6}$$

where *k* is the inactivation rate constant (min^{-1}), Ea is the activation energy (kJ/mol), R is the gas constant (8.31441 J/mol·K) and T is the absolute temperature (K). The magnitude of E_a indicates the temperature dependence of the inactivation reaction.

Plotting the logarithm of the inactivation rate constant as a function of the reciprocal temperature at constant pressure, the activation energy at pressures between 50-400 MPa was determined by linear regression.

For a definition of reference temperature equation 4.6 can be rewritten as

$$k_2 = k_1 \cdot e^{-\frac{E_a}{R}\left(\frac{1}{T_2}-\frac{1}{T_1}\right)} \qquad \text{equation 4.7}$$

where T_1 is the reference temperature and k_1 is the corresponding inactivation rate constant.

The pressure dependence of k is given by the activation volume as expressed in the Eyring relationship:

$$\left(\frac{\partial \ln k}{\partial p}\right)_T = -\frac{\Delta V^{\neq}}{RT} \qquad \text{equation 4.8}$$

where $\Delta V^{\neq}$ is the activation volume (cm^3/mol), R is the gas constant (8.31441 J/mol·K) and T is the absolute temperature (K).

By plotting the logarithm of the inactivation rate constant as a function of pressure at constant temperature, the activation volume at temperatures between 25 and 70°C was determined by linear regression.

For a definition of reference pressure equation 4.8 can be rewritten as

$$k_2 = k_1 \cdot e^{-\frac{\Delta V^{\neq}}{RT}(p_2-p_1)} \qquad \text{equation 4.9}$$

Combining equations 4.7 and 4.9 we get

$$k_{T_2 p_2} = k_{T_1 p_1} \cdot e^{-\frac{\Delta V^{\neq}}{RT}\cdot(p_2-p_1)} \cdot e^{-\frac{E_a}{R}\cdot\left(\frac{1}{T_2}-\frac{1}{T_1}\right)} \qquad \text{equation 4.10}$$

that allows calculation of k values at any temperature / pressure combination.

The inactivation rate constant at reference pressure and reference temperature (k_{ref}), the activation energy and activation volume were used as kinetic parameters characterizing this model.

Hypothesizing first order inactivation kinetics the integral effect of the inactivation process under variable condition can be described by

$$\ln\left(A_t / A_0\right) = -\int_0^t k(T,p)dt \qquad \text{equation 4.11}$$

Rate constants were calculated for the whole come up time including the equilibration period in one second time steps. Using these constants the inactivation effect was calculated by numeric integration:

$$\ln \frac{A_0}{A_t} = \int_0^t k(T,p)dt \qquad \text{equation 4.12}$$

$$\ln \frac{A_0}{A_t} = \left(\frac{k_{t_1}}{2} + (k_{t_2} + k_{t_3} + ...k_{t_{\substack{pressure \\ buildup-1}}}) + \frac{k_{t_{\substack{pressure \\ buildup}}}}{2} \right) \times 1/60 \qquad \text{equation 4.13}$$

where A_0 is the initial spore concentration, A_t spore concentration at time t and k is the first order inactivation rate constant.

All calculations were carried out with Excel, Sigma Plot and Origin software.

4.6. Chemicals

The chemicals used throughout this study are:

Agar – Agar: Fluka, Buchs, Switzerland

Ethanol: 99,9%, Fluka, Buchs, Switzerland

Isopropanol: Chem. purr, Chemikalienlager, Theoretikum, Heidelberg

Mangan(II)-chloride – tetrahydrate: for analysis, Merck, Damstadt

Magnesiumchloride – Hexahydrate: for analysis, J.T. Baker, Deventer, Holland

Natriumchloride: J.T. Baker, Deventer, Holland

Standard I – Nutrient Broth: granulate, for microbiology, Merck, Darmstadt

Polysorbate 80

Chapter 5 Results

5.1 Preliminary remarks

The influence of handling and system-dependent parameters

Kinetics is the study of rates of reactions. The rate can be influenced by many experimental conditions. Because these conditions can have a drastic effect on the rate it is imperative to record and report the path of the process.

Fundamental considerations which apply to all series of measurements are discussed.

5.1.1 Factors related to bacterial spore suspension

Pure spore suspensions of *Bacillus subtilis var. niger* were used throughout this study. It is important to point out this fact because the majority of studies on spore high pressure inactivation, particularly with *Bacillus subtilis*, often uses mutants. It is known that some mutants exhibit altered spore resistance because of alterations in gene expression caused by mutation (Setlow, 2003).

Another factor that influences the inactivation is the sporulation temperature. The sporulation temperature has a large effect on spore resistance and spore composition. *Bacillus subtilis* spores can be prepared at temperatures from 22 to 48°C. The core water content is lower in spores prepared at higher temperature (Beamann and Gerhardt, 1986). Based on literature data 37°C was chosen for this study as sporulation temperature being known that the *Bacillus subtilis* spores prepared at higher temperatures are more resistant to many different stress factors. (Melly et al., 2002; Raso et al., 1998).

The suitability of the work routine was tested concerning the influence of initial spore concentration on inactivation. In order to receive reproducible results it must be guaranteed that the different experiments can be compared with one another. The initial spore number varies with each experiment within certain limits. In order to be able to compare the results it is therefore a condition that the inactivation is independent of the initial spore concentration. Figure 5.1 represents the inactivation of *B. subtilis* spores under identical experimental conditions starting from different spore concentrations.

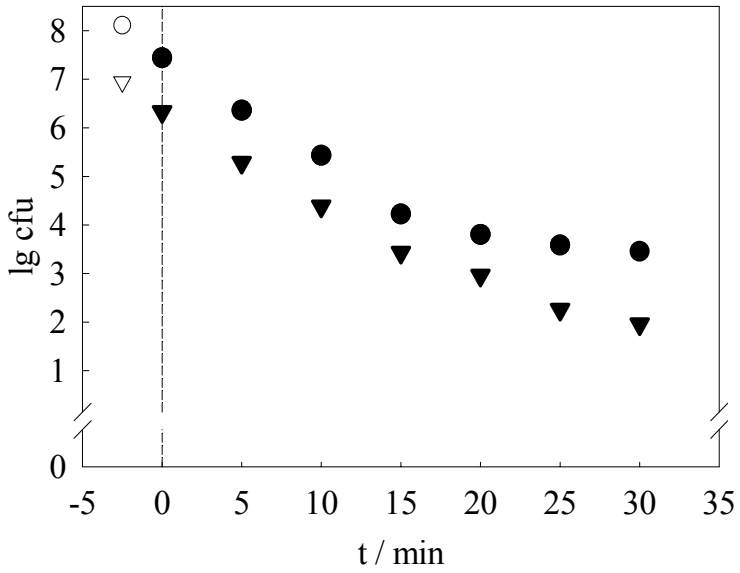

Fig. 5.1 Inactivation of *B. subtilis* spores at 60°C/400 MPa
starting from different spore concentration; (●) 10^8 spores/ml and (▼) 10^7 spores/ml

Fig. 5.1 shows that the initial germ concentration does not influence the inactivation process. For different initial concentrations the shape of the inactivation curve is vertically shifted without changes.

5.1.2 Factors related to experimental conditions

One fundamental parameter which might influence the inactivation is the temperature increase due to compression.

Temperature increases with pressure and decreases with pressure release. Previous works in our research group have shown that temperature alone does not harm the spores.

Sojka (1996) has quantified the effect of temperature alone on the *B. subtilis* spore inactivation by treating the samples at ambient pressure at constantly 90°C, the highest temperature measured after compression to 500 MPa at 70°C. The conclusion was that the spore inactivation is not a result of temperature rise. In the present work the highest temperature level reached after pressurization was 85°C and it was assumed, based on previous works in our group, that this temperature increase has no influence on spore inactivation. Thus the inactivation is achieved by a complicated interplay of temperature and pressure effects on the inactivation process.

Another factor that could influence the inactivation is the type of pressure release. Starting from the fact that the *Bacillus subtilis* strain used is relatively hard to inactivate a rapid

decompression was applied. Rapid decompression results in a fast adiabatic expansion of pressure transmitting fluid and increases the impact force on the spore coat (Hayakawa et al. 1998). Furthermore, the impact force upon rapid decompression is much stronger than the force caused by pressurization alone.

5.2 Quantification of the inactivation effect during fast pressure build-up phase

This subchapter presents the effect of combined pressure and temperature on the inactivation of *Bacillus subtilis* spores over the period of fast pressure increase. The time periods (in seconds) needed to reach the treatment pressure including the equilibration time to constant temperature are presented in Table 6.1. The inactivation kinetics was studied and, starting from the inactivation rate constants obtained, the parameters characterizing the inactivation process were estimated. The reference temperature/pressure were chosen in the middle of the temperature/pressure range used for inactivation determinations. Finally, the inactivation effect during the fast pressure build-up phase was quantified as described in Chapter 4, section 4.5.

	25°C	30°C	40°C	50°C	60°C	70°C
50 MPa					118	
100 MPa				201	172	213
150 MPa					224	
200 MPa		239			269	
300 MPa	450	445	371	452	426	326
400 MPa		677			642	

Table 5.1 Come-up times (in s) for the pressure/temperature combinations studied

5.2.1 Inactivation kinetics of *Bacillus subtilis* spores after fast pressure build-up

The inactivation of *Bacillus subtilis* spores was investigated in the pressure range from 50 MPa to 400 MPa, at temperatures from 25 to 70°C. For all temperature/pressure combinations studied a first order inactivation kinetics has been observed, excepting the combinations 60°C/50MPa, 60°C/100 MPa, 60°C/150MPa, where the inactivation curves bend to biphasic behavior. The inactivation behavior is represented in Figs. 5.2 and 5.3.

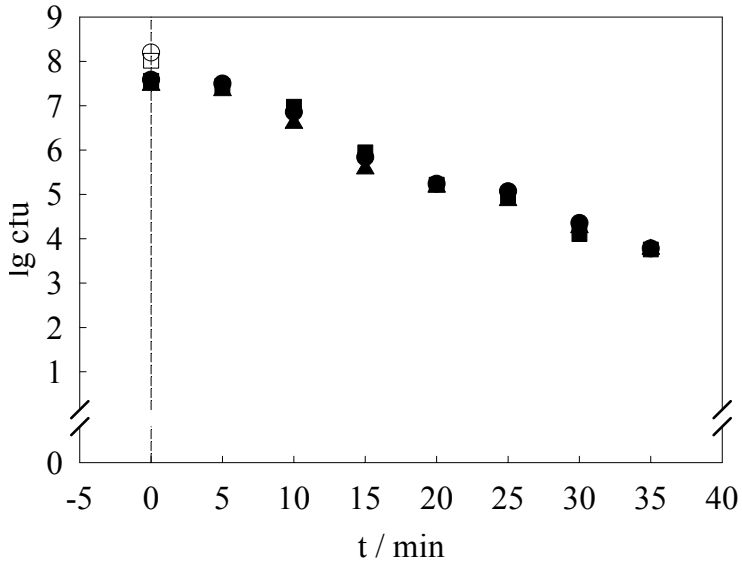

Fig. 5.2 First order inactivation kinetics at 60°C/300 MPa (in triplicate); open symbols are: (□) control and (o) the initial germ number.

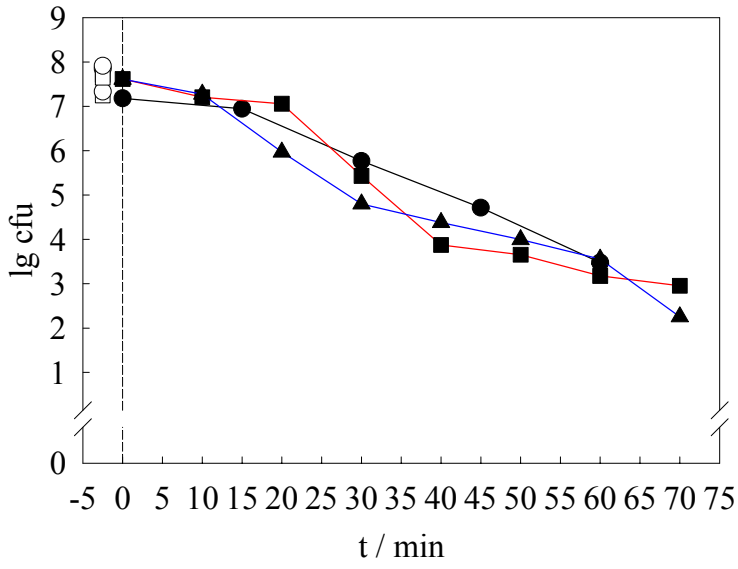

Fig.5.3 Inactivation kinetics of *B. subtilis* spores at 60°C and three different pressures; (●) 50 MPa (■) 100 MPa; (▲)150 MPa); open symbols are: (□) control and (o) the initial germ number.

In Fig.5.3 the inactivation kinetics of *B. subtilis* spores show a typical tailing and an initial lag-phase indicating a certain time-dependent resistance mechanism. In these cases, for an accurate determination of inactivation effect during pressure build-up phase, the inactivation curve was split up into a two-step reaction and the inactivation rate constants were determined for the first part of the curve.

5.2.2 Inactivation kinetics in the pressure range of 50-150 MPa at temperatures between 25-70°C

The determination of the inactivation rate constants was carried out using a design with three pressure levels (50, 100, 150 MPa choosing as reference pressure the 100 MPa level) and three temperature levels. The temperature range studied was divided in 2 domains: 25-40°C and 50-70°C choosing as reference temperatures 30°C and 60°C, respectively. The process time levels were chosen appropriately depending on the expected inactivation rates. Fig.6.4 shows the inactivation kinetics of *Bacillus subtilis* spores at constant pressure (100 MPa) and different temperatures.

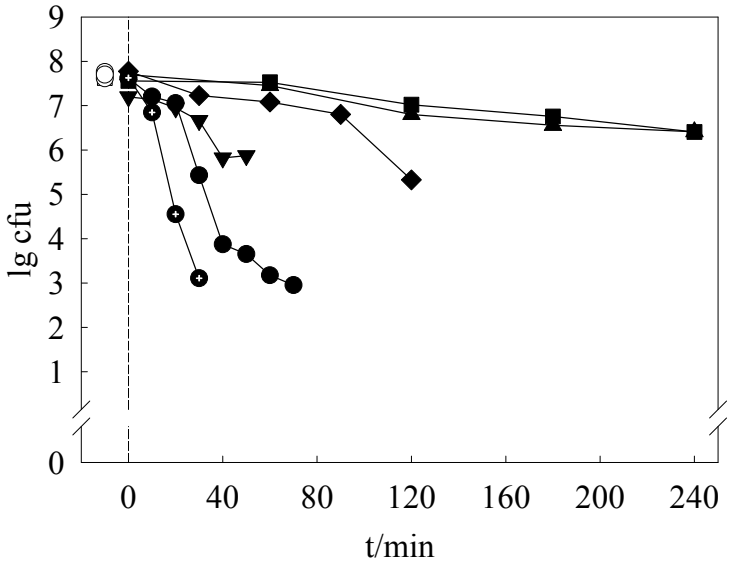

Fig. 5.4 Inactivation of *B. subtilis* spores at 100 MPa and different temperatures; (■) 25°C; (▲)30°C; (♦) 40°C; (▼) 50°C; (●) 60°C; (✪) 70°C; open symbols are: (□) controls and (o) initial germ number.

From Fig. 5.4 follows that increasing the process temperature from 25 to 70°C at constant pressure higher inactivation rates are observed.

The Figures 5.5 and 5.6 represent the inactivation kinetics at the chosen reference temperatures and three different pressure levels.

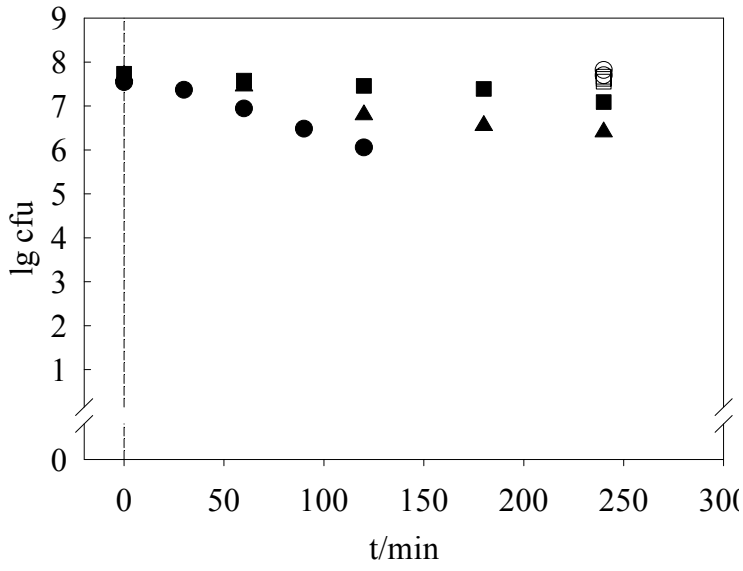

Fig. 5.5 Inactivation kinetics of *B. subtilis* at 30°C and three different pressures: (■) 50 MPa; (▲) 100 MPa and (●) 150 MPa; open symbols are: (□) controls and (o) initial germ numbers.

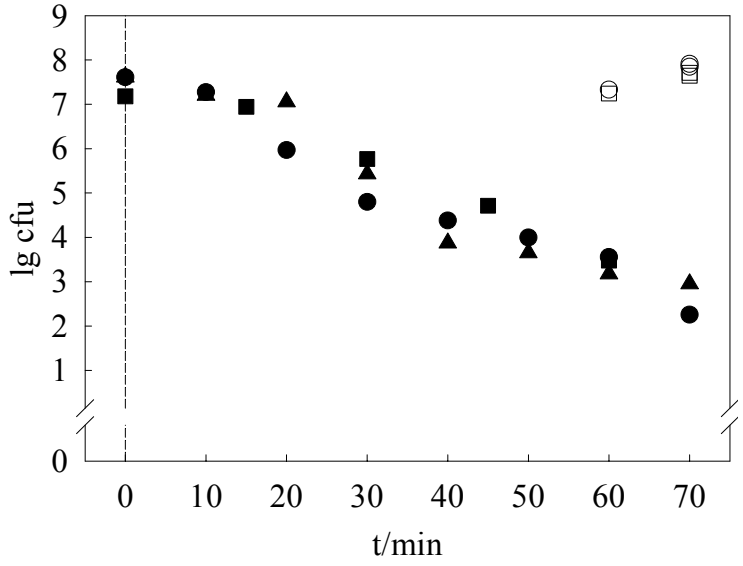

Fig. 5.6 Inactivation kinetics of *B. subtilis* spores at 60°C and three different pressures:(■)50 MPa; (▲) 100 MPa and (●) 150 MPa; open symbols are: (□) controls and (o) initial germ numbers.

Comparing the figures 5.5 and 5.6, it is to observed that at all pressures applied, an improved inactivation effect has been obtain by increasing the temperature from 30 to 60°C.

Also, a comparison of the Fig. 5.4 with Fig.5.6 shows that the shape of the inactivation curve is not altered by using different pressures in contrast to the influence of temperature.

The measured values of the inactivation rate constants (min^{-1}) for the pressure/temperature combinations studied are presented in Table 5.2. The data shown in Table 5.2 represent the mean value of at least three determinations.

Temperature / Pressure	25°C	30°C	40°C	50°C	60°C	70°C
50 MPa		0.0043			0.1070	
100 MPa	0.0100	0.0144	0.0399	0.0746	0.1284	0.3657
150 MPa		0.0319			0.1852	

Table 5.2 Inactivation rate constants values for the temperature/pressure ranges 25-70°C / 50-150 MPa

The values presented in table 5.2 confirm the fact that inactivation rate constants generally increase with increasing pressure and temperature.

5.2.2.1 Estimation of the kinetic parameters

Once the *k* values are estimated, the kinetic parameters characterizing the inactivation process (Ea, $\Delta V^{\neq}$ and k_{ref}) can be calculated. Fig.5.7 gives the determination of the activation energy related to the pressure/temperature range studied.

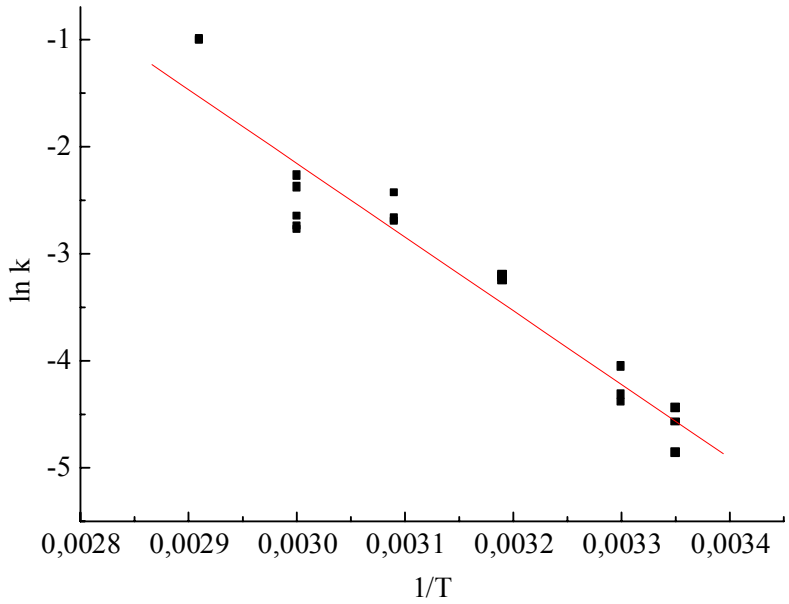

Fig. 5.7 Determination of the activation energy at 100 MPa for temperature between 25-70°C

In Fig 5.7 is to recognized that for each temperature at least three experiments were done. In some cases (for example for 60°C) more than three experiments were necessary to be done in

order to assure an accurate estimation. The value obtained for the activation energy is 57.244 ± 4.443 kJ/mol.

The activation volume at 30°C was determined by plotting ln*k* versus pressure as shown in Fig. 5.8.

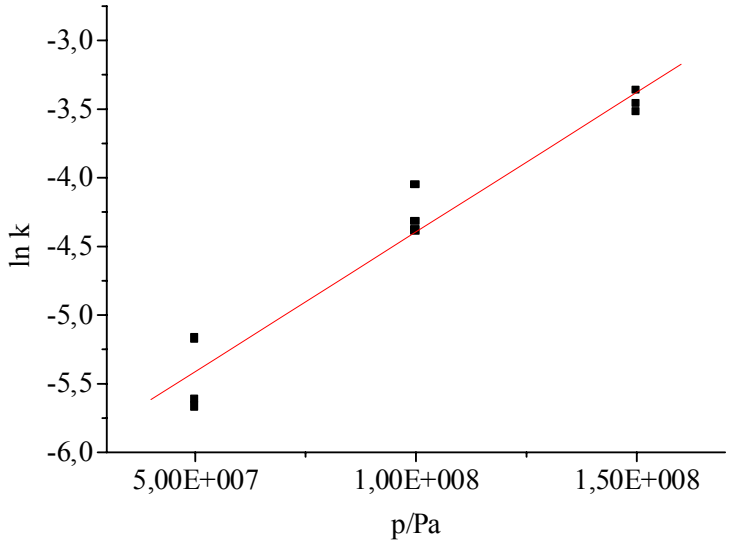

Fig.5.8 Determination of the activation volume at 30°C and three different pressures: 50, 100 and 150 MPa

The determined value of the activation volume is –51.277 ± 4.34 cm^3/mol.

Fig. 5.9 shows the determination of the activation volume at 60°C in the pressure range 50-150 MPa.

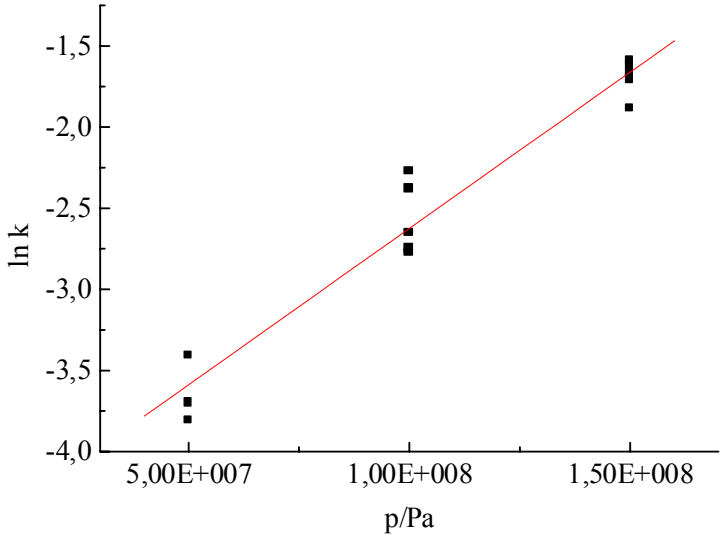

Fig. 5.9 Determination of the activation volume at 60°C and three different pressures: 50, 100 and 150 MPa

The value obtained for the activation volume is $-53.371 cm^3/mol \pm 0.368$.

The negative values of the activation volume show that inactivation of *B. subtilis* spores at the studied temperature/pressure combinations is favored by high pressure.

Using the equations 4.6 and 4.8 presented in Chapter 4, the k values at the reference temperatures and reference pressure were calculated. The values of k_{ref} are: 0.013690875 min^{-1} at 30°C respectively, 0.0937326 min^{-1} at 60°C for the pressure range 50-150 MPa.

The standard deviation of the estimated parameters were situated between 6.8-8.4% and the regression coefficients between 0.90-0.95.

5.2.3 Inactivation kinetics in the pressure range of 200-400 MPa at temperatures between 25-70°C

The determination of the inactivation rate constants was carried out at the pressure levels 200, 300 and 400 MPa (the 300 MPa level is the reference pressure) at temperatures from 25 to 70°C. The temperature range was divided, as before, in 2 domains with the same reference temperatures 30°C and 60°C, respectively. Fig.5.10 shows the inactivation kinetics of *Bacillus subtilis* spores at constant pressure (300 MPa) and different temperatures.

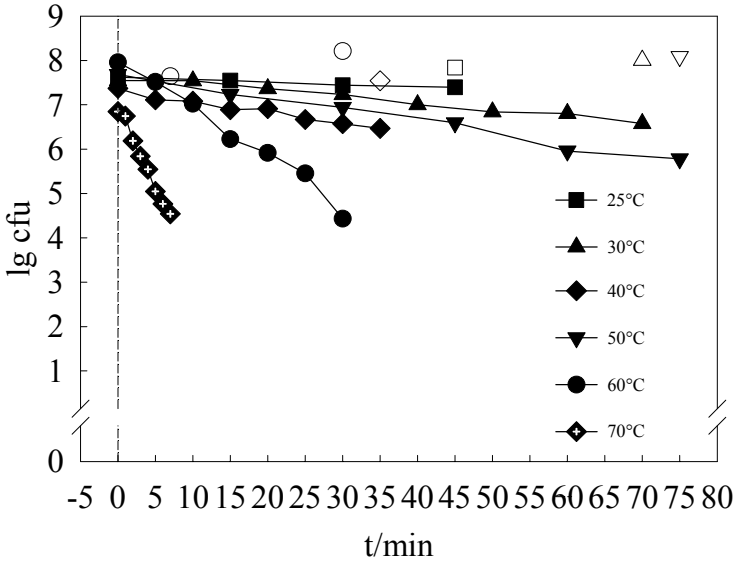

Fig. 5.10 Inactivation kinetic of *B. subtilis* spores at 300 MPa and different temperatures; open symbols are the initial germ numbers.

The inactivation kinetics at reference temperatures (30 and 60°C) and different pressures are represented in figures 5.11 and 5.12.

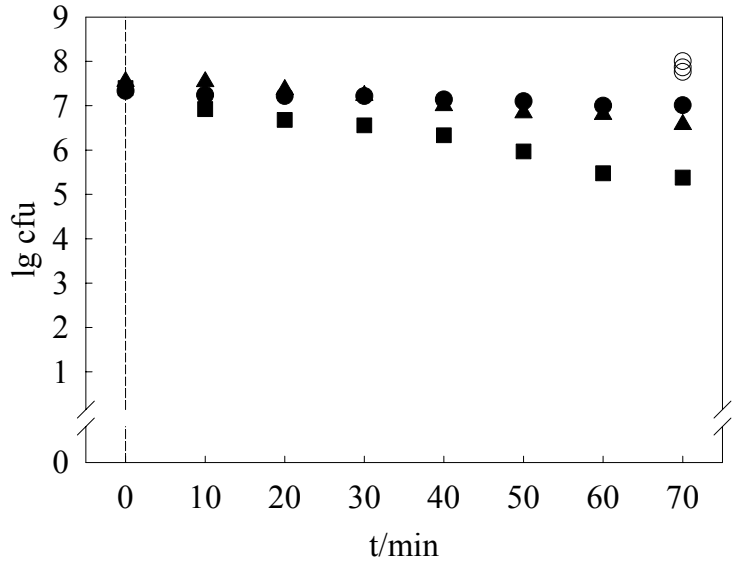

Fig. 5.11 Inactivation kinetics of *B.subtilis* spores at 30°C and three different pressures: (■)200 MPa; (▲) 300 MPa and (●) 400 MPa; open symbols are: (□) controls and (o) initial germ numbers.

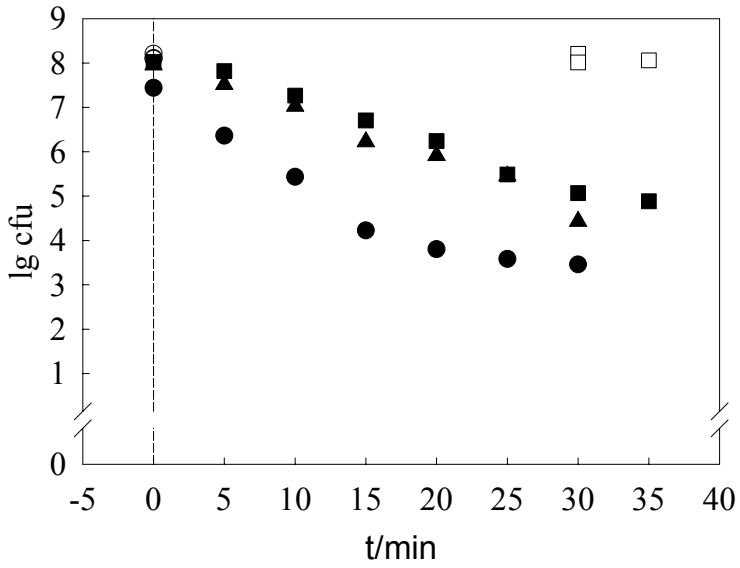

Fig. 5.12 Inactivation kinetics of *B. subtilis* spores at 60°C and three different pressures: (■)200 MPa; (▲) 300 MPa and (●) 400 MPa; open symbols are: (□) controls and (o) initial germ numbers.

Table 5.3 summarizes the measured values of the inactivation rate constants (min^{-1}) for the pressure/temperature combinations studied.

Temperature / Pressure	25°C	30°C	40°C	50°C	60°C	70°C
200 MPa		0.0603			0.244	
300 MPa	0.0109	0.0324	0.0565	0.0694	0.2641	0.8150
400 MPa		0.0122			0.3421	

Table 5.3 Values of the inactivation rate constants for the temperature/pressure range 25-70°C / 200-400 MPa

This table shows that at constant pressure the inactivation rates increased with increasing temperature.

On the contrary to the inactivation rate values obtained for 30°C in low pressure range (50-150 MPa), in the high pressure range these values have decreased with increasing the pressure level.

5.2.3.1 Estimation of the kinetic parameters

Using the estimated *k* values the kinetic parameters characterizing the inactivation process (Ea, $\Delta V^{\neq}$ and k_{ref}) in temperature/pressure ranges studied were calculated. Fig.5.13 represents the determination of the activation energy related to the pressure range 200-400 MPa at the temperatures between 25 to 70°C.

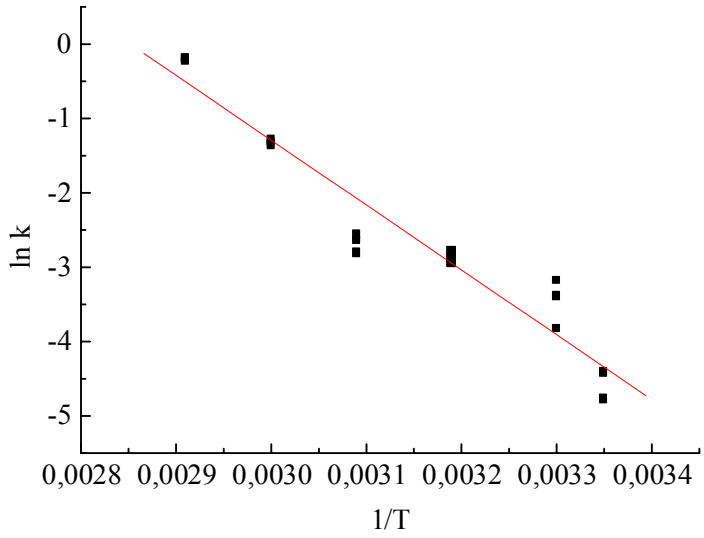

Fig. 5.13 Determination of the activation energy at 300 MPa for the temperature range 25-70°C

The calculated value of the activation energy is 72.508 kJ/mol ± 4.851. This value is higher that the one obtained at 100 MPa for the same temperature range.

The activation volumes at 30°C and 60°C, respectively, were determined by plotting ln*k* versus pressure as shown in figures 5.14 and 5.15.

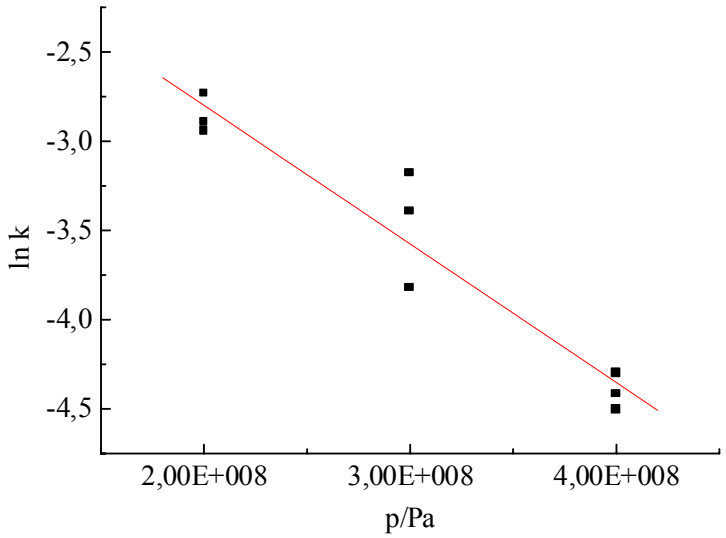

Fig. 5.14 Determination of the activation volume at 30°C and three different pressures: 200, 300 and 400 MPa

The calculated value for the activation volume is 19.565 $cm^3/mol \pm 2.174$.

In the pressure range 200 –400 MPa the value of the activation volume is positive, i.e. by increasing the pressure the velocity of the reaction decreases.

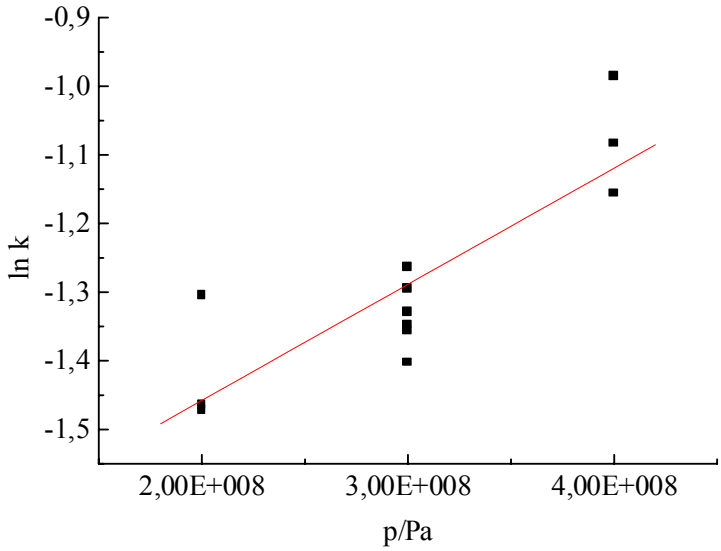

Fig. 5.15 Determination of the activation volume at 60°C and three different pressures: 200, 300 and 400 MPa

The determined value of the activation volume is – 4.687 $cm^3/mol \pm 0.931$.

The values of k_{ref} are: 0.024180589 min^{-1} at 30°C and 0.261461401 min^{-1} at 60°C, respectively, for the pressure range 200-400 MPa.

The standard deviation of the estimated parameters are situated between 6.6-19% and the regression coefficients between 0.71-0.93.

5.2.6 Calculation of rate constants using the first order model

For the calculation of the rate constants using the first order kinetic model, parameter estimates obtained by linear regression on inactivation data were used. These values are reviewed in Table 5.4.

Kinetic parameters	25-40°C 50-150 MPa	50-70°C 50-150 MPa	25-40°C 200-400 MPa	50-70°C 200-400 MPa
E_a (kJ/mol)	57.244		72.508	
$\Delta V^{\neq}$ (cm^3/mol)	-51.277	-53.371	19.565	-4.687
k_{ref} (min^{-1})	0.013691	0.093732	0.024180	0.261461

Table 5.4 Kinetic parameters characterizing the pressure/temperature ranges studied

Using the equation 4.10 presented in Chapter 4 the rate constants corresponding to the pressure/temperature ranges studied were determined. The rate constants were calculated for the whole come-up time including the time needed to compensate the adiabatic heating in one second time steps.

5.2.7 Reliability of the estimated kinetic parameters

A measure of the reliability of the kinetic parameters to predict the inactivation rate constants after pressure-temperature treatment was obtained by comparing the experimentally observed rate constants to the ones predicted by the model. A plot of the experimental *k* values versus *k* values calculated using the estimated parameters was made (Fig.5.16).

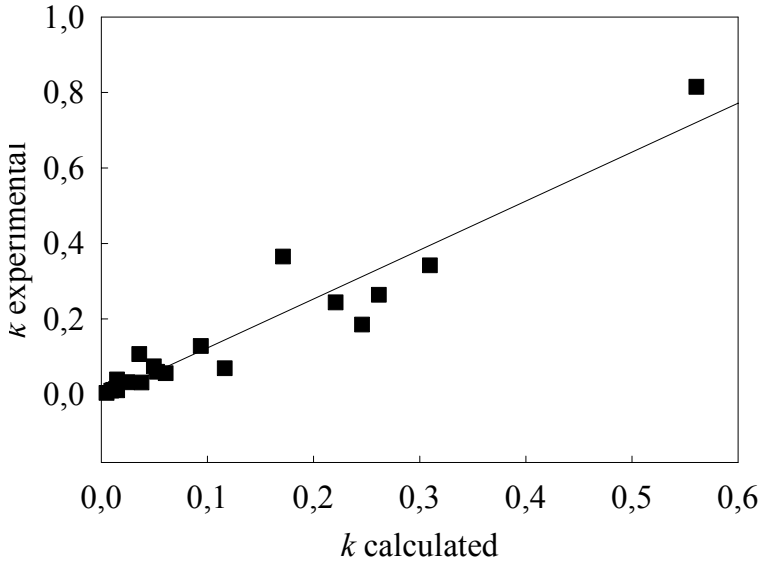

Fig.5.16 Correlation between the experimental and calculated *k* values

The divergence from the diagonal line can be seen as an indicator for the accuracy of the parameter estimation: the more the calculated and estimated values differ, the less precise the parameters are estimated. Fig. 5.16 shows a satisfactory correlation between these values i.e. the model accurately describes the dependence of the inactivation rate constant on pressure and temperature in the pressure-temperature domain studied.

The percentage error of the predicted values was generally situated between 0.9 and 50%. About 30% of the calculated values were higher than the experimental ones.

5.2.8 Estimation of the inactivation effect during the fast pressure build-up phase

The integral effect of the inactivation process under variable conditions over the fast pressure build-up and equilibration period was quantified. Figures 5.17 and 5.18 present the inactivation effect during the fast pressure build up phase at temperatures between 25 to 70°C in the pressure range 50 – 400 MPa.

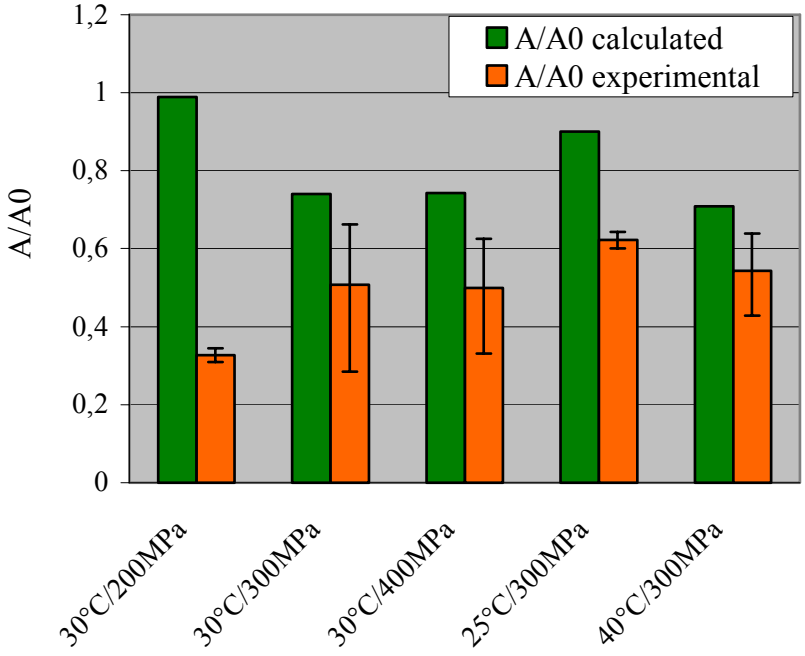

Fig 5.17 The effect of fast pressure build-up on the inactivation of *B. subtilis* spores in the temperature/pressure range 25-40°C and 200-400 MPa; A_0 is the initial spore number and A the surviving spore number at time zero

The ratio between the survivors spore number at *time zero* (the time moment at which the adiabatic heating is compensated and the constant conditions of pressure and temperature are established) and the initial spore number (A/A0) represents the experimental inactivation effect. The calculated inactivation effect was determined by numeric integration of the inactivation rate constant values that were calculated in one second time steps over the entire pressure build-up period.

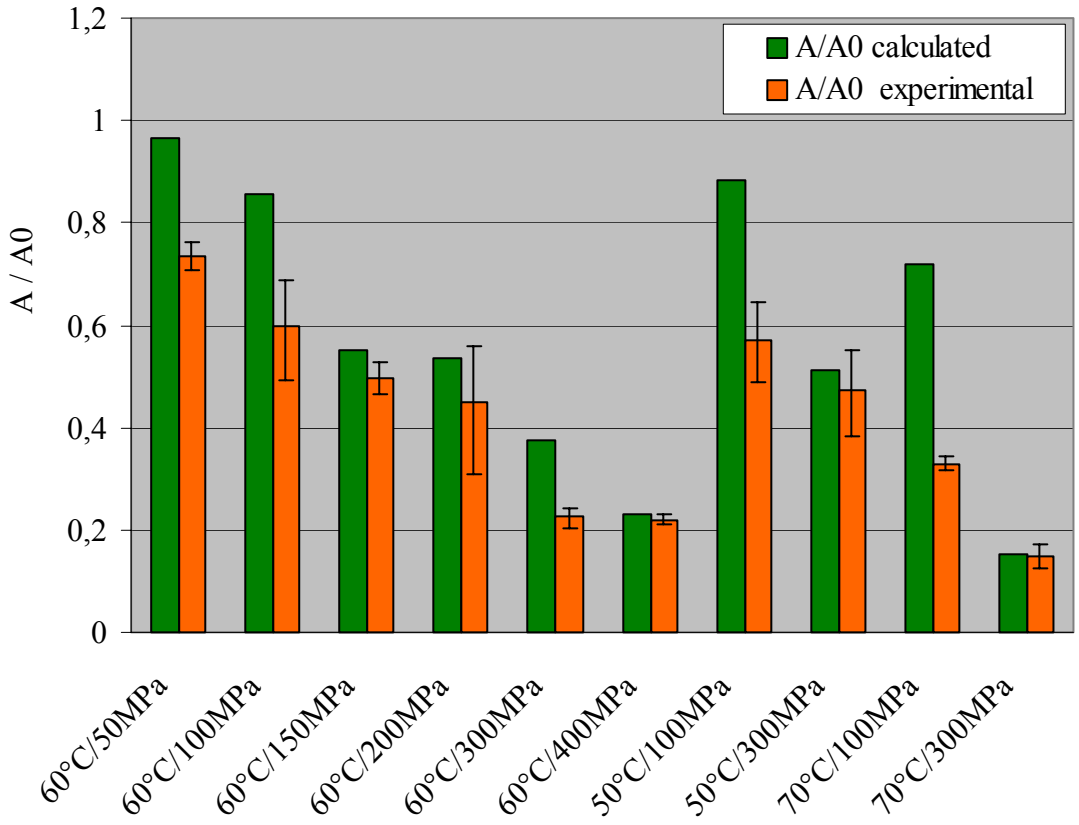

Fig 5.18 The effect of fast pressure build-up on the inactivation of *B. subtilis* spores in the temperature/pressure range 50-70°C and 50-400 MPa; A_0 is the initial spore number and A the surviving spore number at time zero

The asymmetric estimates for the error in A/A_0 represented in Fig. 5.17 and 5.18 were obtained by converting the error in $\lg(A/A_0)$ to an error of A/A_0.

From these figures it could be concluded that the fast pressure build-up has an additional effect on the inactivation of *Bacillus subtilis* spores.

5.3 Quantification of the inactivation effect during slow pressure build-up phase

This subchapter presents the effect of combined pressure and temperature on the inactivation of *B. subtilis* spores over the period of slow pressure increase. The pressure was slowly built-up with a controlled rate of 20 MPa/min. The time periods (in seconds) needed to reach the treatment pressures including the equilibration period are presented in Table 5.5.

	50°C	60°C	70°C
50 MPa		180	
100 MPa	359	346	420
150 MPa		510	
200 MPa		754	
300 MPa	1042	931	1014
400 MPa		1337	

Table 5.5 Come-up times (in s) for the pressure/temperature combinations studied after slow pressure build-up

5.3.1 Inactivation kinetics of Bacillus subtilis spores after slow pressure build-up

The inactivation of *Bacillus subtilis* spores was investigated in the pressure range 50 - 400 MPa and at temperatures from 50 to 70°C. At all temperature/pressure combinations studied the first order inactivation kinetic has occurred, excepting the combinations 60°C/50MPa, 60°C/100 MPa and 60°C/300 MPa, where the inactivation curves bend to biphasic behavior. The inactivation behavior is represented in figures 5.19 and 5.20.

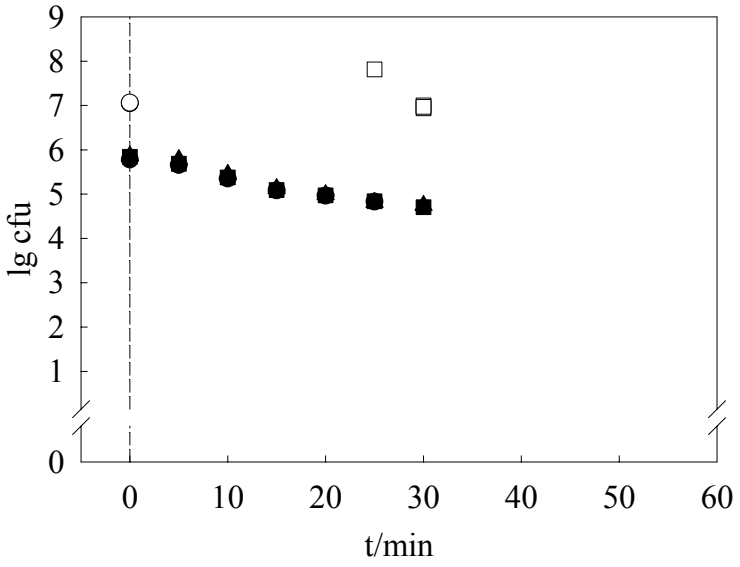

Fig. 5.19 First order inactivation at 50°C/100 MPa (in triplicate); open symbols are: (□) control and (o) the initial germ numbers

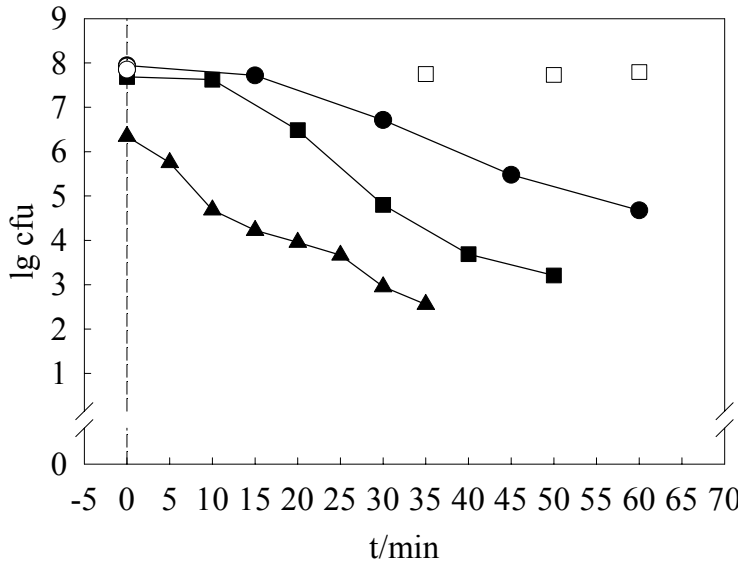

Fig. 5.20 Biphasic inactivation kinetics of *B. subtilis* spores at 60°C and three different pressures: (●) 50 MPa, (■) 100 MPa and (▲) 300 MPa; open symbols are: (□) controls and (o) initial germ numbers

Fig. 5.20 shows clearly the biphasic behaviour which appears in the same pressure range (excepting the pressure level 300 MPa) as by fast pressure build-up process. The curve appears to be composed of two linear segments. As well as for the fast pressure build-up process the inactivation curve was split up into a two step reaction and the inactivation rate constants were determined for the first segment of the curve.

5.3.2 Inactivation kinetics in the pressure range of 50-400 MPa at temperatures between 50-70°C

The determination of the inactivation rate constants was performed at temperatures between 50°C to 70°C in the pressure range 50-400 MPa. The pressure range studied was divided in two domains: the low (50–150MPa) and the high pressure domain (200–400 MPa).

The reference pressure was set at 100 MPa and 300 MPa, respectively, and the reference temperature was 60°C.

Figures 5.21 and 5.22 represent the inactivation kinetics of *B. subtilis* at the reference pressure levels and different temperatures.

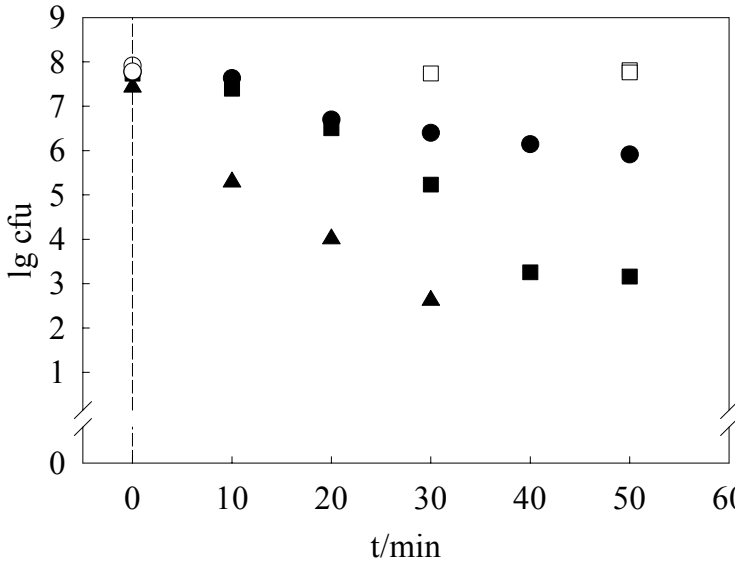

Fig. 5.21 Inactivation kinetics of *B. subtilis* spores at 100 MPa and different temperatures: (●) 50°C, (■) 60°C and (▲) 70°C; open symbols are: (□) controls and (o) initial germ numbers.

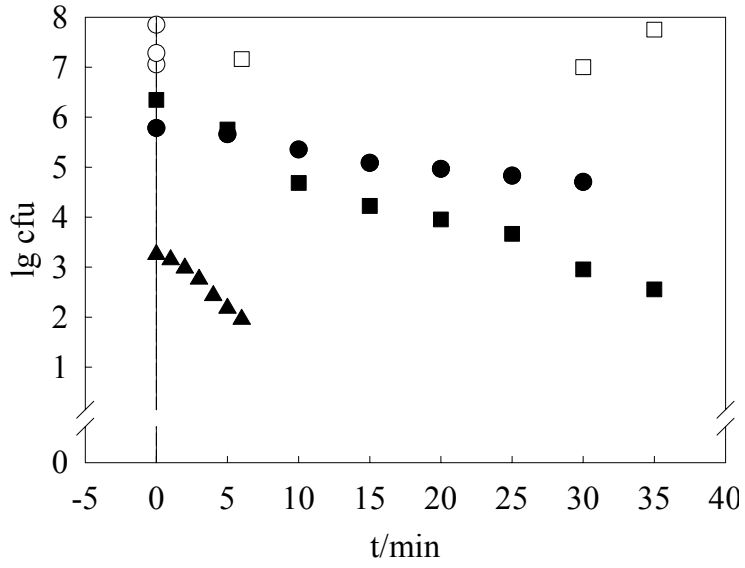

Fig.5.22 Inactivation kinetics of *B. subtilis* spores at 300 MPa and different temperatures: (●) 50°C, (■) 60°C and (▲) 70°C; open symbols are: (□) controls and (o) initial germ number.

Fig.5.23 illustrates the inactivation kinetics at the reference temperature and different pressure levels.

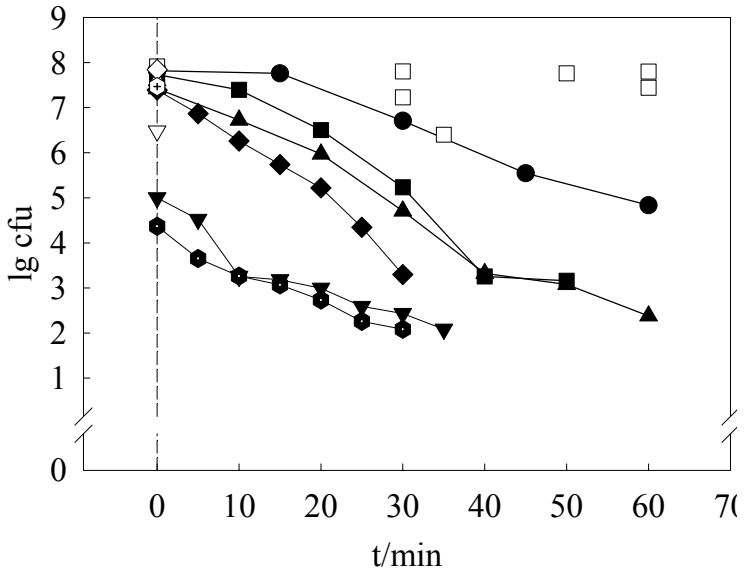

Fig. 5.23 Inactivation kinetics of *B. subtilis* at 60°C and different pressure levels: (●) 50 MPa,(■) 100 MPa (▲) 150 MPa, (♦) 200 MPa, (▼) 300 MPa, (✪)400 MPa; open symbols are: initial germ numbers and (□) controls.

Fig. 5.23 shows a drastic decrease of the initial spores number after the pressure increase period (at t=0). The measured values of the inactivation rate constants (min^{-1}) for the temperature/pressure range 50-70°C /50-400 MPa are presented in Table 5.6. The data shown in Table 5.6 represent the mean value of three determinations.

Temperature / Pressure	50°C	60°C	70°C
50 MPa		0,0871	
100 MPa	0,1120	0,1469	0,3668
150 MPa		0,1996	
200 MPa		0,2896	
300 MPa	0,0901	0,1617	0,5366
400 MPa		0,1388	

Table 5.6.Values of the inactivation rate constants (min^{-1}) for the temperature/pressure range 50-70°C/50-400 MPa

5.3.2.1 Estimation of the kinetic parameters

Using the measured values of the inactivation rate constants, the kinetic parameters characterizing the inactivation process after slow pressure build up (Ea, $\Delta V^{\neq}$ and k_{ref}) were calculated. Fig.5.24 and 5.25 show the determination of the activation energy for the pressure/temperature range studied.

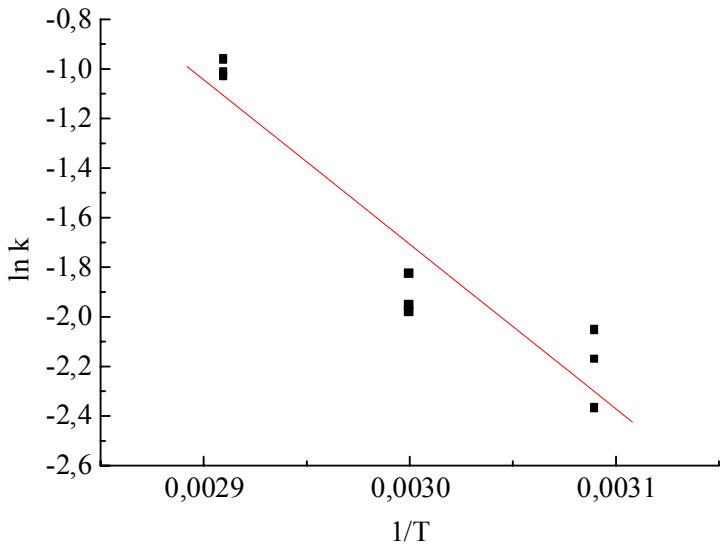

Fig. 5.24 Determination of the activation energy at 100 MPa for the temperature range 50-70°C

The determined value of the activation energy is 55.184 kJ/mol ± 7.421.

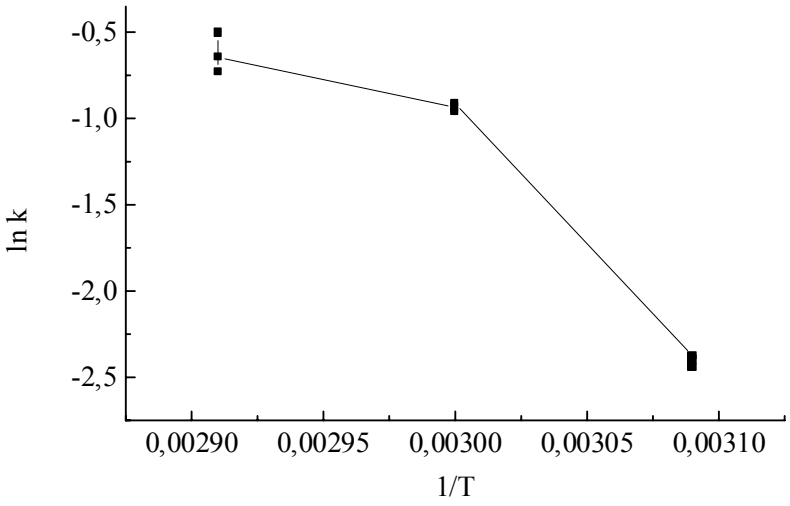

Fig.5.25 Determination of the activation energy at 300 MPa for the temperature range 50-70°C

The values obtained for the activation energy are: 28.650 kJ/mol ± 6,134 for the first part of the regression and 135.848 kJ/mol ± 2,265 for the second part.

Because the Eyring relationship was not valid over the entire pressure range studied, the pressure dependence of k could be described by a third-degree polynomial model:

$$\ln k = a + bP + cP^2 + dP^3$$

This is visualized in Fig.5.26:

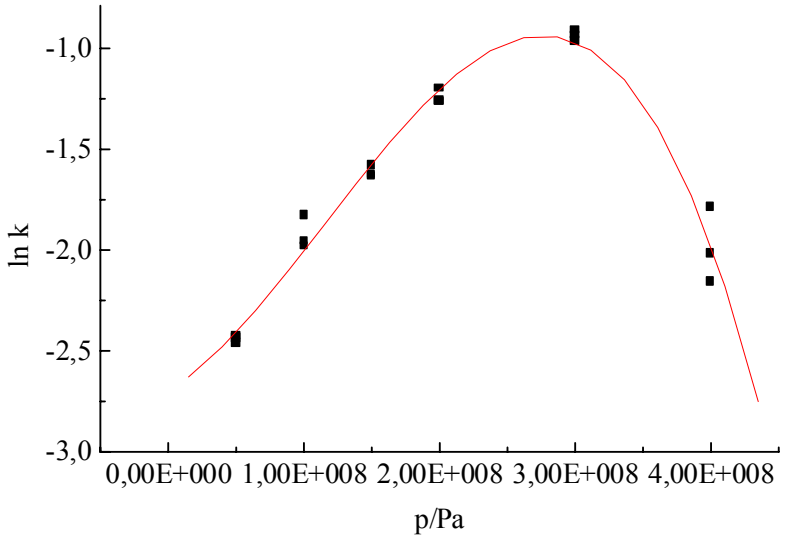

Fig. 5.26 Determination of the activation volume at 60°C in the pressure range 50-400 MPa

The equation derived from the polynomial regression is:

$$y = -2{,}70011 + 4{,}1788\ e^{-9}\ x + 3{,}90461\ e^{-17}\ x^2 - 1{,}1246\ e^{-25}\ x^3$$

Solving the equation above, the values of the activation volume for the entire pressure range were calculated. These values are presented in Table 5.7

Pressure (MPa)	$\Delta V^{\neq}$ (cm^3/mol)
100	-23.860
150	-22.994
250	-7.245
276.2	0
300	7,638
350	27.195
400	51.424

Table 5.7 Values of the activation volume for the pressure range 50-400 MPa

The elliptical curve represented in Fig.5.26 is similar found for the pressure denaturation of proteins.

The values of k_{ref} are: 0.157469924 min^{-1} for the pressure range 50-150 MPa and 0.387650225 min^{-1} for the pressure range 200-400 MPa, respectively.

The standard deviation of the estimated parameters are situated between 8.2 - 13.4% and the regression coefficients between 0.71-0.93

5.3.3 Calculation of rate constants using the first order model

For the calculation of the rate constants using the first order kinetic model, parameter estimates obtained by linear regression on inactivation data were used. These values are reviewed in Table 5.4.

Kinetic parameters	50-70°C 50-150 MPa	50-70°C 200-400 MPa
E_a (kJ/mol)	55.184	28.650 135.848
$\Delta V^{\neq}$ (cm^3/mol)	see Table 5.7	
k_{ref} (min^{-1})	0.157469924	0.387650225

Table 5.8 Kinetic parameters characterizing the pressure/temperature ranges studied

Using the equation 4.10 presented in Chapter 4 the rate constants corresponding to the pressure/temperature ranges studied were calculated. The rate constants were determined for the whole come-up time including the time needed to compensate the adiabatic heating in one second time steps.

5.3.4 Reliability of the estimated kinetic parameters

As well as for the fast pressure build-up process, in order to analyze the quality of the predicted kinetic parameters a plot of the experimental *k* values versus calculated *k* values was made also for the slow pressure build-up process. The correlation degree of these values is represented in Fig. 5.27.

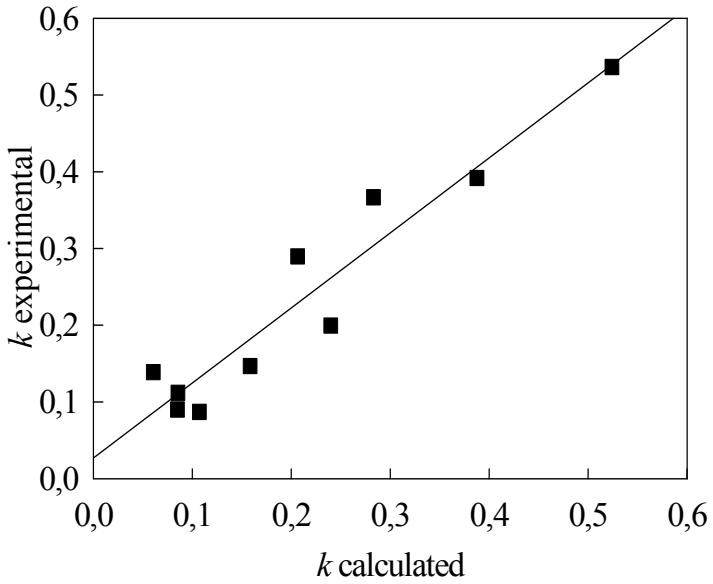

Fig.5.27 Correlation between the experimental and calculated *k* values

The values represented in Fig.5.27 are the mean value of three replications. The correlation between calculated and experimental values is satisfactory, the percentage error was predominantly situated between 0.27 and 38.27%, whereas only two values (60°C/400 MPa; 70°C/300 MPa) characterized by a percentage error higher than 100% were found. It could be concluded that the pressure and temperature dependence of the inactivation rate constants are properly described by the model used.

5.3.5 Estimation of the inactivation effect during the slow pressure build-up phase

The inactivation effect during the slow pressure build-up phase at temperatures between 50 to 70°C in the pressure range 50 – 400 MPa was quantified. In Fig 5.27 the inactivation effect is represented.

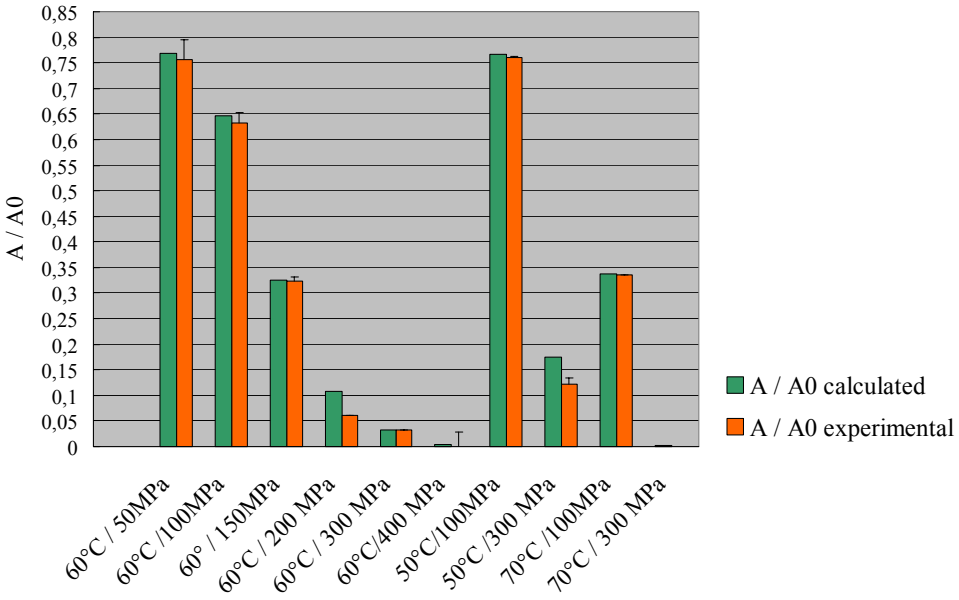

Fig. 5.28 The effect of slow pressure build-up on the inactivation of *B. subtilis* spores at temperatures between 50 and 70°C in the pressure range 50-400 MPa; A_0 is the initial spore number and A the surviving spore number at time zero

Because some values cannot be viewed on the above graphic representation in the next figure (Fig.5.29) the experimental and calculated inactivation effects are represented as lg A_0/A.

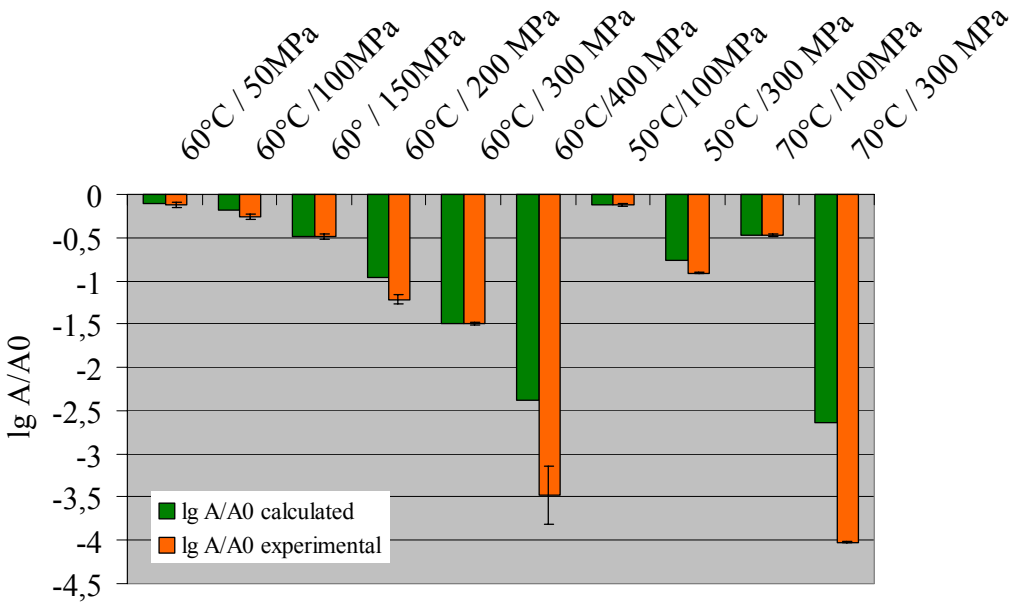

Fig. 5.29 The effect of slow pressure build-up on the inactivation of *B. subtilis* spores at temperatures between 50 and 70°C in the pressure range 50-400 MPa; lgA/A_0 is the amount of inactivated spores during pressure increase.

From Figs. 5.28 and 5.29 follows that a slow rate of pressure build-up has no additional effect on *Bacillus subtilis* spore inactivation. The experiments at 60°C/400 MPa and 70°C/300 MPa are characterized by a high error of the experimental values.

Chapter 6 Discussion

The present work is an attempt to ascertain the impact of the compression time (i.e. time required to attain a given pressure, or come-up time) on the high pressure inactivation of *Bacillus subtilis var. niger* spores in physiological solution at different pressure and temperature levels. The pressure and temperature ranges were selected taking in account a possible further application for an economically feasible process.

Until now, no study concerning the contribution of the dynamic pressure/temperature conditions during pressure increase to the spores inactivation was made. Rodriques et al. (2001) have studied the influence of the come-up time on the inactivation of *Listeria innocua* inoculated into whole milk at four selected pressures 448, 517, 586 and 655 MPa. They found that at 448 MPa the inactivation does not depend on the come-up time whereas at the other three pressures investigated the longer the come-up time the higher the microbial inactivation observed. In their study they do not take in account the compensation period needed for the temperature to evolve to its preset value.

The effect of initial concentration of spore suspension on their inactivation by high pressure was studied. Figure 5.1 shows the kinetics of inactivation for two spore populations with different initial concentration of 10^7 and 10^8 cfu/ml. The number of surviving spores per ml, shown as colony forming units, is plotted logarithmically on the ordinate; the time on the abscissa. The runs are perfectly parallel to each other. Clearly, the initial concentration does not influence the spore inactivation process. In the literature, contradictory statements related to the influence of initial concentration on spore inactivation are made. For example, Furukawa et al. (2002) have found that the inactivation rates of *Bacillus subtilis* spores decreased as the initial concentration of spore suspension increased. This behavior is considered to be caused by the spore clumps which were formed through pressurization. It is known that clumping induces a delay that depends on the number of spores per clump but will not lead to increments in the number of survivors such as those induced by spore activation. To prevent the formation of spore clumps under pressurization an surface-active agent (Tween 80) was added during the preparation of the spore suspension.

Evaluation of the inactivation process

In order to be able to provide an appropriate kinetic model describing the combined effect of pressure and temperature on the inactivation rate constant during high pressure come-up time,

the inactivation curves were evaluated. With some exceptions, at all temperature/pressure combinations studied straight lines can be drawn through the data indicating first order reactions during the time periods investigated (Fig. 5.2 and 5.19). The traditional first-order model for inactivation of microorganisms assumes occurrence of a linear survival curve. At some temperature/pressure combinations studied (60°C/ 50, 100 and 150 MPa after fast pressure build-up; 60°C/50, 100 and 300 MPa after slow pressure build-up) the survival curves appear not to be linear. These inactivation curves (Fig.5.3) showed a sigmoid asymmetric shape when plotted in logarithmic scale. These logarithmic S-shaped curves indicate a certain time dependent resistance mechanism. The inactivation curves can be divided into two parts: an initial lag phase with approximately no inactivation and an inactivation phase accompanied by more or less pronounced tailing.; each part follows a first order inactivation rate. The occurrence of a two-phase inactivating curve is differently explained by the researchers. This phenomenon which is not only limited to the high pressure inactivating but also to thermal processing is mostly explained by the presence of germs in different growth phases (Van Almsick et al., 1995).

Difficulties with this explanation model arise however with inactivating of spores. If the germ are in different growth phases however it might not come then to the occurrence of only two different populations. Therefore it seems more plausible that during the high pressure inactivating two different inactivating mechanisms determine the microorganisms death. Ludwig et. al. (1999) assumed a dependence of the inactivation mechanism on the pressure level applied. Heinz (1996) stated that the transition of the bacterial cell from a stable state A (at the beginning of the lag phase) to a metastable intermediate state B (at the end of the lag phase) is assumed to take place after a certain period of time, primarily dependent on the temperature and the pressure applied. This statement is confirmed by the non-linear survival curves that occurred in both cases (fast and slow pressure build-up) at the same pressure levels (50, 100 and 150 MPa) and always at 60°C.

Summarizing, it could be said that if first order kinetics is found it remains restricted to a part of the whole temperature range only.

Estimation of the kinetic parameters

The most common method to determine kinetic parameters is to assume first-order reaction and conduct three or more isobaric-isothermal tests. The 2-step regression method was used to determine the kinetic parameters. This method is to plot the number of spore survivors (as lg

cfu) versus time to obtain the inactivation rate constants k_{exp} corresponding to each temperature/pressure combination and ln$k$ versus 1/T (absolute temperature) and pressure to obtain the activation energy and activation volume, respectively.

In the cases where the inactivation was not linear the rate constants were calculated from the slope of the first linear part of the curve (the initial lag phase).

For a better overall view, the experimental inactivation rate constants (min^{-1}) obtained are reviewed in the table 6.1. Comparison of the inactivation rate constants values after fast and slow pressure build-up shows that at pressures between 50 and 150 MPa the inactivation rate constants increase with increasing temperature and pressure, i.e. the inactivation occurred more rapidly at higher temperature and higher pressure suggesting the synergistic effect of pressure and temperature.

Treatment conditions	*Fast pressure build-up*						*Slow pressure build-up*		
	25°C	**30°C**	**40°C**	**50°C**	**60 °C**	**70 °C**	**50 °C**	**60 °C**	**70 °C**
50 MPa		0.0043			0.1070			0.0871	
100 MPa	0.0100	0.0144	0.0399	0.0746	0.1284	0.3657	0.1120	0.1468	0.3668
150 MPa		0.0319			0.1852			0.1996	
200 MPa		0.0603			0.2440			0.2896	
300 MPa	0.0109	0.0324	0.0565	0.0694	0.2641	0.8150	0.0901	0.1617	0.5366
400 MPa		0.0122			0.3421			0.1388	

Table 6.1 Experimental rate constants (min^{-1}) obtained after fast/slow pressure build-up

At 30°C in the case of fast pressure build-up and 60°C for slow pressure build-up respectively, in the high pressure range (200-400 MPa) a clearly antagonistic effect of pressure and temperature was observed. Increasing pressure caused a decrease of the inactivation rate constants. The antagonistic effect of temperature can be explained by the fact that higher temperatures cause a volume increase through dilatation. The principle of microscopic ordering states that at constant temperature an increase in pressure increases the degree of ordering of the molecules. On the basis of this principle pressure and temperature are expected to exert an antagonistic effect in molecular terms.

The effect of temperature and pressure on the inactivation rate constants is expressed in terms of activation energy (E_a) and activation volume ($\Delta V^{\neq}$), respectively. Activation

energies at different constant pressures and activation volumes at different constant temperatures, calculated according to the Arrhenius and Eyring equations respectively, are overviewed in Table 6.2.

Fast pressure build-up				
Kinetic parameters	**25-40°C 50-150 MPa**	**50-70°C 50-150 MPa**	**25-40°C 200-400 MPa**	**50-70°C 200-400 MPa**
E_a (kJ/mol)	57.244		72.508	
$\Delta V^{\neq}$ (cm^3/mol)	- 51.277	- 53.371	19.565	-4.687
k_{ref} (min^{-1}) (p_{ref}/T_{ref})	0.013690875 (30°C/100 MPa)	0.0937326 (60°C/100 MPa)	0.024180589 (30°C/300 MPa)	0.261461401 (60°C/300 MPa)
Slow pressure build-up				
E_a (kJ/mol)		55.184		28.650 135.848
$\Delta V^{\neq}$ (cm^3/mol)		See Table 5.7		
k_{ref} (min^{-1}) (p_{ref}/T_{ref})		0.157469924 (60°C/100 MPa)		0.387650225 (60°C/300 MPa)

Table 6.2 Kinetic parameters characterizing the *B. subtilis* spore inactivation after fast/slow pressure increase

Fast pressure build-up

From the Table 6.2 it can be seen that by the fast pressure build-up process the activation energy value increases with increasing pressure. Corroborating these values with the values of the inactivation rate constants it could be concluded that at pressures higher than 150 MPa the inactivation rate constant is more temperature-sensitive.

For the whole temperature range studied (25-70°C), in the low pressure domain the values of the activation volume are negative and seem to be constant (-53.371 to -51.277 cm^3/mol); these negative values show that the inactivation process is favored by high pressure in the temperature/pressure domains investigated. In the high pressure range at temperatures between 25-40°C the value of the activation volume is first positive (19.565 cm^3/mol) afterwards negative (- 4.687 cm^3/mol). The pressure dependence of the ln*k* at 30 and 60°C could be better visualized in the Figures 6.1 and 6.2.

At temperatures between 25-40°C and for the pressure range 50-150 MPa, the value of the activation volume is – 51.277 cm^3/mol, then increases and reaches zero at about 250 MPa

and afterward becomes positive (19.565 cm^3/mol) in the pressure range 200- 400 MPa. Thus, at low temperatures and high pressures the inactivation process is slowed down by pressure.

At temperatures between 50-70°C for the whole pressure range investigated the value of the activation volume is negative but increases drastically from a value of –53.371 to –4.687 cm^3/mol.

All these values of the activation volume are in the order of chemical reactions. It is worth to underline that 200 MPa seems to be the threshold pressure where the kinetic parameters drastically change.

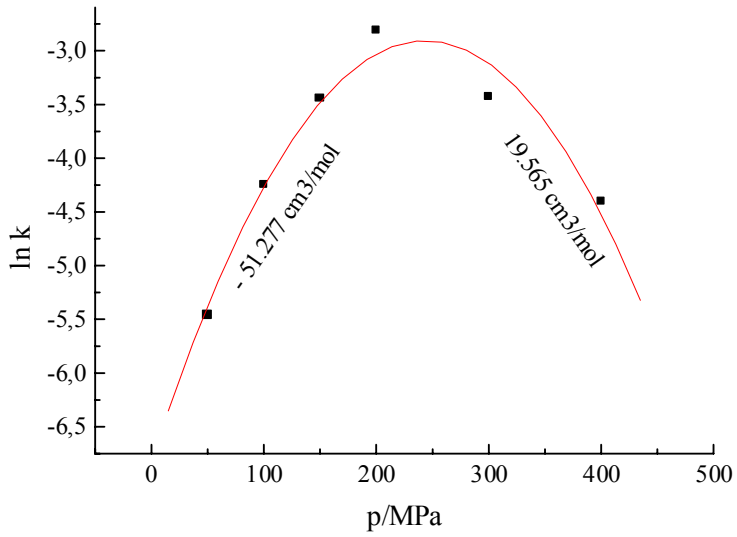

Fig.6.1 Pressure dependence of ln*k* at 30°C after fast pressure build-up

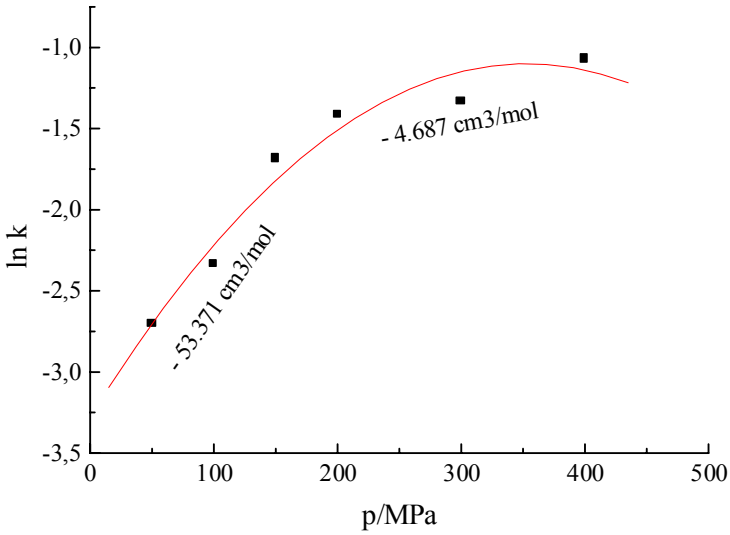

Fig.6.2 Pressure dependence of ln*k* at 60°C after fast pressure build-up

Fig.6.1 and 6.2 have some common features: **(a)** The variation of the activation volume value over the pressure/temperature range studied resemble those found for the pressure denaturation of proteins (see Fig.3.1). This similarity may imply that the inactivation of microorganisms is due to interference with some critical life processes such as enzyme reactions or protein aggregation;

(b) At temperatures between 25 to 70°C, in the low pressure domain it seems that the same life processes are affected, whereas in the high pressure domain different targets are damaged. It can be stated that the decisive step on spore inactivation happens on the molecular level.

Interpretation of thermodynamic constants calculated for biological systems is difficult because they represent the net effect from a possible multiplicity of reactions.

Slow pressure build-up

Table 6.1 gives all the k_{exp} values obtained after slow pressure build-up process. As expected in the low pressure domain the inactivation rates increase with increasing pressure and temperature and the values are more or less different from those obtained after fast pressure build-up process for the same temperature/pressure ranges. The ratio $\lg(A_0/A)$ giving the

amount of the inactivated spores during pressure build-up time is in the low pressure range similar with that obtained after fast pressure build-up whereas in the high pressure range the inactivation is much higher than for the fast pressurization.

The explanation is simple: for example to perform an experiment at 60°C and 400 MPa the time needed to reach the desired pressure including the equilibrating period was 10.7 min after fast pressure increase and 22.2 min after slow pressure increase. It has been proved that pressure-induced germination of *Bacillus subtilis* spores is best when pressures between 60 and 150 MPa are used (Sojka, 1997). Moderate pressure leads to germination of dormant spores whereas high pressure results in inactivation of germinated specimens. Increasing the pressure with a rate only of 20 MPa/min the spores remain for a longer time at pressures that favor the germination and therefore the inactivation. Only the germinated specimens are inactivated since dormant spores are unassailable to high pressure of 500 MPa (Sojka, 1997). It is to presume that for treatments at pressures between 50 and 150 MPa during the period of pressure increase the spores are germinating whereas for treatments at pressures above 150 MPa during the period of pressure build-up also the inactivation process occurred.

This leads to presume that building up slowly the pressure is an important factor for inducing germination.

It is known that pressure-induced germination is followed by the release of dipicolinic acid and amino acids in the surrounding medium. This could be used to test the given explanation.

In contrast to the process of slow pressure increase, for the fast pressure build-up process, germination cannot take place during the period of pressure build-up because it is restricted to the time under pressure. Sojka (1997) has found that at 50°C/110 MPa 6 min are sufficient to release all the dipicolinic acid from the spores. In this work, for the experiment at 50°C the pressure level of 100 MPa is reached in ca. 15 seconds and the equilibrating period is about 3.2 min.

In the high pressure range the same antagonistic effect of pressure and temperature was observed as we had found for the fast pressure build-up process at 30°C, but in this case at 60°C. Increasing pressure caused a decrease of the inactivation rate constants.

The value of the activation energy in the low pressure domain is on the same level as the one calculated after fast pressure build-up process (Table 6.2). In the high pressure domain the activation energy is 28.650 kJ/mol above and 135.848 kJ/mol below 60°C (see Fig.5.24). The threshold temperature is 60°C. It is not easy to define which molecular process is perturbed to give rise of such an abrupt change in the slope of the Arrhenius plot. A change

in activation energy with temperature indicates a shift in the controlling mechanism of reaction. Recalling that a higher activation energy represents a more temperature sensitive reaction, a rise in E_a value with temperature indicates that the controlling mechanism has shifted to an alternate or parallel path. The simplest explanation for such a curvature is that inactivation occurs by more than one mechanism. At temperatures below 60°C, inactivation is the results of some processes such as protein unfolding, with a high activation energy. At temperatures above 60°C some other processes with much lower energy take place.
The occurrence of a sharp break in the slope of the Arrhenius plot for enzymes has been noted in several cases (Dixon &Webb, 1964). Ceuterick et al. (1978) showed that phase transition in lipids are responsible for the abrupt change in the activation energy of *Azotobacter* nitrogenase.

Because of the observed antagonistic effect of pressure and temperature the Eyring relation, expressing the pressure dependence of the inactivation rate constant at constant temperature, was not linear in the high pressure domain. Therefore, for the estimation of the activation volume as illustrated in Fig.5.25 a third-degree polynomial model was used. Table 5.7 (Chapter 5) shows the calculated values of the activation volume for the entire pressure range investigated. The value of the activation volume increases from negative values in the low pressure domain reaches the zero value at 276.2 MPa and is positive in high pressure range.

Reliability of the predicted kinetic parameters

In order to check the accuracy of the predicted kinetic parameters, the experimental *k* values were compared with the *k* values calculated using the estimated parameters. A satisfactory correlation between these values was found for both cases, fast and slow pressure build-up, and therefore the chosen model accurately describes the influence of temperature and pressure on the inactivation rate constant.

Calculation of rate constants during come-up time

Once the isobaric-isothermal parameters are determined they are used to predict the inactivation rate constants during the pressure build-up which is an non-isobaric-isothermal process with a dynamic temperature/pressure profile. The *k* values during the pressure build-up were calculated using the predicted kinetic parameters in one second steps. Using these

constants the inactivation effect during come-up time was quantified by numeric integration of the equation 4.12 (see Chapter 4).

Estimation of the inactivation effect during pressure build-up phase

Fast pressure build-up

From Figures 5.17 and 5.18 it is easy to see that the fast pressure build-up has an additional effect on the *Bacillus subtilis* spore inactivation. In the low temperature domain (25-40°C) for all the temperature/pressure combinations studied this additional effect is observed. It is much more accentuated at pressures below 200 MPa (30°C/200 MPa; 50°C/100 MPa; 70°C/100 MPa). In some cases at pressures above 200 MPa, this effect is within the range of the experimental error (60°C/400 MPa; 50°C/300 MPa; 70°C/300 MPa).

Slow pressure build-up

Figs.5.28 and 5.29 show the inactivation effect during slow pressure build-up. No additional effect due to the type of the pressure increase is observed.

At present it is difficult to explain why and how the dynamic pressure/temperature profile during fast pressure build-up enhances the inactivation of *Bacillus subtilis* spores. This is mainly due to the limited data available for physico-chemical properties of spores under pressure. The literature data show that the bacterial membrane could be the primary target for high pressure by disorganization among membrane lipids. (Wong 1988; Macdonald 1992). Biological membranes consist of a complicated combination of lipids and proteins. Most of the lipids in the membrane are phospholipids.

Phospholipids spontaneously form a bilayer in a watery environment producing a spherical structure. They arrange themselves so that the polar heads are oriented towards the water and the fatty acid tails are contained within the membrane bilayer. The fatty acid tails are flexible, causing the lipid bilayer to be fluid. This makes the cell flexible. The state of the lipid molecules which surround the membrane proteins affect indirectly the protein function. At the transition temperature, pressure converts the liquid crystalline (physiological state) state to a gel state.

It is possible that the fast pressure build-up induces a strong pressure/temperature gradient on the lipid bilayer causing the gel formation which leads to a disruption of biological functions.

Kato and Taniguchi (2002) have studied the pressure-induced changes of biological membranes using Na^+/K^+ -ATPase as a model system of protein and lipid membrane. The activity showed a three-step change: at pressures ≤ 100 MPa a decrease in the membrane fluidity of lipid bilayer and a reversible conformational change in transmembrane protein is induced; pressures of 100-220 MPa cause a reversible phase transition in part of the lipid bilayer from the liquid crystalline phase to the gel phase and dissociation in the protein subunits; pressures ≥ 220 MPa irreversibly destroy and fragment the membrane structure due to protein unfolding.

Ichimori (2000) reached an interesting conclusion that contact faces of lipid and membrane-penetrating proteins are reversibly separated to produce tunnels or holes at 100-250 MPa, followed by disordered breakdown of the membrane system including protein denaturation at 300 MPa or higher.

These findings provide in part an explanation for the drastic changes of the parameter values around 250 MPa for fast pressure increase and 275 MPa for slow pressure increase, respectively.

In the case of slow pressure increase, the pressure was built-up very slowly (20 MPa/min) and the temperature increases due to adiabatic heating was max. 3°C above the treatment temperature and it might be presumed that the phase transition of lipid bilayers and/or proteins during come-up time was reversible and the initial state was someway recovered.

By the fast pressure increase process the temperature increase was ca. 20°C above the treatment temperature and this fact may produce the melting of lipids and subsequently the protein denaturation. It was found that the additional inactivation effect observed by fast pressure increase is much more accentuated at pressures below 200 MPa. Lesser pressures affect the inactivation by mechanisms which are uncertain.

Furthermore, the inactivation kinetics results raise an interesting question: Why are not all the cells destroyed after pressurization if the membrane is affected by pressure?

Although high hydrostatic pressure treatments are considered to be isostatic (i.e., equal pressure at every point of the treatment vessel) the cellular damage is not equally withstood by all the cells, suggesting that more resistant or less damaged cells are present in the pressurized spore population. With regard to membrane integrity the bacterial population might be formed by two different subpopulations: a small one whose cells membrane was not seriously damaged and a second one, more susceptible to high pressure especially to the dynamic changes of pressure/temperature during fast pressure increase.

Chapter 7 Error consideration

The main error sources of the high pressure treatment come of the evaluation of the pressure-treated samples. Different sources of error are to be considered.

Errors by the determination of the viable spore counts

The number of spores surviving the high pressure treatment was determined using the spread plate method. The decimal dilution series prepared were plated on agar dishes in triplicates. The average of these three values was used for the graphic representations. The volume error of the Eppendorf pipettes used to obtain the dilution stages contain the largest inaccuracy. After manufacturer information, the equipment conditioned volume error of the pipettes is maximally 2%. Within an experiment this error remains without consequences, it affects only the comparability of different series of measurements. The error of the germ number determination increases with decreasing germ number and can reach 50%. Additional inaccuracies by platting and counting enter into this value.

Table 7.1 shows the errors corresponding to the temperature dependence of *Bacillus subtilis* spores at 300 MPa and 30 min treatment time. The values 1 to 3 represent the number of viable spores counted on agar plates after the treatment.

T (°C)	Value 1	Value 2	Value 3	Mean value	Standard error	%
Initial germ number	8.04E+07	5.37E+07	1.00E+08	7.80E+07	2.32E+07	29.78285
30°C	3.21E+07	1.68E+07	1.70E+07	2.20E+07	8.78E+06	39.95278
Initial germ number	5.50E+07	3.48E+07	2.80E+07	3.93E+07	1.40E+07	35.76384
40°C	5.10E+06	3.75E+06	2.67E+06	3.84E+06	1.22E+06	31.70566
Initial germ number	1.22E+08	1.16E+08	1.30E+08	1.23E+08	7.02E+06	5.25899
50°C	8.70E+06	1.10E+07	9.69E+06	9.80E+06	1.15E+06	11.7765
Initial germ number	1.50E+08	1.50E+08	1.40E+08	1.47E+08	5.77E+06	3.936479
60°C	1.28E+04	2.20E+04	1.78E+04	1.75E+04	4.61E+03	26.26878

Table 7.1 Error calculation of the inactivation of *B. subtilis* spore at 300 MPa and different temperatures for 30 minutes after fast pressure increase

The error value is between 5 and 40%. The large deviations can be explained by the small number of pair values and include also the error by platting and counting the germs.

In the logarithmic representation of the values these errors are not to be recognized if the initial germ number is high. In the range of low initial germ number this error can easily lead to misinterpretation of the inactivation curves. Here, the possibility exists that in the case of determination of the regression slope for rate constants large differences arise. The error which results from the calculation of the rate constants using the regression of the curves can be measured. The standard deviation of the regression coefficients could be calculated using the following equation:

$$s_k = \frac{s_y}{s_x}\sqrt{\frac{1 - R^2}{n - 2}}$$

s_x/s_y = standard deviations of the x respectively, y values;

R^2 = correlation coefficient of the regression;

n = number of pair values.

The Table 7.2 shows the error values for a few rate constants.

Fast pressure increase Treatment conditions	***k* value**	**s_k**	**s_k / *k* (%)**
25°C/100 MPa	0.0077	0.0005175	6.7201812
30°C/150 MPa	0.0296	0.0010408	3.5162399
40°C/100 MPa	0.0401	0.0026528	6.615366
40°C/300 MPa	0.0559	0.0020195	3.6126701
Slow pressure increase Treatment conditions	***k* value**	**s_k**	**s_k / *k* (%)**
50°C/100 MPa	0.1283	0.0041591	3.241721
60°C/150 MPa	0.2061	0.00611	2.9645974
60°C/400 MPa	0.1674	0.0100251	5.988699
70°C/100 MPa	0.3617	0.0136487	3.77348

Table 7.2 Error values of the determined rate constants

The error value lies between 3 and 7%.

The error values are acceptable and usual for the work with biological systems and have no influence on the interpretation of the results. Despite the deviations, the found inactivation curves allow a clear statement about the behavior of the germ under high pressure.

Chapter 8 Conclusion

This study certifies that the effect of pressure build-up rate on the viability of spores may be significant. It has been stated that a fast pressurization is accompanied by an additional inactivation effect during the pressure increase phase while a slow pressurization with a controlled pressurization rate of 20 MPa/min does not produce a similar effect.

Further research is needed in order to be able to clarify the influence of the dynamic p-T profile during fast pressure increase on the molecular level. To elucidate the mechanisms involved, various properties of model biomembranes such as density, enthalpy change, surface and interfacial tension should be measured. The examination of membrane integrity of the pressurized cells should clarify if the membrane integrity varies between inactivated and non-inactivated cells.

Also, an effort should be made to understand the role of the adiabatic heating on spore inactivation in respect to the rate of pressure build-up.

Furthermore, it should be investigated whether the growth temperature (*Bacillus subtilis*: 22-48°C) of microorganisms has an influence on their pressure sensitivity.

Part V Appendix

Chapter 9 Measured values

The following tables contain the values to the diagrams represented in Chapter 5. The tables have the same numeration as the corresponding figures.

Measured values to 5.1

Inactivation of *B. subtilis* spores at 60°C/400 MPa starting from different spore concentrations

Time (min)	lg cfu	lg cfu
0	7.4405	6.3265
5	6.3622	5.2837
10	5.4357	4.3845
15	4.2257	3.4317
20	3.8037	2.9586
25	3.5847	2.2596
30	3.4598	1.9586
Initial germs number	8.1119	6.9438

Measured values to 5.2

First order inactivation kinetics at 60°C/300 MPa (in triplicate)

Time (min)	lg cfu 1	lg cfu 2	lg cfu 3
0	7.5606	7.5852	7.4592
50	7.4405	7.4985	7.3388
10	6.9758	6.8541	6.6053
15	5.9488	5.8392	5.5744
20	5.2218	5.2373	5.1627
25	4.9827	5.0725	4.8616
30	4.1079	4.3565	4.2523
35	3.7585	3.7825	3.7625
Initial germs number	8.1999	8.1999	8.1480
Control	8.0125	8.0566	8.0193

Measured values to 5.3

Inactivation kinetics of *Bacillus subtilis* spores at 60°C and three different pressures: 50, 100 and 150 MPa

Time (min)	50 MPa lg cfu	100 MPa lg cfu	150 MPa lg cfu
0	7.1804	7.6150	7.6118
10		7.2057	7.2738
15	6.9444		
20		7.0578	5.9689
30	5.7692	5.4317	4.7997
40		3.8754	4.3806
45	4.7144		
50		3.6536	3.9960
60	3.4775	3.1765	3.5567
70		2.9546	2.2557
Initial germs number	7.3327	7.8469	7.9112
Control	7.2449	7.7119	7.6458

Measured values to 5.4

Inactivation of *Bacillus subtilis* spores at 100 MPa and different temperatures: 25, 30 and 40°C

Time (min)	25°C lg cfu	30°C lg cfu	40°C lg cfu
0	7.5603	7.6976	7.7759
30			7.2269
60	7.5267	7,4552	7.0835
90			6.8037
120	7.0216	6.7997	5.3265
180	6.7563	6.5567	
240	6.4069	6.4120	
Initial germs number	7.7585	7.7002	7.7889
Control	7.6236	7.6174	7.7868

Inactivation of *Bacillus subtilis* spores at 100 MPa and different temperatures: 50, 60 and 70°C

Time (min)	50°C lg cfu	60°C lg cfu	70°C lg cfu
0	7.1974	7.6150	7.6276
10	7.1442	7.2057	6.8469
20	6.9488	7.0578	4.5531
30	6.6633	5.4317	3.1106
40	5.8219	3.8754	
50	5.8737	3.6536	
60		3.1765	
70		2.9546	
Initial germs number	7.5026	7.8469	8.1229
Control	7.3265	7.7119	8.0867

Measured values to 5.5

Inactivation kinetics of *Bacillus subtilis* spores at 30°C and three different pressures: 50, 100 and 150 MPa

Time (min)	50 MPa lg cfu	100 MPa lg cfu	150 MPa lg cfu
0	7.7343	7.6976	7.5421
30			7.3679
60	7.5767	7.4552	6.9438
90			6.4814
120	7.4592	6.7997	6.0573
180	7.3899	6.5567	
240	7.0882	6.4120	
Initial germs number	7.8209	7.7002	7.6734
Control	7.5533	7.6174	7.6678

Measured values to 5.6 are the same with those for 5.3.

Measured values to 5.7

Determination of the activation energy at 100 MPa for temperatures between 25 and 70°C

T (°C)	1/T (K^{-1})	ln *k*
25	0.003354	-4,8632
25	0.003354	-4.5729
25	0.003354	-4.4388
30	0.003298	-4.3188
30	0.003298	-4.3834
30	0.003298	-4.0533
40	0.003193	-3.1973
40	0.003193	-3.2469
40	0.003193	-3.2175
50	0.003094	-2.6944
50	0.003094	-2.6759
50	0.003094	-2.4367
60	0.003001	-2.3771
60	0.003001	-2.2720
60	0.003001	-2.7465
60	0.003001	-2.7721
60	0.003001	-2.6502
70	0.002914	-1.0082
70	0.002914	-1.0038
70	0.002914	-1,0079

Measured values to 5.8

Determination of the activation volume at 30°C for the pressure range 50-150 MPa

p / MPa	ln *k*
50	-5.1715
50	-5.6106
50	-5.6662
100	-4.3188
100	-4.3884
100	-4.0533
150	-3.5200
150	-3.4635
150	-3.3616

Measured values to 5.9

Determination of the activation volume at 60°C for the pressure range 50-150 MPa

p / MPa	ln *k*
50	-3,4082
50	-3.8083
50	-3.6988
100	-2.3771
100	-2.2720
100	-2.7465
100	-2.7721
100	-2.6502
150	-1.6224
150	-1.5834
150	-1.6652
150	-1.7056
150	-1.8802

Measured values to 5.10

Inactivation kinetics of *Bacillus subtilis* spores at 300 MPa and different temperatures: 25, 30, 40 and 50°C

Time (min)	25°C lg cfu	30°C lg cfu	40°C lg cfu	50°C lg cfu
0	7.6236	7.5457	7.3696	7.6843
5			7.1110	
10		7.5420	7.0828	
15	7.5459		6.8891	7.2334
20		7.3640	6.9121	
25			6.6706	
30	7.4405	7.2334	6.5744	6.9399
35			6.4687	
40		7.0026		
45	7.3952			6.5954
50		6.8430		
60		6.8059		5.9586
70		6.5779		
75				5.7825
Initial germs number	7,8392	8.0038	7.5420	8.0882

Inactivation kinetics of *Bacillus subtilis* spores at 300 MPa and different temperatures: 60 and 70°C

Time (min)	60°C lg cfu	70°C lg cfu
0	7.9557	6.8486
1		6.7470
2		6.1876
3		5.8411
4		5.5457
5	7.5153	5.0496
6		4.7670
7		4.5421
10	7.0265	
15	6.2257	
20	5.9153	
25	5.4552	
30	4.4317	
Initial germs number	8.2106	7.6448

Measured values to 5.11

Inactivation kinetics of Bacillus subtilis spores at 30°C in the pressure range 200-400 MPa

Time (min)	200 MPa lg cfu	300 MPa lg cfu	400 MPa lg cfu
0	7.3966	7.5457	7.3348
10	6.9247	7.5420	7.2399
20	6.6816	7.3640	7.2179
30	6.5567	7.2334	7.2179
40	6.3348	7.0026	7.1403
50	5.9689	6.8430	7.1008
60	5.4775	6.8059	7.0000
70	5.3806	6.5779	7.0129
Initial germs number	7.8649	8.0038	7.7631

Measured values to 5.12

Inactivation kinetics of *Bacillus subtilis* spores at 60°C in the pressure range 200-400 MPa

Time (min)	200 MPa lg cfu	300 MPa lg cfu	400 MPa lg cfu
0	8.0243	7.9643	7.4405
5	7.8189	7.352	6.3622
10	7.2668	7.0374	5.4357
15	6.7054	6.2138	4.2257
20	6.2409	5.8354	3.8037
25	5.4861	5.5914	3,5847
30	5.0686	4.5567	3.4598
35	4.8392		
Initial germs number	8.0963	8.1856	8.1119

Measured values to 5.13

Determination of the activation energy at 300 MPa for temperatures between 25 and 70°C

T (°C)	1/T (K^{-1})	ln *k*
25	0.003354	-4.7738
25	0.003354	-4.4115
25	0.003354	-4.4158
30	0.003298	-3.1801
30	0.003298	-3.8202
30	0.003298	-3.3939
40	0.003193	-2.7870
40	0.003193	-2.8835
40	0.003193	-2.9547
50	0.003094	-2.8135
50	0.003094	-2.6380
50	0.003094	-2.5665
60	0.003001	-1.2945
60	0.003001	-1.3292
60	0.003001	-1.3570
70	0.002914	-0.2271
70	0.002914	-0.2037
70	0.002914	-0.1833

Measured values to 5.14

Determination of the activation volume at 30°C for the pressure range 200-400 MPa

p / MPa	ln *k*
200	-2.7335
200	-2.8879
200	-2.9425
300	-3.1801
300	-3.8202
300	-3.3939
400	-4.2999
400	-4.4174
400	-4.5051

Measured values to 5.15

Determination of the activation volume at 60°C for the pressure range 200-400 MPa

p / MPa	ln *k*
200	-1.4720
200	-1.3043
200	-1.4644
300	-1.2945
300	-1.3292
300	-1.3570
300	-1.3473
300	-1.4031
300	-1.2637
400	-1.1563
400	-0.9858
400	-1.0833

Measured values to 5.16

Correlation between the experimental and calculated *k* values

k calculated	*k* experimental
0.0494	0.0746
0.1163	0.0694
0.0358	0.1070
0.0937	0.1284
0.2456	0.1852
0.2208	0.2440
0.l2615	0.2641
0.3097	0.3421
0.1712	0.3657
0.5607	0.8150
0.0093	0.0100
0.0149	0.0109
0.0049	0.0043
0.0137	0.0144
0.0379	0.0319
0.0526	0.0603
0.0242	0.0324
0.0111	0.0122
0.0147	0.0399
0.0606	0.0565

Measured values to 5.17

The effect of fast pressure build-up on the inactivation of *Bacillus subtilis* spores in the temperature/pressure range 25-40°C and 200-400 MPa

	A/A0 calculated	A/A0 experimental
30°C/200MPa	0.9888	0.3276
30°C/300MPa	0.7405	0.5072
30°C/400MPa	0.7434	0.4991
25°C/300MPa	0.8998	0.6224
40°C/300MPa	0.7091	0.5438

Measured values to 5.18

The effect of fast pressure build-up on the inactivation of *Bacillus subtilis* spores in the temperature/pressure range 50-70°C and 50-400 MPa

	A/A0 calculated	A/A0 experimental
60°C/50MPa	0.9665	0.7364
60°C/100MPa	0.8578	0.5991
60°C/150MPa	0.5516	0.4984
60°C/200MPa	0.5336	0.4506
60°C/300MPa	0.3744	0.2250
60°C/400MPa	0.2312	0.2195
50°C/100MPa	0.8852	0.5719
50°C/300MPa	0.5119	0.4744
70°C/100MPa	0.7183	0.3299
70°C/300MPa	0.1508	0.1500

Slow pressure build-up

Measured values to 5.19

First order inactivation at 50°C/100 MPa (in triplicate)

Time (min)	lg cfu 1	lg cfu 2	lg cfu 3
0	5.7825	5.8432	5.8616
5	5.6575	5.6856	5.7825
10	5.3526	5.3751	5.4460
15	5.0860	5.0903	5.1249
20	4.9661	4.9728	4.9866
25	4.8297	4.8375	4.8160
30	4.7042	4.7067	4.7463
Initial germs number	7.0531	7.0531	7.0635
Control	7.0013	6.9393	6.9700

Measured values to 5.20

Biphasic inactivation kinetics of *Bacillus subtilis* at 60°C and different pressures: 50, 100 and 300 MPa

Time (min)	50 MPa lg cfu	100 MPa lg cfu	300 MPa lg cfu
0	7.8525	7.6856	6.3448
5			5.7540
10		7.6245	4.6856
15	7.7195		4.2257
20		6.4858	3.9558
25			3.6661
30	6.7130	4.7997	2.9546
35			2.5567
40		3.6893	
45	5.4771		
50		3.2095	
60	4.6773		
70			
Initial germs number	7.9453	7.9160	7.8488
Control	7.7924	7.7319	7.7509

Measured values to 5.21

Inactivation kinetics of *Bacillus subtilis* at 100 MPa and different temperatures: 50, 60 and 70°C

Time (min)	50°C lg cfu	60°C lg cfu	70°C lg cfu
0	7.8016	7.7343	7.4308
10	7.6337	7.3952	5.2943
20	6.6989	6.5026	4.0090
30	6.4005	5.2334	2.6236
40	6.1442	3.2557	
50	5.9128	3.1587	
Initial germs number	7.9139	7.9160	7.8890
Control	7.8139	7.8521	7.7993

Measured values to 5.22

Inactivation kinetics of *Bacillus subtilis* spores at 300 MPa and different temperatures: 50, 60 and 70°C

Time (min)	50°C lg cfu	60°C lg cfu	70°C lg cfu
0	5.7825	6.3448	3.2557
1			3.1496
2			2.9827
3			2.7602
4			2.4317
5	5.6575	5.7540	2.1804
6			1.9586
10	5.3526	4.6856	
15	5.0860	4.2257	
20	4.9661	3.9558	
25	4.8297	3.6661	
30	4.7042	2.9546	
35		2.5567	
Initial germs number	7.0531	7.8488	7.2808
Control	7.0013	7.7509	7.1627

Measured values to 5.23

Inactivation kinetics of *Bacillus subtilis* spores at 60°C and different pressures:50, 100 and 150 MPa

Time (min)	50 MPa lg cfu	100 MPa lg cfu	150 MPa lg cfu
0	7.8179	7.7343	7.4210
10		7.3952	6.7255
15	7.7602		
20		6.5026	5.9728
30	6.7080	5,2334	4.7080
40		3.2557	3.3226
45	5.5459		
50		3.1587	3.0796
60	4.8355		2.3806
Initial germs number	7.8432	7.9160	7.5987
Control	7.8014	7.7625	7.4405

Inactivation kinetics of *Bacillus subtilis* spores at 60°C and different pressures:200, 300 and 400 MPa

Time (min)	200 MPa lg cfu	300 MPa lg cfu	400 MPa lg cfu
0	7.3899	5.0000	4.3696
5	6.8670	4.5228	3.6536
10	6.2596	3.2557	3.2628
15	5.7376	3.1804	3.0686
20	5.2218	2.9960	2.7328
25	4.3467	2.5914	2.2596
30	3.2970	2.4317	2.0835
35		2.0835	
Initial germs number	7.8432	6.4818	7.4682
Control	7.8037	6.4005	7.2296

Measured values to 5.24

Determination of the activation energy at 100 MPa for the temperature range 50-70°C

T (°C)	1/T (K^{-1})	ln *k*
50	0.003094	-2.0531
50	0.003094	-2.3698
50	0.003094	-2.1709
60	0.003001	-1.9795
60	0.003001	-1.9533
60	0.003001	-1.8275
70	0.002914	-1.0170
70	0.002914	-0.9639
70	0.002914	-1.0288

Measured values to 5.25

Determination of the activation energy at 300 MPa for the temperature range 50-70°C

T (°C)	1/T (K^{-1})	ln *k*
50	0.003094	-2.4442
50	0.003094	-2.4014
50	0.003094	-2.3764
60	0.003001	-0.9121
60	0.003001	-0.9622
60	0.003001	-0.9362
70	0.002914	-0.6448
70	0.002914	-0.7288
70	0.002914	-0.5065

Measured values to 5.26

Determination of the activation volume at 60°C in the pressure range 50-400 MPa

p / MPa	ln *k*
50	-2.4365
50	-2.4628
50	-2.4230
100	-1.9795
100	-1.9533
100	-1.8275
150	-1.5793
150	-1.6316
150	-1.6235
200	-1.1975
200	-1.2623
200	-1.2598
300	-0.9121
300	-0.9622
300	-0.9362
400	-1.7875
400	-2.0147
400	-2.1578

Measured values to 5.27

Correlation between the experimental and calculated *k* values

k calculated	*k* experimental
0.0855	0.1120
0.0850	0.0901
0.1071	0.0871
0.1584	0.1469
0.2399	0.1996
0.2064	0.2896
0.3877	0.3920
0.0606	0.1388
0.2831	0.3668
0.5240	0.5366

Measured values to 5.28

The effect of slow pressure build-up on the inactivation of *Bacillus subtilis* spores at temperatures between 50 and 70°C in the pressure range 50-400 MPa

	lg A/A0 calculated	lg A/A0 experimental
60°C/50MPa	-0.1138	-0.1206
60°C/100MPa	-0.1895	-0.2652
60°C/150MPa	-0.4868	-0.4900
60°C/200MPa	-0.9669	-1.2143
60°C/300MPa	-1.4932	-1.4952
60°C/400MPa	-2.3863	-3.4741
50°C/100MPa	-0.1154	-0.1190
50°C/300MPa	-0.7579	-0.9107
70°C/100MPa	-0.4706	-0.4746
70°C/300MPa	-2.6321	-4.0235

Complementary Part

This part contains measured values not given in the figures.

A. Fast pressure build-up

Inactivation kinetics at 25°C and 100 MPa

	Experiment 1	Experiment 2	Experiment 3
t/min	lg cfu	lg cfu	lg cfu
0	7.6046	7.5228	7.5603
60	7.4413	7.4987	7.5267
120	7.1210	7.1496	7.0216
180	6.9689	6.7997	6.7563
240	6.9247	6.5267	6.4069
Initial germ number	7.6950	7.7585	7.7585
k	0.0077	0.0103	0.0118
Mean value *k*		0.0100	
R^2	0.9334	0.9542	0.9582

Inactivation kinetics at 25°C and 300 MPa

	Experiment 1	Experiment 2	Experiment 3
t/min	lg cfu	lg cfu	lg cfu
0	7.6046	7.6236	7.6046
15	7.5421	7.5459	7.5149
30	7.4985	7.4405	7.4210
45	7.4357	7.3952	7.3735
Initial germ number			
k	0.0084	0.0121	0.0121
Mean value *k*		0.0109	
R^2	0.9952	0.9797	0.9818

Inactivation kinetics at 30°C and 50 MPa

	Experiment 1	Experiment 2	Experiment 3
t/min	lg cfu	lg cfu	lg cfu
0	7.7343	7.7042	7.6428
60	7.5767	7.6458	7.5783
120	7.4592	7.5886	7.4592
180	7.3899	7.4858	7.4005
240	7.0882	7.3075	7.2808
Initial germ number	7.8209	7.8139	7.8139
k	0.0057	0.0037	0.0035
Mean value *k*		0.0043	
R^2	0.9450	0.9341	0.9881

Inactivation kinetics at 30°C and 100 MPa

	Experiment 1	Experiment 2	Experiment 3
t/min	lg cfu	lg cfu	lg cfu
0	7.6976	7.5948	8.0167
60	7.4552	7.4308	7.8880
120	6.7997	6.6239	7.1804
180	6.5567	6.6014	6.6276
240	6.4120	6.3913	6.3845
Initial germ number	7.7002	7.7002	8.1179
k	0.0133	0.0124	0.0174
Mean value *k*		0.0144	
R^2	0.9442	0.8842	0.9605

Inactivation kinetics at 30°C and 150 MPa

	Experiment 1	Experiment 2	Experiment 3
t/min	lg cfu	lg cfu	lg cfu
0	7.5421	7.6053	7.5533
30	7.3679	7.4727	7.4546
60	6.9438	6.9438	6.6856
90	6.4814	6.4357	6.1935
120	6.0573	6.0835	5.9247
Initial germ number	7.6734	7.6734	7.6734
k	0.0296	0.0313	0.0347
Mean value *k*		0.0319	
R^2	0.9807	0.9742	0.9558

Inactivation kinetics at 30°C and 200 MPa

	Experiment 1	Experiment 2	Experiment 3
t/min	lg cfu	lg cfu	lg cfu
0	7.3966	7.3640	7.6816
10	6.9247	6.8577	7.2699
20	6.6816	6.6816	7.1496
30	6.5567	6.4775	7.0686
40	6.3348	6.7343	6.9657
50	5.9689	6.2668	6.9614
60	5.4775	5.6276	6.8897
70	5.3806	5.4814	6.8507
Initial germ number	7.8649	7.8649	7.9308
k	0.0650	0.0557	0.0230
Mean value *k*		0.0603	
R^2	0.9771	0.8827	

Inactivation kinetics at 30°C and 300 MPa

	Experiment 1	Experiment 2	Experiment 3
t/min	lg cfu	lg cfu	lg cfu
0	7.7105	7.5881	7.5457
10	7.6014	7.3640	7.5420
20	7.6205	7.3288	7.3640
30	7.5069	7.2257	7.2334
40	7.0290	7.0584	7.0026
50	6.7493	6.9947	6.8430
60	6.7253	6.9632	6.8059
70	6.6110	6.8990	6.5779
Initial germ number	7.9947	7.7304	8.0038
k	0.0416	0.0219	0.0336
Mean value *k*		0.0324	
R^2	0.9148	0.9525	0.9766

Inactivation kinetics at 30°C and 400 MPa

	Experiment 1	Experiment 2	Experiment 3
t/min	lg cfu	lg cfu	lg cfu
0	7.3226	7.4818	7.3348
10	7.4643	7.3036	7.2699
20	7.3966	7.2484	7.2179
30	7.2837	7.1910	7.2179
40	7.1842	7.1587	7.1403
50	7.1431	7.1210	7.1008
60	7.0780	7.1627	7.0000
70	7.0142	7.0129	7.0129
Initial germ number	7.4987	7.7829	7.7631
k	0.0136	0.0121	0.0111
Mean value *k*		0.0122	
R^2	0.8424	0.8472	0.9633

Inactivation kinetics at 40°C and 100 MPa

	Experiment 1	Experiment 2	Experiment 3
t/min	lg cfu	lg cfu	lg cfu
0	7.7759	7.7245	7.7647
30	7.2296	7.2668	7.3622
60	7.0835	7.0725	6.9128
90	6.8037	6.8794	6.6276
120	5.3265	5.3845	5.5228
Initial germ number	7.7868	7.7868	7.7868
k	0.0409	0.0389	0.0401
Mean value *k*		0.0399	
R^2	0.8391	0.8190	0.9348

Inactivation kinetics at 40°C and 300 MPa

	Experiment 1	Experiment 2	Experiment 3
t/min	lg cfu	lg cfu	lg cfu
0	7.4598	7.3696	7.1210
5	7.3696	7.1110	7.0338
10	7.1468	7.0828	6.9786
15	6.9758	6.8891	6.6014
20	6.9517	6.9121	6.7470
25	6.6536	6.6706	6.5149
30	6.7080	6.5744	6.4269
35	6.5420	6.4687	6.3751
Initial germ number	7.7470	7.5420	7.4552
k	0.0616	0.0559	0.0521
Mean value *k*		0.0565	
R^2	0.9600	0.9659	0.9144

Inactivation kinetics at 50°C and 100 MPa

	Experiment 1	Experiment 2	Experiment 3
t/min	lg cfu	lg cfu	lg cfu
0	7.1974	7.1047	7.2668
10	7.1442	6.9866	6.9728
20	6.9488	6.7205	6.8616
30	6.6633	6.1899	6.0725
40	5.8219	5.9089	5.7807
50	5.9934	5.7647	5.4814
Initial germ number	7.5026	7.2943	7.5108
k	0.0676	0.0688	0.0875
Mean value *k*		0.0746	
R^2	0.8627	0.9657	0.9609

Inactivation kinetics at 50°C and 300 MPa

	Experiment 1	Experiment 2	Experiment 3
t/min	lg cfu	lg cfu	lg cfu
0	7.6843	7.7540	7.8794
15	7.2334	7.2218	7.2449
30	6.9399	7.0612	6.9866
45	6.5954	6.6856	6.7119
60	5.9586	5.9586	5.6575
75	5.7825	5.3265	5.3845
Initial germ number	8.0882	8.0675	8.1337
k	0.0600	0.0715	0.0768
Mean value *k*		0.0694	
R^2	0.9850	0.9627	0.9588

Inactivation kinetics at 60°C and 50 MPa

	Experiment 1	Experiment 2	Experiment 3	Experiment 4
t/min	lg cfu	lg cfu	lg cfu	lg cfu
0	7.1804	7.189	7.1804	6.7602
15	6.9444	6.8258	6.9121	6.4918
30	5.7692	5.8507	5.7463	5.3272
k	0.1073	0.1027	0.1101	0.1100
Mean value *k*	0.1070			
Initial germ number	7.3327	7.3075	7.3203	6.8811
30	5.7692	5.8507	5.7463	5.3272
45	4.7144	4.717	4.691	4.5804
60	3.4775	3.3845	3.5567	3.2257
k	0.1759	0.1893	0.1681	0.1613

Inactivation kinetics at 60°C and 100 MPa

	Experiment 1	Experiment 2	Experiment 3	Experiment 4	Experiment 5	Experiment 6
t/min	lg cfu	lg cfu	lg cfu	lg cfu	lg cfu	lg cfu
0	7.9986	7.8981	7.6118	7.605	7.5088	7.5106
10	7.5886	7.3952	7.2738	7.2051	6.9401	7.0021
20	7.1924	7.0026	5.9689	6.2178	6.5438	6.5819
k	0.0928	0.1031	0.1892	0.1597	0.1111	0.1069
Mean value *k*	0.1284					
Initial germ number	8.1189	8.0669	7.9112	7.8369	7.7266	7.8095
20	7.0922	7.0026	5.9689	6.2178	6.5438	6.5819
30	4.8925	5.2837	4.7997	5.4317	5.2904	5.7631
40	3.2557	3.9586	4.3806	3.8754	3.8925	4.3348
50	2.9286	3.5914	3.9960	3.6536	3.5914	3.7328
60	2.7367	2.1765	3.5567	3.1765	3.0796	3.1765
70	1.4814	2.0796	2.2557	2.9546	2.9960	2.8925
k	0.2293	0.2257	0.1492	0.1533	0.1623	0.1764

Inactivation kinetics at 60°C and 150 MPa

	Experiment 1	Experiment 2	Experiment 3	Experiment 4	Experiment 5
t/min	lg cfu	lg cfu	lg cfu	lg cfu	lg cfu
0	7.3507	8.0142	7.6118	7.1346	7.5421
10	6.4814	7.4345	7.2738	6.9438	7.3075
20	5.6359	6.3667	5.9189	5.4567	6.2171
k	0.1974	0.1897	0.1949	0.1932	0.1526
Mean value *k*	0.1852				
Initial germ number	7.6575	8.2917	7.9112	7.4814	7.8239
20	5.6359	6.3667	5.9189	5.4567	6.2171
30	3.8754	4.6389	4.7997	3.9546	4.7563
40	3.2553	3.3522	4.3806	3.6536	4.5603
50	2.4317	2.8239	3.9960	3.4317	4.0573
60	2.2557	2.2596	3.5567	3.1765	3.7130
70	1.7785	1.7825	2.2557	2.0796	2.1765
k	0.1643	0.2012	0.1476	0.1279	0.1568

Inactivation kinetics at 60°C and 200 MPa

	Experiment 1	Experiment 2	Experiment 3
t/min	lg cfu	lg cfu	lg cfu
0	8.0243	8.2752	7.9468
5	7.8189	7.9208	7.6718
10	7.2668	7.5886	7.3139
15	6.7054	7.0496	6.5189
20	6.2409	6.5420	6.1403
25	5.4861	5.6205	5.3162
30	5.0686	4.7563	5.0457
35	4.8392	4.3806	4.7080
Initial germ number	8.3963	8.4912	8.3974
k	0.2295	0.2714	0.2312
Mean value *k*	0.2440		
R^2	0.9876	0.9746	0.9837

Inactivation kinetics at 60°C and 300 MPa

	Experiment 1	Experiment 2	Experiment 3
t/min	lg cfu	lg cfu	lg cfu
0	7.5606	7.5852	7.4592
5	7.4405	7.4985	7.3388
10	6.9758	6.8541	6.6053
15	5.9488	5.8392	5.5744
20	5.2218	5.2373	5.1627
25	4.9827	5.0725	4.8616
30	4.1079	4.3565	4.2523
35	3.7585	3.7825	3.7625
Initial germ number	8.1999	8.1999	8.148
k	0.2740	0.2647	0.2574
Mean value *k*	0.2651		
R^2	0.9778	0.9783	0.9782

Inactivation kinetics at 60°C and 400 MPa

	Experiment 1	Experiment 2	Experiment 3
t/min	lg cfu	lg cfu	lg cfu
0	7.4405	6.2596	6.3265
5	6.3622	5.3139	5.2837
10	5.4357	4.3265	4.3845
15	4.2257	2.7825	3.4317
20	3.8037	2.4357	2.9586
25	3.5847	2.0835	2.2596
30	3.4598	1.4814	1.9586
35	6.2596	6.2692	6.2678
Initial germ number	8.1119	6.8964	6.9938
k	0.3146	0.3731	0.3385
Mean value *k*	0.3421		
R^2	0.9114	0.9517	0.9736

Inactivation kinetics at 70°C and 100 MPa

	Experiment 1	Experiment 2
t/min	lg cfu	lg cfu
0	7.6276	7.6428
10	6.8469	6.9756
20	4.5531	5.0828
30	3.1106	2.9685
Initial germ number	8.1229	8.1108
k	0.3649	0.3665
Mean value *k*	0.3657	
R^2	0.9699	0.9566

Inactivation kinetics at 70°C and 300 MPa

	Experiment 1	Experiment 2	Experiment 3
t/min	lg cfu	lg cfu	lg cfu
0	6.8449	6.8486	6.8720
1	6.8079	6.7470	6.8595
2	5.6622	6.1876	6.3583
3	5.9618	5.8411	5.9399
4	5.5069	5.5457	5.7698
5	5.3483	5.0496	5.3068
6	4.5886	4.7670	4.7563
7	4.4771	4.5421	4.5109
Initial germ number	7.6448	7.7448	7.6478
k	0.7968	0.8157	0.8325
Mean value *k*		0.8150	
R^2	0.9178	0.9886	0.9806

B. Slow pressure build-up

Inactivation kinetics at 50°C and 100 MPa

	Experiment 1	Experiment 2	Experiment 3
t/min	lg cfu	lg cfu	lg cfu
0	7.8118	7.8016	7.8098
10	7.5886	7.6337	7.6488
20	6.5642	6.6989	6.7195
30	6.2738	6.4005	6.4159
40	5.6359	6.1442	5.7563
50	5.1403	5.9128	5.5382
Initial germ number	7.9194	7.9139	7.9469
k	0.1283	0.0935	0.1141
Mean value *k*		0.1120	
R^2	0.9782	0.9373	0.9679

Inactivation kinetics at 50°C and 300 MPa

	Experiment 1	Experiment 2	Experiment 3
t/min	lg cfu	lg cfu	lg cfu
0	5.7825	5.8432	5.8616
5	5.6575	5.6856	5.7825
10	5.3526	5.3751	5.4460
15	5.0860	5.0903	5.1249
20	4.9661	4.9728	4.9866
25	4.8297	4.8375	4.8616
30	4.7042	4.7067	4.7463
Initial germ number	7.0531	7.0531	7.0635
k	0.0868	0.0906	0.0929
Mean value *k*		0.0901	
R^2	0.9727	0.9702	0.9610

Inactivation kinetics at 60°C and 50 MPa

	Experiment 1	Experiment 2	Experiment 3
t/min	lg cfu	lg cfu	lg cfu
0	7.8525	7.8179	7.6856
15	7.7195	7.7602	7.5149
30	6.7130	6.7080	6.5306
k	0.0875	0.0852	0.0887
Mean value *k*		0.0871	
Initial germ number	7.9453	7.9432	7.8294
30	6.7130	6.7080	6.5306
45	5.4771	5.5459	5.6718
60	4.6773	4.8355	4.7509
k	0.1563	0.1437	0.1366

Inactivation kinetics at 60°C and 100 MPa

	Experiment 1	Experiment 2	Experiment 3
t/min	lg cfu	lg cfu	lg cfu
0	7.6856	7.7343	7.7294
10	7.6245	7.3952	7.5345
20	6.4858	6.5026	6.3327
k	0.1381	0.1418	0.1608
Mean value *k*	0.1469		
Initial germ number	7.916	8.013	8.016
20	6.4858	6.5026	6.3327
30	4.7997	5.2334	4.6816
40	3.6893	3.2557	3.6294
50	3.2095	3.1587	3.0212
k	0.2519	0.2765	0.2530

Inactivation kinetics at 60°C and 150 MPa

	Experiment 1	Experiment 2	Experiment 3
t/min	lg cfu	lg cfu	lg cfu
0	7.4210	7.6428	7.2523
10	6.7255	6.8776	6.7911
20	5.9728	5.4413	5.6575
30	4.7080	4.5818	4.7579
40	3.3226	3.5533	3.1804
50	3.0796	3.2876	3.1346
60	2.3806	2.7367	2.5228
Initial germ number	7.9035	8.1102	7.7724
k	0.2061	0.1956	0.1972
Mean value *k*	0.1996		
R^2	0.9772	0.9661	0.9648

Inactivation kinetics at 60°C and 200 MPa

	Experiment 1	Experiment 2	Experiment 3
t/min	lg cfu	lg cfu	lg cfu
0	7.3899	7.2943	7.2596
5	6.8670	6.7119	6.9811
10	6.2596	6.0903	5.8297
15	5.7376	5.8392	5.7304
20	5.2218	4.8037	5.2138
25	4.3467	4.4775	4.4357
30	3.2970	3.4775	3.4120
Initial germ number	7.8432	7.8355	7.8239
k	0.3019	0.2830	0.2837
Mean value *k*	0.2896		
R^2	0.9801	0.9820	0.9654

Inactivation kinetics at 60°C and 400 MPa

	Experiment 1	Experiment 2	Experiment 3
t/min	lg cfu	lg cfu	lg cfu
0	4.3696	3.8754	3.4317
5	3.6536	3.4317	3.2837
10	3.2628	3.2018	2.9399
15	3.0686	3.0796	2.5189
20	2.7328	2.6816	2.3806
25	2.2596	2.1804	2.2596
30	2.0835	2.1804	1.9586
Initial germ number	7.4682	7.4682	7.1627
k	0.1674	0.1334	0.1156
Mean value *k*	0.1388		
R^2	0.9708	0.9681	0.9744

Inactivation kinetics at 70°C and 100 MPa

	Experiment 1	Experiment 2	Experiment 3
t/min	lg cfu	lg cfu	lg cfu
0	7.4308	7.4637	7.3265
10	5.2943	5.4357	4.9438
20	4.0090	3.9689	3.9689
30	2.6236	2.4317	2.4775
Initial germ number	7.8890	7.9449	7.8109
k	0.3617	0.3814	0.3574
Mean value *k*	0.3668		
R^2	0.9851	0.9942	0.9691

Inactivation kinetics at 70°C and 300 MPa

	Experiment 1	Experiment 2	Experiment 3
t/min	lg cfu	lg cfu	lg cfu
0	3.2557	3.3164	3.2904
1	3.1496	3.1765	3.1587
2	2.9827	2.9546	3.0216
3	2.7602	2.4317	2.6536
4	2.4317	2.7785	2.3806
5	2.1804	2.1804	2.0835
6	1.9586	2.0835	1.7785
Initial germ number	7.2808	7.3448	7.3075
k	0.5247	0.4825	0.6026
Mean value *k*	0.5366		
R^2	0.9815	0.8781	0.9806

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Hiermit erkläre ich an Eides statt,
daß ich die vorliegende Arbeit selbständig
und ohne unerlaubte Hilfsmittel durchgeführt habe.