SITEC-Sieber Engineering AG
Head Office
Aschbach 7
8124 Maur (Zurich) | SWITZERLAND
Phone +41 44 982 2070
Fax +41 44 982 2079
E-mail info@sitec-hp.ch

SITEC-Sieber Engineering AG
Engineering, Production, Logistics
Lohwisstrasse 46-50
8123 Ebmatingen | SWITZERLAND
Phone +41 44 982 1070
Fax +41 44 982 2089
Web www.sitec-hp.ch

SITEC
High-Pressure Technology

High-Pressure Sterilization

- Desk-top and pilot units for high-pressure sterilization
- Sterilization cells with easy to handle quick opening closures
- 7,000 bar and 10,000 bar models

High-pressure sterilization unit (7,000 bar, 80 °C, 100 ml sterilization cell with 70 ml basket insert).

SITEC-Sieber Engineering AG
Head Office
Aschbach 7
8124 Maur (Zurich) | SWITZERLAND
Phone +41 44 982 2070
Fax +41 44 982 2079
E-mail info@sitec-hp.ch

SITEC-Sieber Engineering AG
Engineering, Production, Logistics
Lohwisstrasse 46-50
8123 Ebmatingen | SWITZERLAND
Phone +41 44 982 1070
Fax +41 44 982 2089
Web www.sitec-hp.ch

Advantages

- no thermal damage of the product
- applicable to thermo labile components
- no influence on vitamin content of the product
- no change of the natural flavour
- no adulteration of food taste
- no influence on product colour
- no effect on amino acids
- selective treatment of large molecules
- no adding of preservatives required
- alternative food preparation method
- new food design options available

Applications

Sterilization of food	e.g.	fruit juices, fruits, spices, milk
Sterilization of pharmaceutical products	e.g.	liposome, drugs
Inactivation of micro organisms	e.g.	bacteria, bacteria spores, mould, yeast
Inactivation of enzymes		
Structure changes of biological macromolecules	e.g.	starch (cold-bonding)
Denaturation and coagulation of proteins	e.g.	meet curing
Phase modification of lipids	e.g.	crystallisation of fats

SITEC-Sieber Engineering AG
Head Office
Aschbach 7
8124 Maur (Zurich) | SWITZERLAND
Phone +41 44 982 2070
Fax +41 44 982 2079
E-mail info@sitec-hp.ch

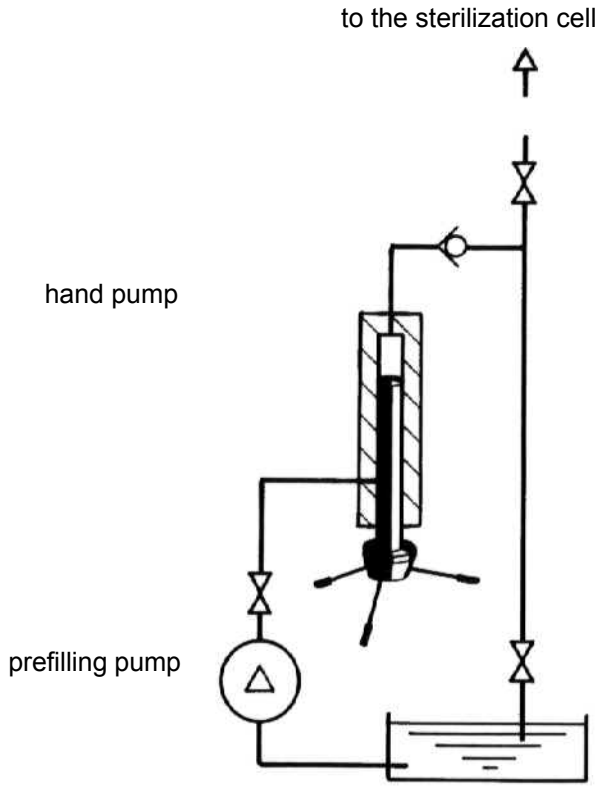
SITEC-Sieber Engineering AG
Engineering, Production, Logistics
Lohwisstrasse 46-50
8123 Ebmatingen | SWITZERLAND
Phone +41 44 982 1070
Fax +41 44 982 2089
Web www.sitec-hp.ch

High-Pressure Technology

Desktop model for high-pressure sterilization

Main features of standard **model 760.0537**:

- sterilization pressure max.: **7,000 bar**
- for solid and liquid products
- hand operated pressure generation
- **25 ml** high pressure sterilization cell with **quick opening closure**
- 18 ml basket insert
- **Heating/cooling thermostat** for sterilization cell
- temperature measurement inside the basket insert (digital indication)
- digital indication of sterilization pressure
- **turn-key unit** (completely piped up and wired)
- different sterilization cell capacities on request



SITEC-Sieber Engineering AG
Head Office
Aschbach 7
8124 Maur (Zurich) | SWITZERLAND
Phone +41 44 982 2070
Fax +41 44 982 2079
E-mail info@sitec-hp.ch

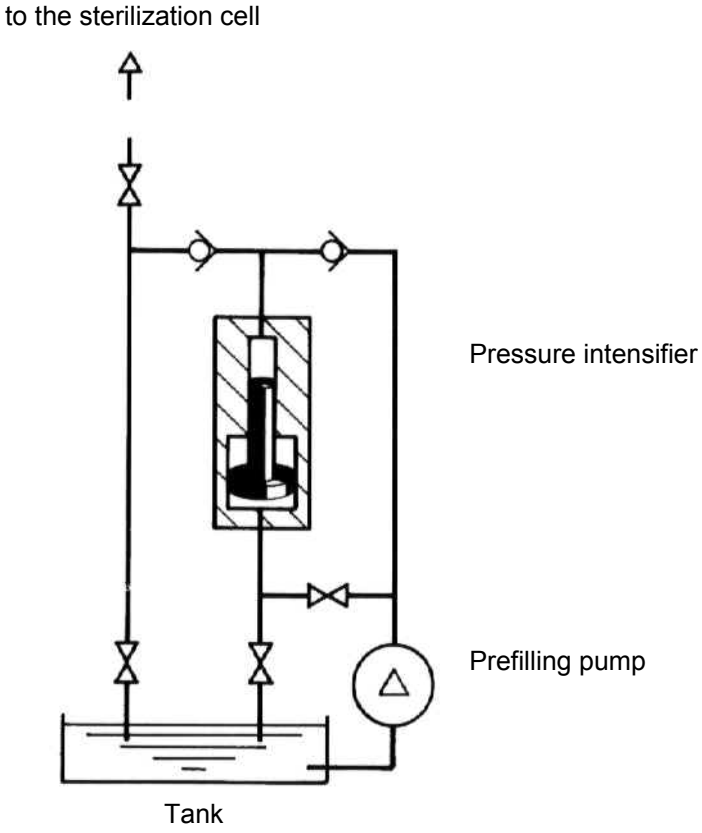
SITEC-Sieber Engineering AG
Engineering, Production, Logistics
Lohwisstrasse 46-50
8123 Ebmatingen | SWITZERLAND
Phone +41 44 982 1070
Fax +41 44 982 2089
Web www.sitec-hp.ch

High-Pressure Technology

Pilot unit for high-pressure sterilization

Main Features of standard **models 760.0118 / 760.0119**:

- max. sterilization pressure: **7,000 bar / 10,000 bar**
- for solid and liquid products
- **pressure intensifier** with external piston-position indicator
- **25 ml** high pressure sterilization cell with **quick opening closure**
- 18 ml basket insert
- **Heating/cooling thermostat** for sterilization cell
- temperature measurement inside the basket insert (digital indication)
- digital indication of sterilization pressure
- **turn-key unit** (completely piped up and wired)
- different sterilization cell capacities on request



SITEC-Sieber Engineering AG
Head Office
Aschbach 7
8124 Maur (Zurich) | SWITZERLAND
Phone +41 44 982 2070
Fax +41 44 982 2079
E-mail info@sitec-hp.ch

SITEC-Sieber Engineering AG
Engineering, Production, Logistics
Lohwisstrasse 46-50
8123 Ebmatingen | SWITZERLAND
Phone +41 44 982 1070
Fax +41 44 982 2089
Web www.sitec-hp.ch

High-Pressure Technology

Questionnaire for high-pressure sterilization units

Operating pressure max.:

	7'000 bar	10'000 bar
Pressure generation by hand pump	☐	
Pressure generation by intensifier	☐	☐

Operating temperature max.: ☐ **80 °C** ☐ 120 °C

☐ .....

Pressure fluid: ☐ **water (H2O)** ☐ .....

……

Sterilization cell capacities:
☐ **25 ml with 18 ml basket insert**
☐ 50 ml with 35 ml basket insert
☐ 100 ml with 70 ml basket insert

☐ .....

Number of sterilization cells: ☐ **1** ☐ 2 ☐ 3 ☐ .....

Options:

☐ Data acquisition system by PC
☐ PLC control with integrated batch documentation
☐ Window units for sterilization cell (2 x Ø 6 mm; 7000 bar maximum!)
☐ Colour camera system with endoscope

SITEC-Sieber Engineering AG
Head Office
Aschbach 7
8124 Maur (Zurich) | SWITZERLAND
Phone +41 44 982 2070
Fax +41 44 982 2079
E-mail info@sitec-hp.ch

SITEC-Sieber Engineering AG
Engineering, Production, Logistics
Lohwisstrasse 46-50
8123 Ebmatingen | SWITZERLAND
Phone +41 44 982 1070
Fax +41 44 982 2089
Web www.sitec-hp.ch

Contact Details

Last name: .....

First name: .....

Title/gender: .....

Company: .....

Department: .....

Street: .....

P.O. Box: .....

Zip code: .....

Town / city: .....

Country: .....

Phone: .....

Fax: .....

E-mail: .....

www. .....

Please fill in this form and return it to SITEC-Sieber Engineering AG.

SITEC-Sieber Engineering AG
Head Office
Aschbach 7
8124 Maur (Zurich) | SWITZERLAND
Phone +41 44 982 2070
Fax +41 44 982 2079
E-mail info@sitec-hp.ch

SITEC-Sieber Engineering AG
Engineering, Production, Logistics
Lohwisstrasse 46-50
8123 Ebmatingen | SWITZERLAND
Phone +41 44 982 1070
Fax +41 44 982 2089
Web www.sitec-hp.ch

High-Pressure Technology

Data acquisition and online visualisation

As a useful addition to the SITEC high-pressure pilot units, we are able to offer you a simple and also a very flexible data acquisition program. This program allows to visualise your process data online during the experiments and to save it on your hard disk for a later interpretation.

The data acquisition and visualisation program will be completely integrated in your high pressure pilot unit and is configured for a specific application. It is also possible to upgrade an existing high pressure pilot unit, but with a bigger expenditure.

Based on the SCADA Software SpecView from EUROTHERM (see overview hereafter) SITEC provides several windows specifically programmed for a certain application.

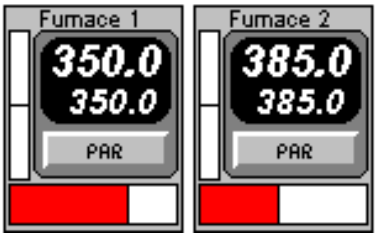
One window shows all the controller symbols and allows set-point adjustment or also alteration of control parameters. In addition, all monitoring signals are shown. On an additional screens all the data are graphically and digitally displayed.

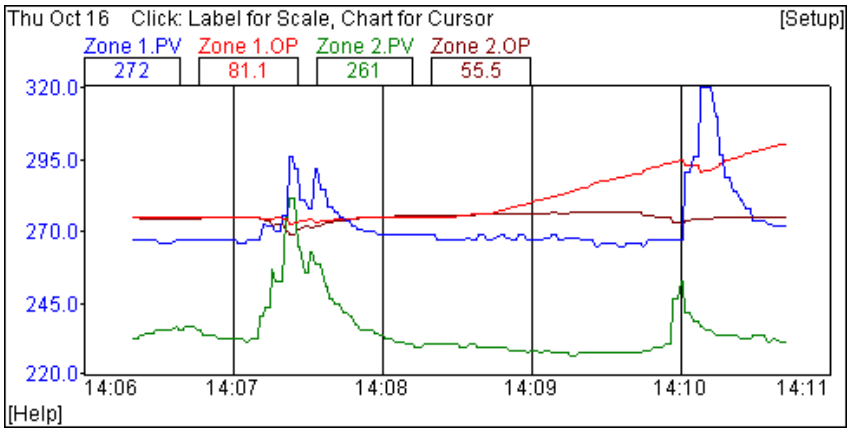
All the data are automatically stored always the data acquisition program is started. On request a certain section (time interval) can be extracted and exported to Microsoft Excel.

The full development package of SpecView which is also supplied allows to change an existing or to create a new user interface using easy to handle “drag and drop” methods. On request, we will gladly send you a more detailed description of this development package.

The communication PC <-> Pilot Unit is made via USB. The controllers are interconnected by a RS485 interface.

The system requirements for the installation of SpecView: Windows PC with Windows 95, 98, Me, NT, 2000, XP or 2003 Server operating system, 64MB RAM minimum (128MB recommended), USB interface.

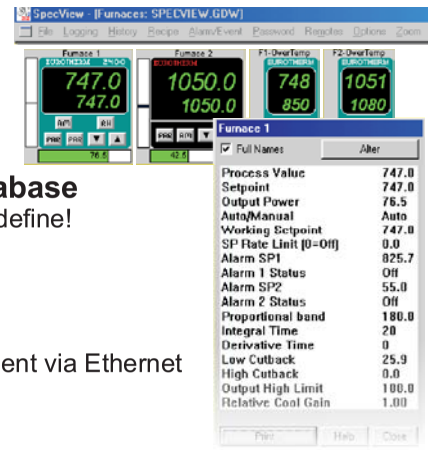




CONNECT to Control and Measuring Instruments

Simple Display and Logging Applications setup in minutes!

Auto-Detection of Connected Instruments

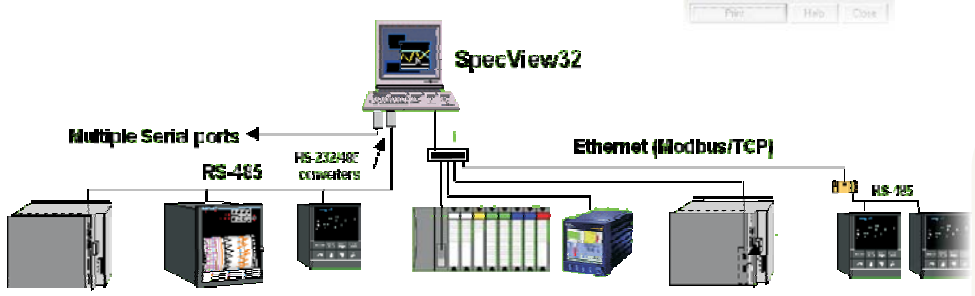


Pre-built Instrument View Database

Over 1000 instruments - no tags to define!
Access all instrument parameters

Concurrent Connections

RS232/RS485 (up to 40 ports)
TCP/IP, connect any RS485 instrument via Ethernet
OPC



- Temperature
- Pressure
- Flow
- Level
- Vacuum
- Speed
- Status
- Power

- Controls
- Systems
- Meters
- Indicators
- Remote I/O
- Data Acquisition
- PLCs

- RS232 / RS485
- Ethernet
- Internet
- OPC
- Radio

SpecView

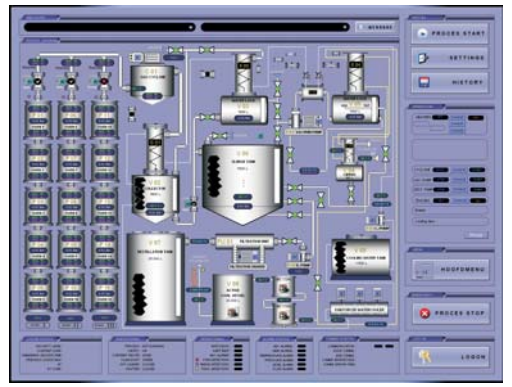
SCADA software for people with other jobs!

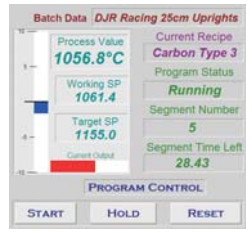
Operator Interface (HMI) OPERATE

Create easy to use HMI

Unambiguous screens ensure error free operation

Visualize your process at a glance





Touch Screen Compatible

Passwords

Multiple password levels
Restrict access to specified items
Record user login

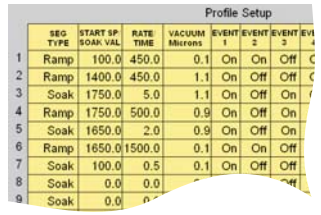
Events & Alarms

All operator actions recorded
Pop-up alarm window
Remote notification: pager / E-mail / SMS

Recipes

Multiple recipe screens
Process snapshot
Flexibility:
- Full Access: select / review / edit / send
- Read-Only: select / review / send
- Single button send

Bar code recipe selection



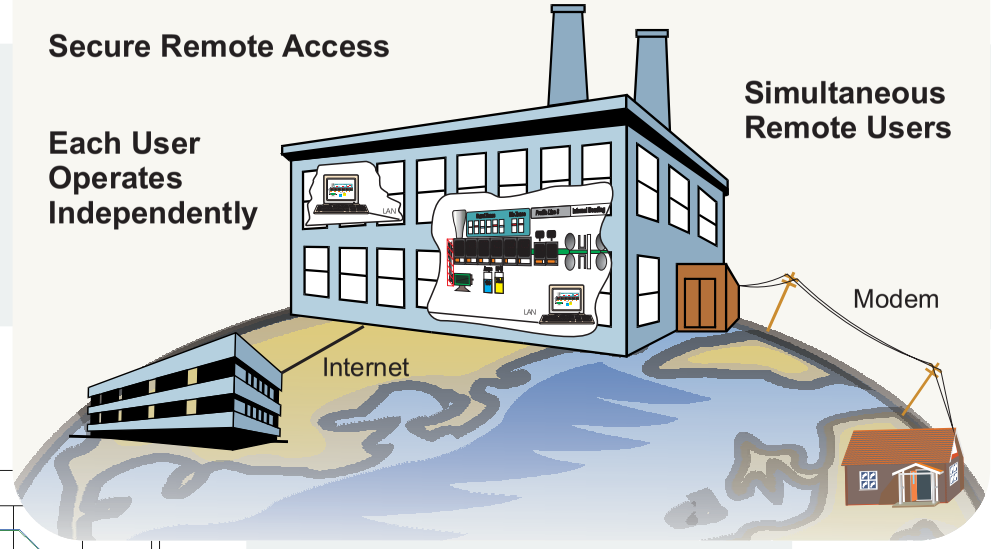
NETWORK Use SpecView from Office or Home

Multiple Operator Stations for Large Machines

Secure Remote Access

Each User Operates Independently

Simultaneous Remote Users



> SpecView is unique. Many of my customers configure the program unaided. Some choose to have some assistance and for those that want a turn-key installation SpecView gives me the tools I need.
>
> Rick Sabo, AQI Engineering

> Loy Instrument, Inc. has installed close to one hundred copies of SpecView. I have found that SpecView is unmatched by anything else on the market. Its versatility has allowed us to use it in everything from straight data logging to full scale SCADA systems. For the money, no other software on the market can touch it.
>
> Mark McDaniel, Loy Instrument Inc.

RECORD Data Management

Logging

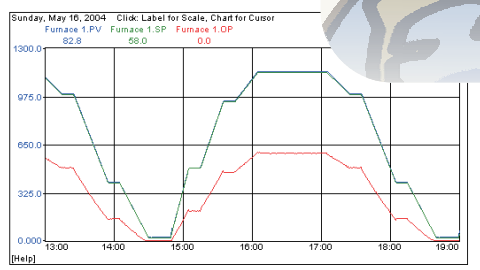
Record any item
By time or event

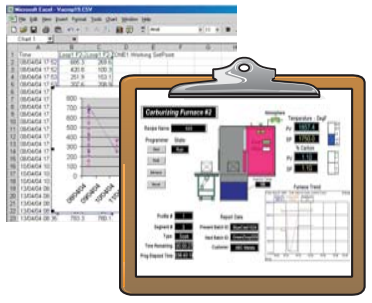
User Variables

Batch data, comments etc
Integrate test data

Trends

Unlimited pens
Unlimited charts
Multiple time axes
Automatic printing



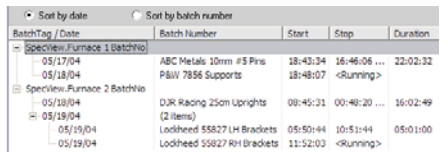


Reports

Log reports for spreadsheet analysis
Batch "Clipboard" report
Automatic process / shift reports

Batch Records

Find data quickly and easily
Multiple parallel batches



Alarm / Event

Start, acknowledge & clear timestamps
Alarm history
All operator actions recorded

Historical Replay

'Video' replay of whole screen
Review your process in 'Fast Forward'

> I received SpecView as a CD and manual. In a short time I had a configured the program to provide both data logging and an operator interface to a number of different control devices. I had no previous experience with this kind of software. SpecView made it easy.
>
> Lisa Reep, Corning

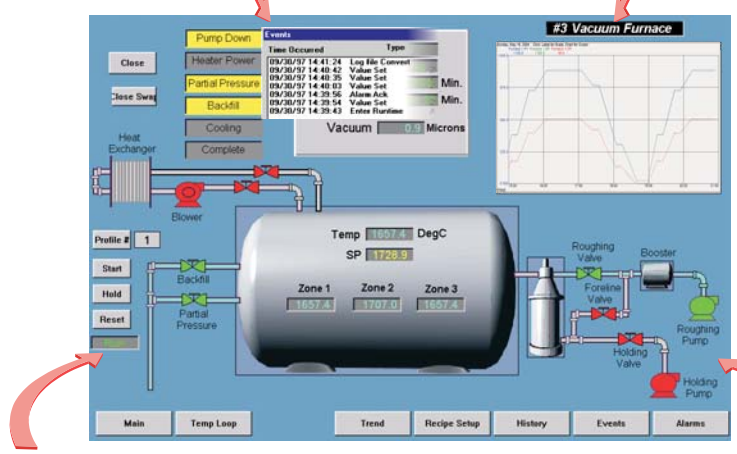
Explore and Troubleshoot ANALYSE

Event Window

What did the operator do?
...and when?

Integrate

Test measurements
Operator comments
Peripheral data



Visual Feedback

"I wish I had been standing here when..."
"Did the purge valve open correctly?"

Performance

How many parts?
Percentage downtime
Correlate run-to-run data

SITEC-Sieber Engineering AG
Head Office
Aschbach 7
8124 Maur (Zurich) | SWITZERLAND
Phone +41 44 982 2070
Fax +41 44 982 2079
E-mail info@sitec-hp.ch

SITEC-Sieber Engineering AG
Engineering, Production, Logistics
Lohwisstrasse 46-50
8123 Ebmatingen | SWITZERLAND
Phone +41 44 982 1070
Fax +41 44 982 2089
Web www.sitec-hp.ch

High-Pressure Technology

Process control with integrated batch documentation

This control allows you to master your processes by combing display, regulation, control and even batch documentation – all in one.

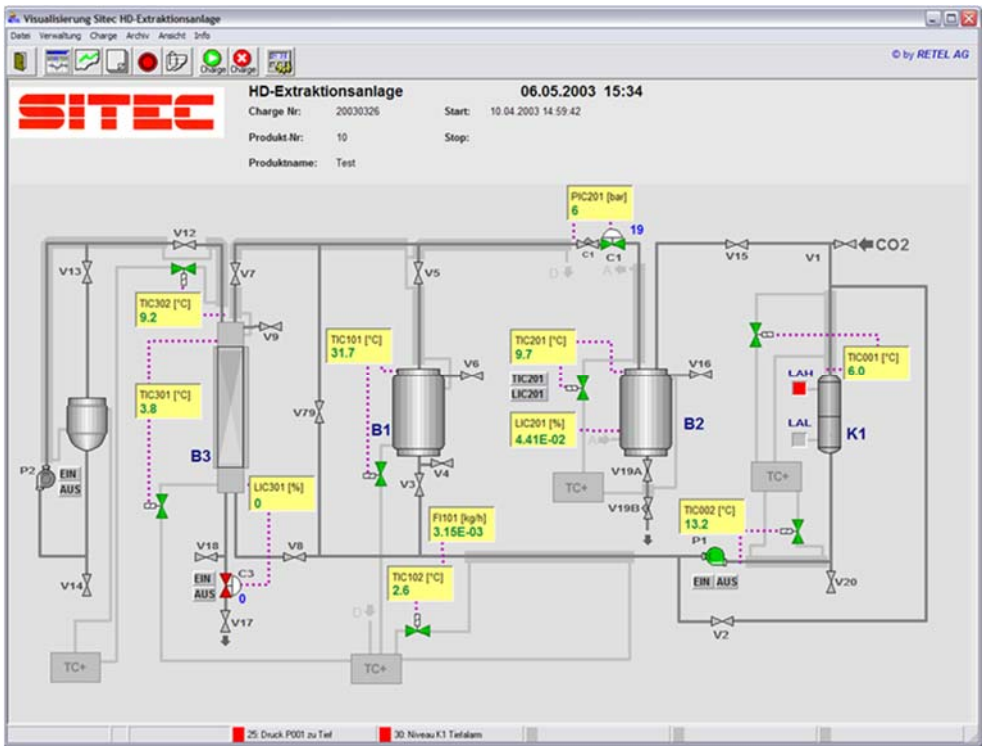
Advantages:

- **complete batch documentation**
- **trend display of process flow**
- **dynamic overview of the installation**
- **easy input of nominal values**
- **exportable into standard formats**
- **protocols and diagrams accessible over network**

The user-friendliness of the system operation is achieved by clearly arranged displays. This control system includes functions which otherwise are integrated in process control systems only.

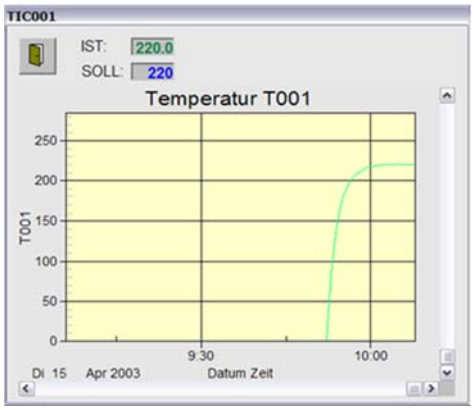
The control system allows manual and – as an option – automatic control of the installation. All process parameters can be displayed.

The data of the current batch as well as the system overview is displayed on the main window. The operator can control the process over the display picture.

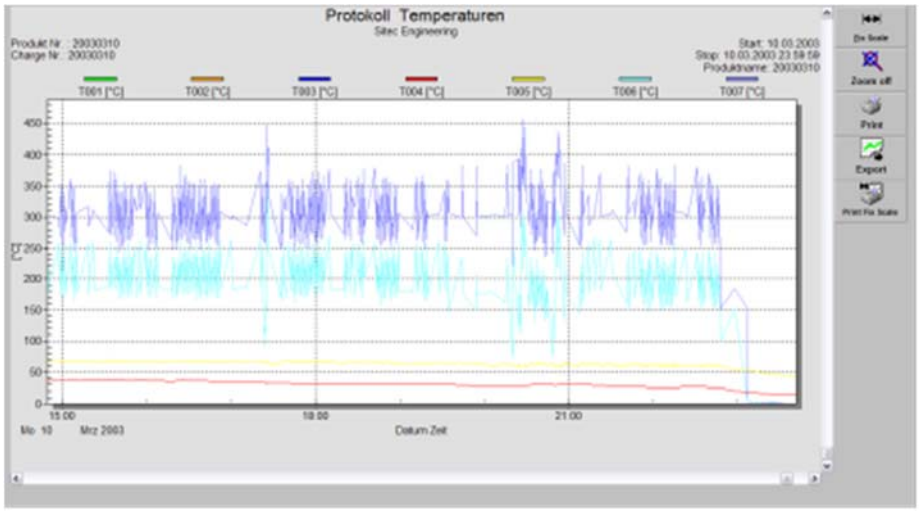


SITEC-Sieber Engineering AG
Head Office
Aschbach 7
8124 Maur (Zurich) | SWITZERLAND
Phone +41 44 982 2070
Fax +41 44 982 2079
E-mail info@sitec-hp.ch

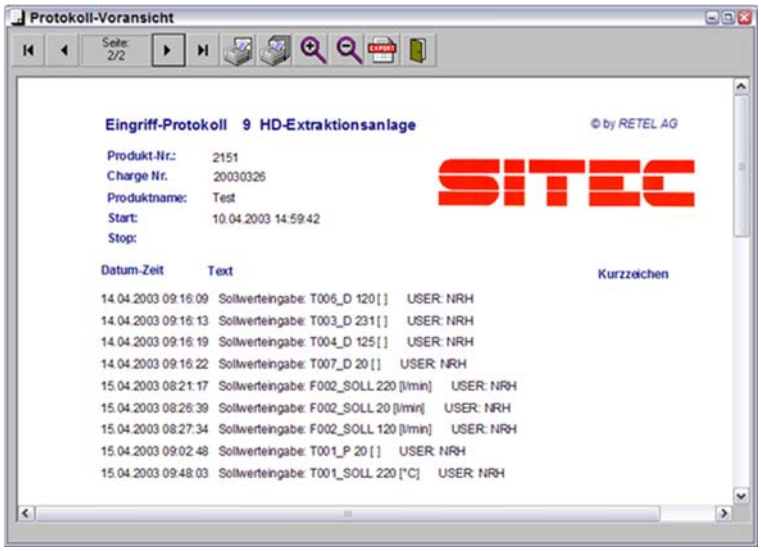
SITEC-Sieber Engineering AG
Engineering, Production, Logistics
Lohwisstrasse 46-50
8123 Ebmatingen | SWITZERLAND
Phone +41 44 982 1070
Fax +41 44 982 2089
Web www.sitec-hp.ch



The automatic control circuits have special display windows with integrated diagrams and nominal value input.



Important process parameters are shown in a trend display. The trend display has a zoom and Fix Scale function and can be printed or exported into bmp, wmf, or jpg graphic formats.



With the help of the integrated logging program the process can be documented completely. All protocols are exportable in pdf, html, or txt formats.

- data protocol
- error protocol
- event protocol
- comment protocol

The batches recorded can be retrieved from the archive. All protocols and diagrams can be exported and re-printed as desired.

SITEC-Sieber Engineering AG
Head Office
Aschbach 7
8124 Maur (Zurich) | SWITZERLAND
Phone +41 44 982 2070
Fax +41 44 982 2079
E-mail info@sitec-hp.ch

SITEC-Sieber Engineering AG
Engineering, Production, Logistics
Lohwisstrasse 46-50
8123 Ebmatingen | SWITZERLAND
Phone +41 44 982 1070
Fax +41 44 982 2089
Web www.sitec-hp.ch

High-Pressure Technology

Reference List for Pilot Plants

Rhône Poulenc, France	Chemical
University of Delft, Holland	Process engineering
University Wageningen, Holland	Agricultural research
BASF Ludwigshafen, Germany	Chemical
Salzgitter, Germany	Chemical
Hüls Chemie, Germany	Chemical
ENI, Italy	Petrochemical
Givaudan, Switzerland	Flavours and fragrances
Research Centre Karlsruhe	Environmental
Reemtsma, Hamburg, Germany	Tobacco
CNRS, France	Food research
TUBITAK, Turkey	Food research
SASOL, South Africa	Waxes
DEGUSSA-SKW, Trostberg, Germany	Hops, spices
Guinness, Ireland	Brewery research
English Hop Products, Great Britain	Hops
Fraunhofer Institute Pfinzthal, Germany	Process engineering
University of Bremerhaven, Germany	Food research
Novartis, Switzerland	Chemical
Firmenich, Switzerland	Flavours and fragrances
Haarmann & Reimer, Germany	Flavours and fragrances
University of Messina, Italy	Chemical engineering
MERCK, Germany	Chemical
University of Bari, Italy	Research
LIPI, Indonesia	Natural products
F.Hoffmann-La Roche, Switzerland	Reactions
University of Tübingen, Germany	Pharmaceutical research
National Technical University of Athens, Greece	Research
Inst. for “Nichtklassische Chemie”, Leipzig, Germany	Research
University of Halle-Wittenberg, Germany	Research
Janssen Pharmaceutica, Beerse, Belgium	Drug delivery research
MAINELAB, Angers, France	Drug delivery research
Semnan University, Semnan, Iran	Research
JSC “Interbridge”, Moscow, Russia	Research
Ecole des Mines d’Albi, Albi, France	Research
KRAFT Foods, UK	Coffee
Hochschule Niederrhein, Germany	Research
Solvay Solexis, Italy	Research (polymers)
EPFL, Switzerland	Research
University of Alicante, Spain	Research
King Fahd University of Petroleum, Saudi Arabia	Research
Inst. Nawozow Sztucznych Pulawy, Poland	Research
TU Bergakademie Freiberg, Germany	Research
3M, Seefeld, Germany	Research
FAPEX, Salvador de Bahia, Brazil	Research
University of Copenhagen, Frederiksberg, Denmark	Research
University Duisburg-Essen, Essen, Germany	Research
C. Illies, Hamburg, Germany (for China)	Research
AiFame GmbH, Wald-Schönengrund, Switzerland	Natural products

SITEC-Sieber Engineering AG
Head Office
Aschbach 7
8124 Maur (Zurich) | SWITZERLAND
Phone +41 44 982 2070
Fax +41 44 982 2079
E-mail info@sitec-hp.ch

SITEC-Sieber Engineering AG
Engineering, Production, Logistics
Lohwisstrasse 46-50
8123 Ebmatingen | SWITZERLAND
Phone +41 44 982 1070
Fax +41 44 982 2089
Web www.sitec-hp.ch

High-Pressure Technology

Additional units in following countries:

Denmark
Turkey
Belgium
Italy
Canada
Greece
Spain
Brazil
Germany
Holland
Switzerland
India
Bulgaria
Iran
Saudi Arabia
China
South Africa
France
Great Britain
Ireland
Indonesia
Russia
Poland

Activities:

Flavours and Fragrances
Biotechnology
Chemical Industry
Coal Industry
Food Industry
Pharmaceutical Industry
Oil/Gas Industry

Sonderdruck / Reprint

die pharmazeutische industrie

Pharm. Ind. **56**, 7, 660–663 (1994)
ECV · Editio Cantor Verlag · Aulendorf (Fed. Rep. of Germany)

From the Institut für pharmazeutische Technologie und Biopharmazie, Gruppe Physikalische Chemie, Universität Heidelberg (Fed. Rep. of Germany)

Pressure-induced Germination and Inactivation of Bacillus subtilis Spores

By B. Sojka and H. Ludwig

Summary

The ratio between dormant and germinated bacterial spores in an aqueous solution could be estimated using a temperature treatment at 80 °C. Using this concept, an optimized pretreatment at pressures of 600 to 1500 bar was developed. The result was an almost quantitative germination of the former dormant spores. Subsequent inactivation was the same whether 80 °C or 3000 bar was used and in both cases an identical number of remaining viable spores was detected.

The pressure inactivation of bacterial spores was studied in the range from 600 to 6000 bar at temperatures of 40 and 50 °C. At 40 °C the number of germs could be reduced by the factor of 10^6 after 210 min of pretratment at 600 bar and following inactivation at 5000 bar for some minutes. Raising the temperature to 50 °C, an alternating pressurization at 600 and 5000 bar in intervals of 30 min led to the complete inactivation of spores after an overall time of 180 min.

Zusammenfassung

Druckinduzierte Auskeimung und Inaktivierung von Bacillus subtilis-Sporen

Das Verhältnis zwischen den in einer wäßrigen Lösung inaktiv ruhenden und den ausgekeimten Bakteriensporen konnte mit einer Temperaturbehandlung bei 80 °C bestimmt werden. Mit Hilfe dieser Methode wurde eine optimierte Druckvorbehandlung mit 600 bis 1500 bar ausgearbeitet, womit eine nahezu vollständige Auskeimung der vorher ruhenden Bakteriensporen erreicht wurde. Die anschließende Inaktivierung der ausgekeimten Sporen verlief dann unabhängig davon, ob hierzu 80 °C oder 3000 bar verwendet wurden. In beiden Fällen blieben gleich viele lebensfähige Sporen übrig.

Die Druckinaktivierung von Bakteriensporen wurde im Bereich von 600 bis 6000 bar untersucht, wobei Temperaturen von 40 und 50 °C zur Anwendung gelangten. Bei 40 °C konnte nach einer 210minütigen Vorbehandlung mit 600 bar und einer kurzen Inaktivierung bei 5000 bar die Keimzahl um den Faktor 10^6 verringert werden. Nachdem die Temperatur auf 50 °C erhöht worden war, konnten nach insgesamt 180 min sterile Proben erhalten werden, wenn bei einer Intervallänge von 30 min abwechselnd 600 und 5000 bar angewandt wurden.

Key words: *Bacillus subtilis · Bacterial spores, hydrostatic pressure, germination, inactivation*

1. Introduction

Destruction of microorganisms is one of the most important effects of high pressure treatment of food or drugs and the first experiments in this area were done nearly one hundred years ago [1]. Later, Zobell [2, 3, 4] examined the effects of high hydrostatic pressure on various forms of bacteria. Timson and Short [5] were the first to study the inactivation of bacterial spores, which turned out to be much less pressure-sensitive than their vegetative forms. Gould and Sale [6, 7] proved the existence of an optimal hydrostatic pressure for the inactivation of spores. This can be explained by the fact that moderate pressures on the one hand induce the germination of spores and on the other hand lead to inactivation of the then germinated spores.

In order to establish high pressure processing as a universal method of sterilisation, an intensive examination of bacterial spores is necessary considering that bacterial spores are the microorganisms most difficult to inactivate. The effects of pressure on Bacillus subtilis and Bacillus stearothermophilus spores have therefore been studied by Butz et al. [8] and Ludwig et al. [9]. Their work proved that the inactivation of spores follows complicated kinetic curves, whereas the same treatment often leads to simple first order reactions in their vegetative forms.

In the first part of this article, some detailed studies of the germination process of Bacillus subtilis spores are reported. Later, inactivation is investigated in its relation to various pressures and temperatures. Emphasis was focused on studies of alternating low and high pressure treatment which promised to result in fast and quantitative inactivation.

2. Materials and methods

2.1. The high pressure equipment

All high pressure experiments were carried out using a unit built by the Dunze Hochdrucktechnologie Co. (Bad Homburg, FRG), capable of pressures up to 7000 bar. The device consists of 10 vessels which can be thermostated in 2 groups of 5 each. It is possible to aerate each autoclave separately to allow kinetic studies.

The spores were pressurized in tubes made of polyethylene whose ends were sealed using silicon plugs. The content of each vessel was 1–5 ml according to the length of the tube used.

2.2. Cultivation of spores

The strain used was ATCC 9372 and was purchased from Deutsche Sammlung von Mikroorganismen und Zellkulturen, Braunschweig, FRG. Bacteria were incubated overnight in standard-1-nutrient broth (E. Merck, Darmstadt, FRG) and afterwards plated in Petri dishes on nutrient agar supplemented with 500 mg of $MnCl_2$ and 500 mg of $MgCl_2$ (Merck) [10]. After 8 days of incubation at 35 °C the spores were harvested with a scalpel and transferred into a 0.9 % solution of NaCl in water. The suspension was washed several times with the NaCl solution and centrifugated at 4000 g. Heating the suspension at 80 °C for 30 min had no effect on the number of germs and it was therefore proved that the preparation was free of vegetative cells. A dilution with 0.9 % NaCl in water gave a concentration of $10^{9.5}$ spores per ml.

2.3. Preparation of samples

1 ml of spore suspension was added to 4.5 ml of 0.9 % NaCl solution and 4.5 ml of germination medium to give a 1:10 dilution. The germination medium consisted of 6 g NaH_2PO_4, 6 g K_2HPO_4, 1 g NaCl, 10 g D-Glucose, 100 mg $MgCl_2$, 80 mg $MnCl_2$ and 400 mg L-alanine (all Merck) per 1 l of water. This solution was shown to accelerate germination of Bacillus subtilis spores [11].

2.4. Viable counts

The number of spores surviving the different treatments was evaluated after serial dilutions and subsequent incubation on nutrient agar for 24 h. In case no colonies were found on the petri dishes, the samples were tested for sterility. This was achieved by suspending the sample in standard-1-nutrient broth and incubating at 37 °C for 10 days. If the suspension remained clear after that time, sterility was proved [12].

3. Results

3.1. Germination as prerequisite for inactivation

The success of every high pressure treatment depends mainly on the ratio of germinated to dormant spores. Since dormant spores are almost unassailable, it is of great importance to initiate germination before applying high pressure for inactivation. There are several methods of estimating the number of already germinated spores [8], but they all fail if almost entire germination has occured. According to Gould and Dring [13], spores absorb large quantities of water while germinating. This should make them sensitive to rising temperatures, whereas dormant spores keep their low water content and remain therefore unaffected by elevated temperatures.

In Fig. 1, spores were caused to germinate at 600 bar and 40 °C for 0 to 480 min and then the germinated spores were inactivated by heating for 15 to 60 min at 80 °C and 1 bar. The temperature-inactivation is a fast process that leads very quickly to a steady state. The longer the samples are pretreated by 600 bar, the smaller the remaining number of viable spores after an inactivation treatment. Incubation at 80 °C without previous pressure-treatment (curve at 0 min) does not result in a decrease of the number of germs, since almost no spores were allowed to germinate.

It is informative to compare temperature inactivation at 1 bar and 80 °C with high pressure inactivation at 3000 bar and 40 °C. Assuming that in both cases only germinated spores are affected, both treatments should show the same kinetics and result in the same number of colony counts. Fig. 2 shows the correlation of the different inactivation techniques. It can be seen that high pressure is as effective as high temperature processing, and it should be kept in mind that high pressures allow for a far more gentle treatment of sensitive products.

To estimate the lower limit for a pressure-induced germination, samples were treated with pressures of 200, 400 or 600 bar and later, inactivation was effectuated by 5000 bar. From Fig. 3 it can be seen that the best results were obtained with 600 bar and it can therefore be concluded that this pressure should be maintained to achieve an effective germination.

To find the optimal pressure for pretreatment, the samples were first incubated at 40 °C and 600, 1000 or 1500 bar for 15 to 60 min and then treated at 80 °C for 15 min. The results are displayed in Fig. 4. There are no significant differences in the number of remaining germs when germination inducing pressures between 600 and 1500 bar are used prior to the inactivation step. Germination may therefore be initiated by pressures between 600 and 1500 bar.

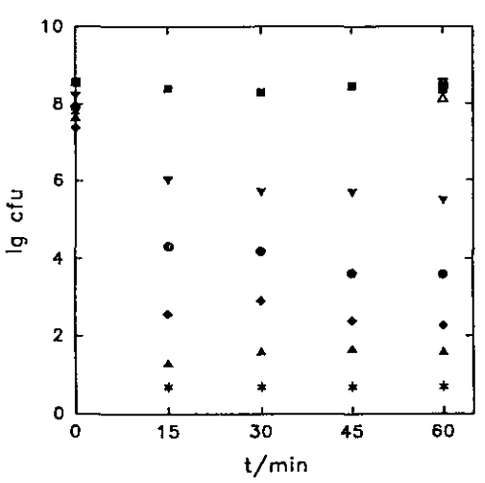


Fig. 1: Temperature inactivation of B. subtilis spores at 80 °C after pretreatment with 600 bar at 40 °C for different times: ■ 0, ▼ 30, ● 60, ◆ 120, ▲ 240, * 480 min, resp.; open symbols: controls.

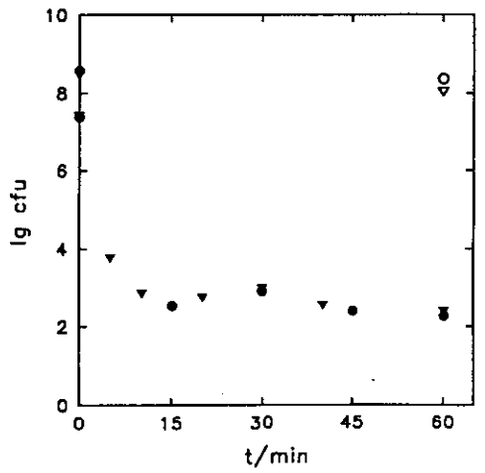


Fig. 2: Comparison of pressure inactivation and temperature inactivation of B. subtilis spores after pretreatment with 600 bar at 40 °C for 120 min: ▼ 3 kbar at 40 °C, ● 80 °C; open symbols: controls.

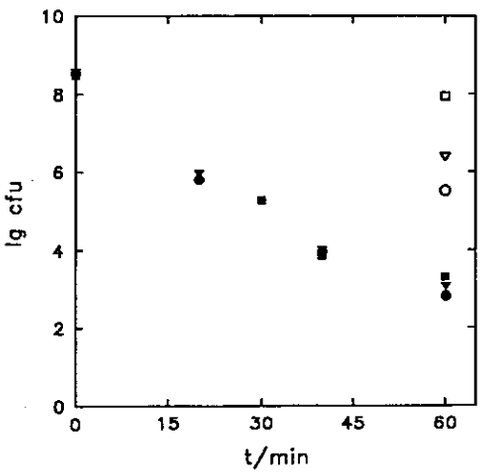


Fig. 4: Pretreatment of B. subtilis spores with variable pressure at 40 °C for different times and subsequent inactivation for 15 min at 80 °C: ■ 600, ▼ 1000, ● 1500 bar, resp.; open symbols: controls (effect of pretreatment).

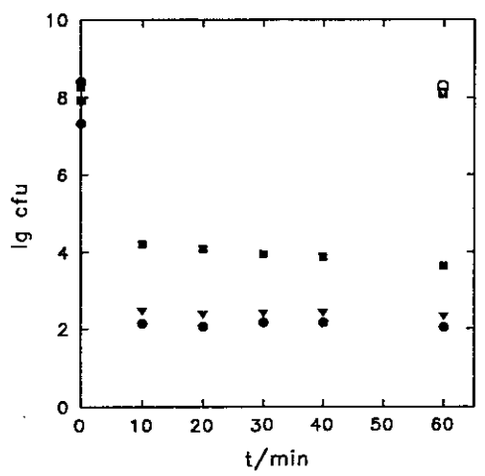


Fig. 3: Pressure inactivation of B. subtilis spores by 5 kbar at 40 °C after pretreatment with different pressures at 40 °C for 210 min: ■ 200, ▼ 400, ● 600 bar, resp.; open symbols: controls.

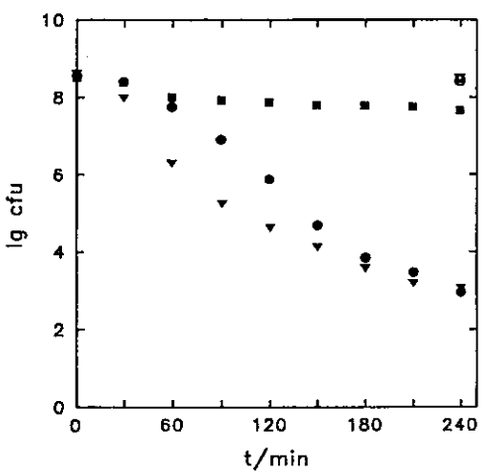


Fig. 5: Multiple step process at 40 °C for pressure inactivation of B. subtilis spores: ■ 600 bar constantly, ▼ 1500 bar constantly, ● 600/1500 bar alternatingly; open symbols: controls.

3.2. Inactivation by high pressures

From Fig. 2 it appears that the inactivation reaches a plateau already after 5 to 10 min. A longer treatment by 3000 bar does not lead to any further killing of spores because no additional germination occurs. Butz et al. [8] showed that the utilization of repeated germination intervals may result in an increased reduction of the germ concentration. It was therefore studied, under which conditions (temperature, pressure, length of intervals) the optimal pasteurization effect is achieved.

3.2.1. Experiments at 40 °C

The results of multi-step processes are shown for inactivations with alternating pressures of 600/1500 bar (Fig. 5) and 600/5000 bar (Fig. 6) using intervals of 30 min for every period of constant pressure.

Fig. 5 confirms the observation that 600 bar and 1500 bar both induce germination in the same degree. Since a constant treatment with 1500 bar could also be interpreted as a multistep process with 1500/1500 bar, it results in the same plateau of about 10^3 germs per ml as with alternating 600/1500 bar. The treatment with 600/5000 bar in Fig. 6 represents the optimal combination of two pressures, since decreasing (3000 bar) or increasing (6000 bar) of inactivation pressure yielded poorer results.

A comparison of Fig. 6 with the 600 bar curve of Fig. 3 allows an evaluation of the efficiency of a multi-step process at 40 °C. The 30-min sample of Fig. 3 represents an overall processing time of 240 min and yields almost the same result as the 240-min sample of Fig. 6. It may therefore be presumed that a multi-step treatment at 40 °C has no significant advantages over a two-step process.

3.2.2. Experiments at 50 °C

To accelerate inactivation, the temperature was raised to 50 °C and the best results were achieved by using a multi-step process with alternating 600 and 5000 bar. The kinetics is given in Fig. 7. The 180-min sample was tested for sterility as described in 2.4. and was found to contain no viable germs. An analog experiment using 1500 bar to induce germination also gave sterile samples after three hours of treatment (Fig. 8). This again confirms the finding that pressures of 600 and 1500 bar are capable of the stimulation of germination in the same degree.

Analog to paragraph 3.2.1., an easy evaluation of the multi-step process is possible when Fig. 7 is compared to Fig. 9. After 180 min of treatment no sterility could be obtained in a two-step process whereas repeated application of low and high pressures led to sterile samples.

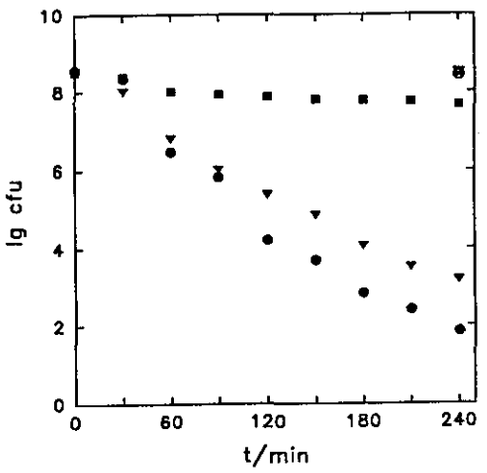


Fig. 6: Multiple step process at 40 °C for pressure inactivation of B. subtilis spores: ■ 600 bar constantly, ▼ 5000 bar constantly, ● 600/5000 bar alternatingly; open symbols: controls.

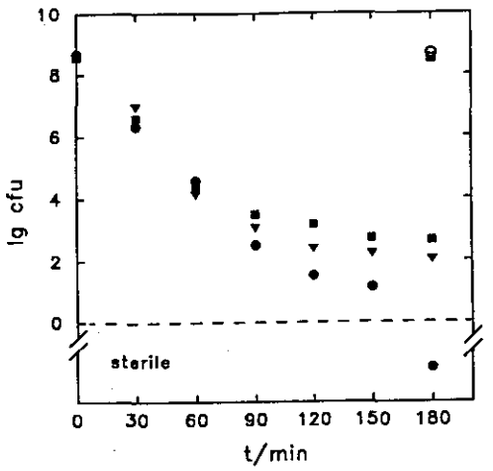


Fig. 8: Multiple step process at 50 °C for pressure inactivation of B. subtilis spores: ■ 1500 bar constantly, ▼ 5000 bar constantly, ● 1500/5000 bar alternatingly; open symbols: controls.

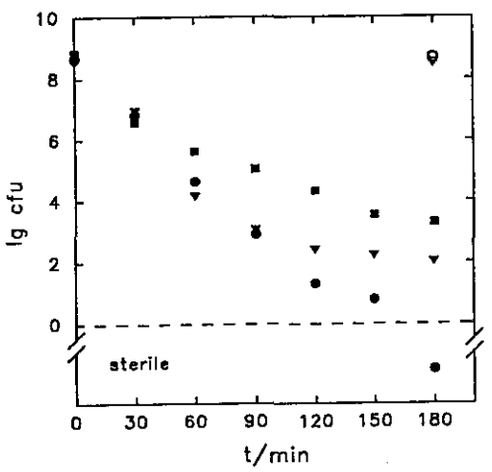


Fig. 7: Multiple step process at 50 °C for pressure inactivation of B. subtilis spores: ■ 600 bar constantly, ▼ 5000 bar constantly, ● 600/5000 bar alternatingly; open symbols: controls.

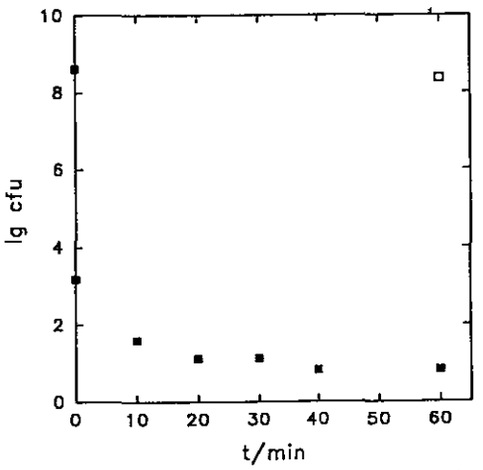


Fig. 9: Pressure inactivation of B. subtilis spores by 5 kbar at 50 °C after pretreatment with 600 bar at 50 °C for 150 min (■); open symbol: control.

4. Conclusion

A short treatment with 80 °C represents an easy and effective method to estimate the number of already germinated spores, even when the number of still dormant ones is very small. It is remarkable that a longer processing time at 80 °C does not lead to further germ kill and it may be assumed that germination is not achieved by temperatures in this order of magnitude. In the latter case no plateau would occur and the number of germs would constantly decrease during the temperature treatment since any germinated spore would quickly be inactivated. Ideal germination is not achieved below 600 bar. Pressures between 600 and 1500 bar are best suited to initiate germination.

At 40 °C the number of viable spores could be reduced by the factor of 10^6 during a processing time of 4 h. Most of this time the sample can be exposed to 600 bar and only a few minutes at a higher pressure is necessary. The resulting number of less than 100 spores per ml is particularly convenient for applicaitons in the field of foodstuffs. For example, canned foods are allowed to contain up to 1000 germs per g dry weight [13].

To achieve sterile solutions, work had to be carried out at 50 °C. After 3 h no germs could be found in samples that originally had contained $10^{8.5}$ spores per ml. In this case a multi-step process yielded much better results than a treatment with only one germination interval followed by a single high pressure application.

5. References

[1] Hite, B. H., Bulletin of the West Virginia University Agricultural Experiment Station No. 59 (1899) – [2] Zobell, C. E., Johnson, F. H., J. Bacteriol. **57**, 159 (1949) – [3] Zobell, C. E., Morita, P. Y., J. Bacteriol. **73**, 563 (1957) – [4] Zobell, C. E., Cobet, A. B., J. Bacteriol. **84**, 1228 (1962) – [5] Timson, M. W., Short, A. J., Biotechnol. Bioengineering **7**, 139 (1965) – [6] Gould, G. W., Sale, A. J. H., J. Gen. Microbiol. **60**, 335 (1970) – [7] Gould, G. W., Sale, A. J. H., Hamilton, W. A., J. Gen. Microbiol. **60**, 323 (1970) – [8] Butz, P., Ries, J., Traugott, U., Weber, H., Ludwig, H., Pharm. Ind. **52**, 487 (1990) – [9] Ludwig, H., Bieler, C., Hallbauer, K. Scigalla, W., High Pressure and Biotechnology Colloque INSERM **224**, 25 (1992) – [10] Moran, L., Rowe, M. T., Hagan, J. A., Lett. Appl. Microbiol. **10**, 43 (1990) – [11] Woese, C., Morowitz, H. J., J. Bacteriol. **76**, 81 (1958) – [12] Wallhäuser, K. H., Praxis der Sterilisation, Georg Thieme Verlag, Stuttgart – New York (1984) – [13] Gould, G. W., Dring, G. J. Nature **258**, 402 (1975)

For the authors: Prof. Dr. H. Ludwig, Institut für pharmazeutische Technologie und Biopharmazie, Gruppe Physikalische Chemie, Universität Heidelberg, Im Neuenheimer Feld 346, D-69120 Heidelberg (Fed. Rep. of Germany)

Editor: Viktor Schramm. Publisher: ECV · Editio Cantor Verlag für Medizin und Naturwissenschaften GmbH, P.O.B. 12 55, D-88322 Aulendorf (Fed. Rep. of Germany), phone: (0 75 25) 9 40-0, fax: (0 75 25) 9 40-1 80. Printed by Vereinigte Buchdruckereien A. Sandmaier u. Sohn, D-88422 Bad Buchau (Fed. Rep. of Germany).

Delivery conditions: The journal is published monthly. It is supplied by the publisher and by booksellers. Annual subscription rate (minimum 12 issues): 285.– DM plus postage, foreign annual subscription rate: 338.– DM plus postage. Sale per copy: 36.– DM plus postage. VAT always included. Free specimen copies are supplied by the publisher upon request.

Printed in the Federal Republic of Germany · ISSN 0031-711 X

High Pressure and Biotechnology. Eds C. Balny, R. Hayashi, K. Heremans & P. Masson.
Colloque INSERM/John Libbey Eurotext Ltd. ©1992. Vol. 224, pp. 25-32.

Inactivation of microorganisms by hydrostatic pressure

Horst Ludwig, Christian Bieler, Kai Hallbauer and Wilhelm Scigalla

Institut für pharmazeutische Technologie und Biopharmazie, Gruppe Physikalische Chemie, Universität Heidelberg, Im Neuenheimer Feld 346, 6900 Heidelberg, Federal Republic of Germany

SUMMARY

The pressure inactivation of E. coli is studied up to 5 kbar in the temperature range between 1 and 50 °C in different aqueous solutions. The inactivation rate increases with pressure and is minimal at room temperature. It does not depend on the composition of the solvent as long as some water is present.
The germination of bacterial spores is investigated in dependence on pressure, temperature and additives. The best conditions are medium pressure, high temperature and some additives like salts, amino acids and glucose. The germinated spores can then be inactivated.

INTRODUCTION

Destruction of microorganisms is one of the most important effects in high pressure treatment of food or drugs. First experiments in this area have been done nearly hundred years ago. Later on, research was concentrated upon pressure tolerant or barophilic microorganisms taken from the deep sea and on high pressure physiology of cells and animals (Zimmerman et al.,1970; Sleigh et al.,1972; Jannasch et al.,1987). Also, pressure effects on various biochemical systems have been investigated (Heremans,1978 and 1987; Wong,1987; Weber,1987). Recently a lot of work has been published in Japan in respect of food processing (Hayashi,1989 and 1990).
The kinetics of pressure inactivation has been studied for bacteria, bacterial spores, yeasts, moulds including their spores (Butz et al.,1986;1990;1991), and viruses (Brauch et al.,1990; Carl et al.,1991). Simple first order reactions were found for the inactivation of vegetative bacteria only, whereas all the other microorganisms and viruses resulted in complicated kinetic curves.
In the first part of this report it will be shown that deviations of the first order might also occur in the case of vegetative bacteria, and in the second part some new results will be given about bacterial spores which are the most difficult microorganisms to inactivate.

INACTIVATION OF COLI BACTERIA

Freshly prepared E. coli were used in each experimental run. The preparations were always cultured from a single organism (one plaque) and grown until the stationary phase was reached. The experimental device (Butz et al.,1990) consisted of ten small pressure vessels that were filled with the samples, simultanously pressurized and thermostated. The single vessels could be opened independently in order to determine the number of viable organisms at any given time.

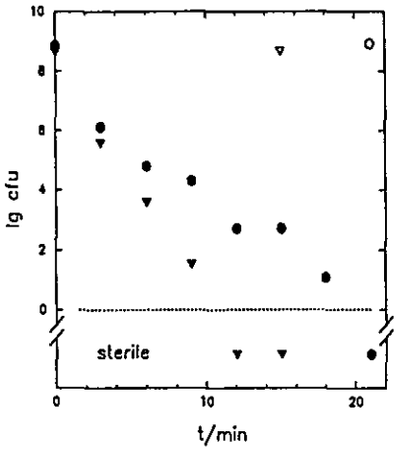


Fig.1 Pressure inactivation of E.coli at 2.5 kbar, ● 40 °C,▼ 50 °C, open symbols: controls.

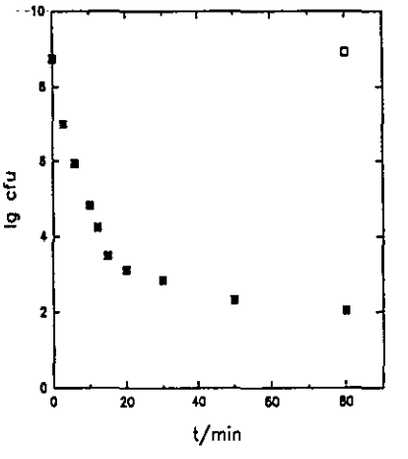


Fig.2 Pressure inaktivation of E.coli at 2.5 kbar and 25 °C, □ control.

Figure 1 gives results. The logarithm of colony forming units, i.e. the number of living bacteria per ml, is plotted on the ordinate and the time on the abscissa. Starting with 10^9 bacteria per ml and under an applied pressure of 2.5 kbar sterile solutions were obtained in 10 and 20 min at temperatures of 50 or 40 °C. Straight lines can be drawn through the data indicating first order reactions. This changes drastically when the temperature drops below 30 °C. Figure 2 gives an example at 25 °C of how the curve now looks. This holds for all the lower temperatures down to 0 °C. The shape of this inactivation curve does not depend on the initial concentration. For different initial concentrations it can vertically be shifted without changes. A small fraction of all bacteria seems to be less sensitive to pressure when the temperature is lower than 30 °C. This could be explained by an altered membrane composition of these bacteria with the consequence of a liquid-gel transformation in the membrane near 30 °C. Erbe (1990) reports on such phase transitions in E. coli membranes.

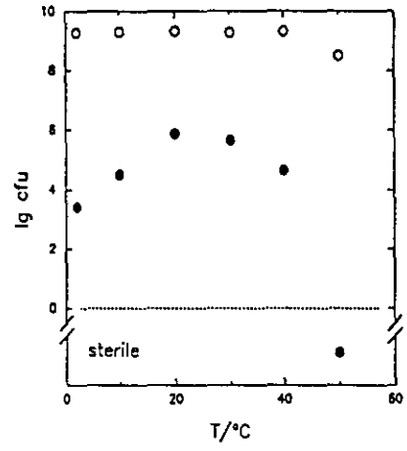


Fig.3 Temperature dependence of E.coli inactivation, 15 min at 2 kbar, o control.

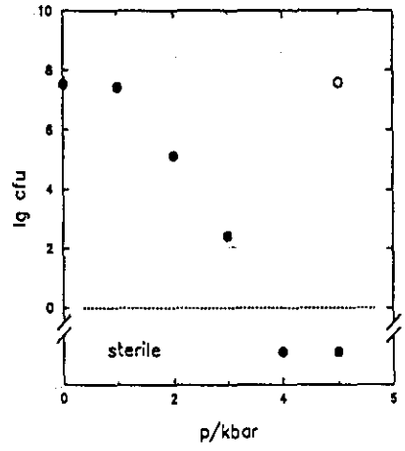


Fig.4 Pressure dependence of E.coli inactivation, 9 min at 4 °C, o control.

Figure 3 gives the temperature dependence of inactivation at 2 kbar. The inactivation rate has a minimum at room temperature. The faster inactivation at lower temperature might be caused by the participation of hydrophobic forces and of membrane processes.
The inactivation of E. coli is strongly dependent on pressure. This is shown in Fig.4, which gives data for 9 min of pressure treatment at 4 °C, and in Fig.5 where the inactivation course at 25 °C is presented for pressures between 2 and 5 kbar. The numbers on the ordinate hold for 5 kbar the other curves being vertically shifted each by two logarithmic units which is allowed as described above.

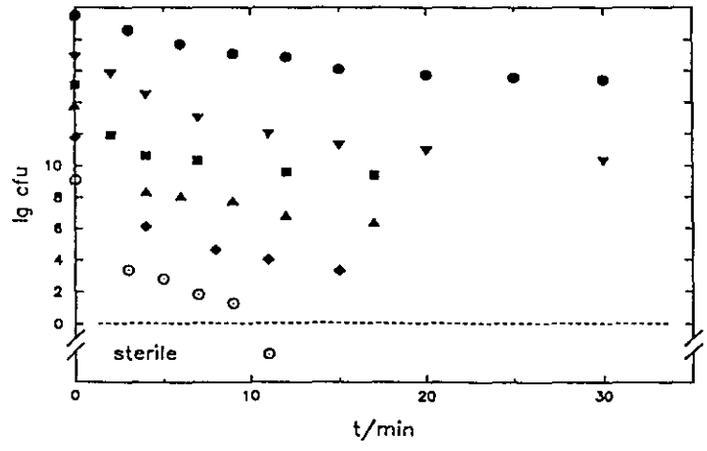


Fig.5 Pressure inactivation of E.coli at 25 °C and different pressures of ● 2, ▼ 2.5, ■ 3, ▲ 3.5, ◆ 4 and ○ 5 kbar.

The rate of inactivation is the same in nutrient solution, physiological NaCl solution and in a 1:1 mixture of NaCl solution with glycerol. The rate, as well as the amount of bacteria that is more pressure sensitive, is reduced to about 60 per cent in pure glycerol, possibly because the membrane is influenced (Fig.6).
For practical purposes it can be stated that aqueous solutions of various compositions containing 10^9 coli bacteria per ml can be sterilized in 10 min using 4 kbar at 4 °C or 5 kbar at 25 °C or 2.5 kbar at 50 °C.

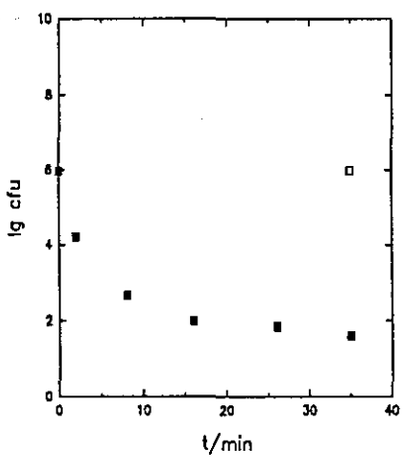


Fig.6 Pressure inactivation of E.coli in Glycerol at 2.5 kbar and 25 °C, □ control.

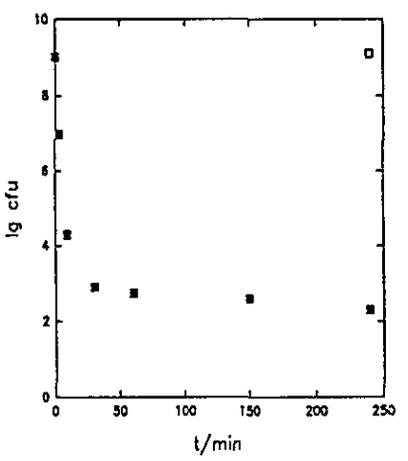


Fig.7 Pressure inactivation of B. stearothermophilus at 2.5 kbar and 60 °C, □ control.

INACTIVATION OF SPORE FORMING BACTERIA

Figure 7 shows the kinetics of Bacillus stearothermophilus inactivation. A fast decrease of viable cells in the first ten minutes is followed by a much slower inactivation process; several hours are needed to obtain sterile solutions. Whereas in the fast step the vegetative Bacilli are killed, the slow step is caused by the spores. In Fig.8 and 9 the temperature and pressure dependence for the inactivation of vegetative forms can be seen; it looks similar to the diagrams of E. coli (Fig.3 and 4) with the only exception that plateaus are reached at high temperature and at high pressure. These plateaus again indicate the spore fraction. Similar results are obtained for B. subtilis, but its spores are more sensitive to pressure than those of B. stearothermophilus (Fig.10).

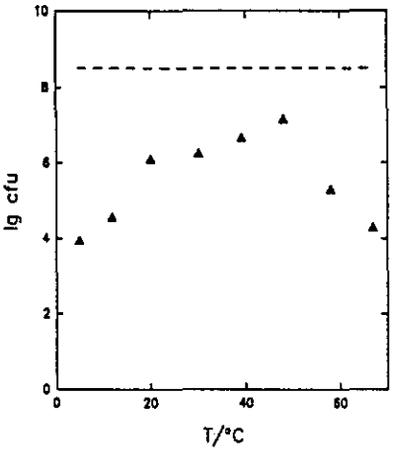


Fig.8 Temperature dependence of B. stearothermophilus inactivation, 15 min at 2 kbar; – – – control.

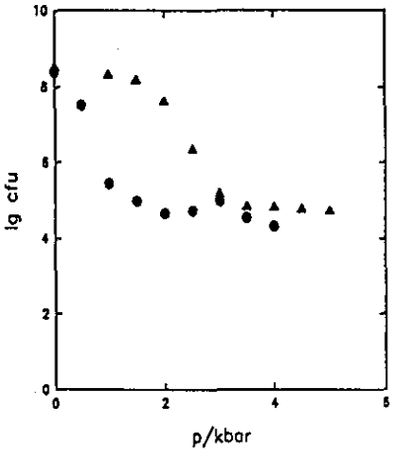


Fig.9 Pressure dependence of B. stearothermophilus inactivation, 15 min, ● at 4 °C and ▲ at 25 °C.

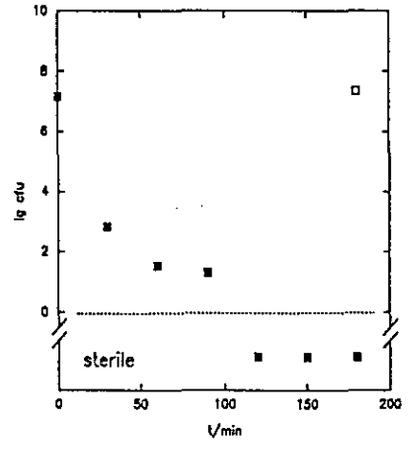


Fig.10 Pressure inactivation of B. subtilis at 2 kbar and 30 °C; □ control.

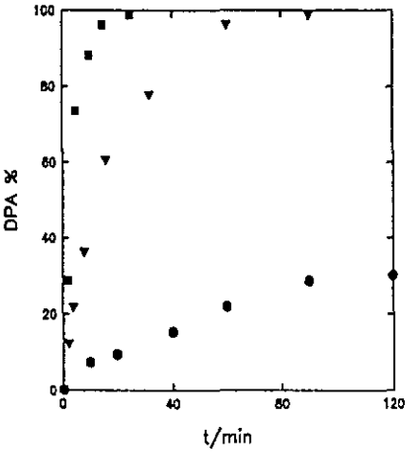


Fig.11 Kinetics of DPA release from B. subtilis spores in 0.9% NaCl solution at 1 kbar, ● 20 °C, ▼ 30 °C, ■ 40 °C.

It has been shown that spores can be killed by a combined pressure and temperature treatment (Seyderhelm et al.,1992; Butz et al.,1990). Medium pressures of about 2 kbar seem better than very high pressures; the best results are obtained when the germination process which runs fastest at low pressure is decoupled from the inactivation of the germinated spores that is done at high pressure (Butz et al.,1990).

Since pressure only kills the germinated forms of spores it seems necessary to study the germination process in detail. Fig.11 shows the kinetics of germination of B. subtilis spores, as measured by the release of Dipicolinic acid (DPA) which quantitatively describes the germination process. The time needed for full germination (100 per cent DPA) depends strongly on the temperature. There is optimal DPA release at about 2 kbar (Fig.12). The germination rate depends on the salt content of the solution (Fig.13). Most DPA is released

near an ionic strength of 0.14 M. This corresponds to 0.14 M NaCl (physiological solution) or to 0.05 M $CaCl_2$; no special ion effect can be seen. The germination induced at normal pressure by an appropriate medium is further improved by pressures between 1 and 2.5 kbar (Fig.14 and 15).

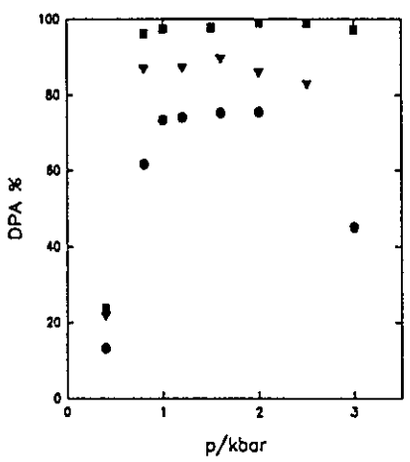


Fig.12 Pressure dependence of DPA release from B. subtilis spores in 0.9% NaCl solution at 40 °C,
● 5 min, ▼ 10 min; ■ 30 min.

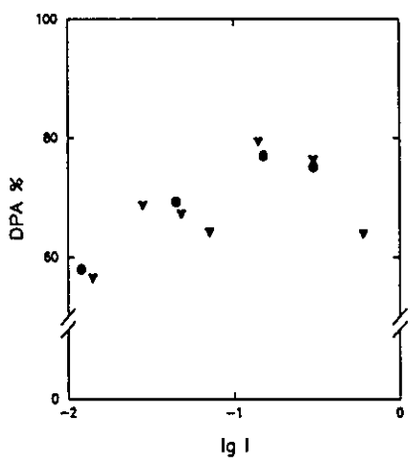


Fig.13 Pressure induced DPA release from B. stearothermophilus spores at different ionic strenght I, 1 kbar and 40 °C,
● $CaCl_2$, ▼ NaCl.

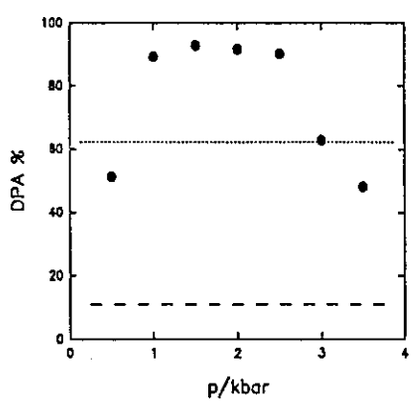


Fig.14 Pressure dependence of DPA release from B. subtilis spores in a germination inducing medium at 40°C (NaCl, $MgCl_2$, $MnCl_2$, L-Alanine, Phosphatbuffer of pH 7), 5 min at normal pressure followed by 10 min under the pressure indicated, DPA release after – – –5 min or ····· 15 min without pressure.

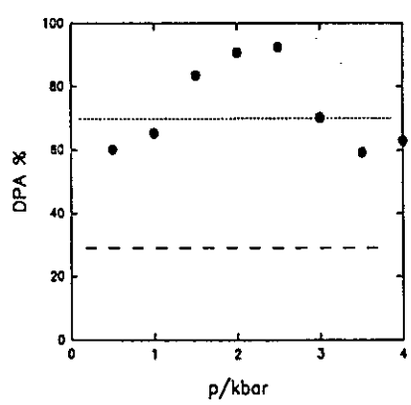


Fig.15 Pressure dependence of DPA release from B. stearothermophilus spores in a germination inducing medium at 65 °C (L-Arginine instead of L-Alanine in the medium), 10 min at normal pressure followed by 10 min under pressure, DPA release after – – – 10 min or ····· 20 min without pressure.

CONCLUSION

Inactivation of vegetative bacteria by pressure presents no diffulty in principle, but careful kinetic studies have to be done to reach full safety. Sterilisation times can be greatly extended when deviations from first order kinetics occur. Inactivation is slowest at room temperature; high and low temperatures give better results. Spores can only be inactivated when they are caused to germinate. This is best achieved by medium pressures, high temperatures and suitable solvents containing salts, amino acids and glucose. Either one or better several of these conditions must be given. Water seems to be necessary for an effective inactivation of microrganisms.

REFERENCES

Brauch, G., Hänsler, U. and Ludwig, H. (1990): The effect of pressure on bacteriophages. High Pressure Research 5:767-769.

Butz, P. and Ludwig, H. (1986): Pressure inactivation of microorganisms at moderate temperatures. Physica 139 u.140B:875-877.

Butz, P., Ries, J., Traugott, U., Weber, H. and Ludwig, H. (1990): Hochdruckinaktivierung von Bakterien und Bakteriensporen. Pharm. Ind.52 (4):487-491.

Butz, P. and Ludwig, H. (1991): Hochdruckinaktivierung von Hefen und Schimmelpilzen. Pharm. Ind.53 (6):584-586.

Carl, K. and Ludwig, H. (1991): Pressure effects on bacteriophage T4. High Pressure Research 7:176-178.

Eze, M.O. (1990): Consequences of the lipid bilayer to membrane-associated reactions. J. Chem. Educ. 67:17-20.

Hayashi, R., ed. (1989): Use of high pressure in food. Kyoto: SAN-EI Pub. Co., Japan.

Hayashi, R., ed. (1990): Pressure processed food. Research and development. Kyoto: SAN-EI Pub. Co., Japan.

Heremans, K. (1978): Pressure effects on biochemical systems. In High Pressure Chemistry, NASI series: Series C, Vol.41, ed. H. Kelm, pp.467-487. Dordrecht, The Netherlands.

Heremans, K. (1987): Pressure effects on the reactions of heme proteins. In High Pressure Chemistry and Biochemistry, NASI series. Series C, Vol.197, ed. R. van Eldik and J. Jonas, pp.421-445.

Jannasch, H.W., Marquis, R.E. and Zimmerman, A.M., ed. (1987): Current Perspectives in High Pressure Biology. London: Academic Press.

Sleigh, M.A. and Macdonald, A.G., ed. (1972): The effects of pressure on organisms. Symposia of the society for experimental biology Nr.26. Cambridge: University Press.

Weber, G. (1987): Dissociation of oligomeric proteins by hydrostatic pressure. In High Pressure Chemistry and Biochemistry, NASI series. Series C, Vol.197, ed. R. van Eldik and J. Jonas, pp.401-420.

Wong, P.T.T. (1987): High pressure studies of biomembranes by vibrational spectroscopy. In High Pressure Chemistry and Biochemistry, NASI series. Series C, Vol.197, ed. R. van Eldik and J. Jonas, pp.381-400. Dordrecht, The Netherlands.

Zimmerman, A.M., ed. (1970): High pressure effects on cellular processes. New York and London: Academic Press.

高圧による微生物の不活性化

大腸菌（ *E. coli* ）の圧力による不活性化を1 ～50℃の温度範囲で、溶媒を変え、5 kbarまでの圧力をかけて検討した。その結果、不活性化速度は圧力と共に増加し、室温では最低となる。いく分の水が存在する限り、不活性化速度は溶媒の組成に依存しない。圧力、温度および添加物を変化させ細菌芽胞の発芽を調べたところ、最良の条件は中程度の圧力、高温、添加物（塩、アミノ酸、グルコース）の存在のときであった。このようにすれば発芽した胞子を不活性化することができる。

Résumé (traduction des éditeurs)

L'inactivation par la pression d'E. coli dans différentes solutions aqueuses est étudiée jusqu'à 5 kbar, dans une gamme de température allant de 1 à 50 °C. La vitesse de l'inactivation augmente avec la pression et est minimale à température ordinaire. Cette vitesse ne dépend pas de la composition du solvant, à condition que de l'eau soit présente.

La germination des spores bactériennes est étudiée en fonction de la pression, de la température et de divers composés. Les meilleures conditions trouvées sont une pression moyenne, une température élevée avec l'addition de produits comme des sels, des acides aminés et du glucose. Dans ce cas, la germination des spores peut être inactivée.