Non-Thermal Bacteria Inactivation With Dense CO_2

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Abstract: The use of CO_2 under pressure (dense CO^2) is one of the most promising techniques to achieve cold pasteurization and/or sterilization of liquid and solid materials, and is likely to replace or partially substitute currently and widely applied thermal processes. Although the ability of CO_2 to inactivate microorganisms has been known since the 1950s, only within the last 15 years it has received special attention, and the scientific and economic interest towards practical applications is presently growing more and more. Here we collect and discuss the relevant current knowledge about the potentials of dense CO_2 as a non-thermal technology in the field of microbial inactivation. We summarize the state of the art, including definitions, description of the equipment, relevant applications, in both simple suspensions and complex media, for the treatment of a wide range of microorganisms in both liquid and solid substrates. Finally, we also summarize and discuss the different hypotheses about the mechanisms of inactivation. © 2003 Wiley Periodicals, Inc. *Biotechnol Bioeng* **84**: 627–638, 2003.

Keywords: CO_2; high pressure; microorganisms; inactivation

INTRODUCTION

Non-thermal processes have gained increasing importance in recent years as a potentially valuable technology to replace or at least complement the traditional processes currently applied for microbial inactivation. Compared to traditional techniques, they avoid drawbacks such as retention of flavor, denaturation of nutrients, production of side toxic reactions as well as changes in physical, mechanical, and optical properties of the material involved in the treatment (Dempsey and treatment Thirucote, 1989; Konig et al., 1997; Nair, 1995).

Among them, the use of CO_2 under pressure seems to be the most promising technique. This possibility was first addressed in the 1950s by Fraser (Fraser, 1951) and Foster (Foster et al., 1962) who reported about the disruption of bacteria cells by the rapid release of CO_2 gas from a pressure of 500 lbf/in^2 (about 34 atm) to ambient pressure. The proposed process involved a pressurization step, to make the applied gas able to penetrate the microbial cells, and subsequent explosive decompression resulting in rapid gas expansion within the cells. Even though this hypothesis of inactivation now has been overtaken, as it will be discussed in a later section, Fraser and Foster et al. were the first to evidence the phenomenon.

In 1969 Swift & Co. (Chicago, IL) obtained the first U.S. patent for food product sterilization with CO_2 (Kauffman et al., 1969). They claimed that a sterile food product could be produced without flavor degradation by subjecting foodstuffs to an atmosphere of CO_2 at ''super-atmospheric'' pressure and by exposing them to relatively low radiation dosages. In addition, the inhibitory effect of CO_2 increased when CO_2 was applied under pressure (Kauffman et al., 1969).

Since 1980 others have reported the bacteriostatic action and inhibitory effect of CO_2 on growth and metabolism of some microorganisms. *Pseudomonas* were found to be very sensitive while other types, such as *Lactobacillus* and *Clostridium* reacted less sensitively (Doyle, 1983; Enfors and Molin, 1980; Jones and Greenfield, 1982; Molin, 1983). In 1981 Blickstad et al reported that a CO_2- modified packaging atmosphere extended the shelf-life of perishable foods, in particular pork meat, (Blick Stad, 1981) and in 1983 Shibata and Anpo obtained a Japanese patent on the sterization of lever sheets in CO_2 atmosphere (Shibata and Anpo, 1983). Although these early authors did not address the effect of CO_2 on microbes under a pressure as high as its critical value (which is 73.8 bar), they can be considered as the pioneers of research activity in this field.

The use of pressurized CO_2 was also studied to combat pest insects and mites. In the early 1980s a process for pest control was developed, at pressure up to 40–50 bar and ambient temperature (Gerard et al., 1988; Quirin, 1988). The efficiency of this process was demonstrated on *Lasioderma serricorne* (Cigarette bettle), *Tribolium confusum*, *Stegobium paniceum* (drugstore bettle), *Plodia interpunctella*

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(Indian meal moth), and *Acarus siro* (flour mite). A 100% mortality of different pest insects was guaranteed after a treatment time in the range of 5–120 minutes, depending on the pressure applied and the stage of the insect's development. Quirin commenting on several articles published in the early 1980s (Quirin et al., 1988), pointed out the antimicrobial effect of dense CO_2 on different kind of insect pests, bacteria, viruses, and fungi and underlined the potential of this technique both in pest control of food storage and in the sterilization in pharmaceutical and foodstuff processing.

However, it is with the work published in 1987 by Kamihira (Kamihira et al., 1987), that the inhibitory effect of CO_2 under pressure started to be addressed systematically. These authors tested the sterilizing effect of CO_2 at supercritical (SC), liquid (L), and gaseous (G) states towards wet and dry *Escherichia coli*, *Staphylococcus aures*, and conidia of *Aspergillus niger* by using a supercritical fluid extraction apparatus.

Following this work, number of publications on this topic steadily increased over the years: In this review we examine more than 50 articles from the open literature (half of which were published in 1999–2002) and include 15 patents.

We have structured this review as follows: First, the effects of dense CO_2 on vegetative and latent forms of microorganism are thoroughly reported and discussed, considering all significant articles published in the field. A brief description follows about the high-pressure equipment needed to perform the process with dense CO_2. Then, we summarize the most relevant hypotheses drawn by different authors concerning microbial inactivation mechanisms. In conclusion, we focus on potential industrial applications and future research needs.

EFFECTS OF DENSE CO_2 ON MICROORGANISMS

In this section we deal with the CO_2 effects on vegetative forms and spores separately, considering both simple suspensions and complex media.

Vegetative Forms

Kamihira et al. (1987) reports that Baker's yeast, *E. coli* and *S. aureus* were completely sterilized by SC-CO_2 at 200 atm and 35°C while no sterilizing effect was detached with G- and L-CO_2. The water content within the cell was found to play a major role in the sterilization process, as dry cells (i.e., with a water content less than 10%) were not inactivated even with SC-CO_2.

After Kamihira's work, especially in recent years, there have been many publications and patents on the effects of dense CO_2 on microorganisms. Table I is a compilation of the experimental results that can be found in the literature from 1987 on, with indication of the substrate, the type of microorganism and the conditions of CO_2 treatment. The most relevant studies will be briefly discussed in this section.

It is clear that CO_2 is, in general, able to substantially reduce the microbial activity of any microorganism at relatively mild operating conditions. Most researches found that higher treatment time, pressure, and temperature increase the inactivation effect. This could be further enhanced by using specific entrainer. For instance, trace amounts of sulphur dioxide (30 ppm in CO_2) were found to be a good additive to improve the activity reduction of wet cells of *Saccharomyces cerevisiae* (Lin et al., 1992a). In any case, moisture plays a fundamental role in inactivation efficiency, as dried cells were particularly recalcitrant to SC-CO_2 antimicrobial action. Industrial researchers were also soon attracted by the bacteriostatic action of dense CO_2. For example, Smelt and Rijke (1992) confirmed the strong synergism between temperature and pressure on various microorganisms. Even more interestingly, these authors compared the hydrostatic pressure (HP) treatment, with the SC-CO_2 treatment and reported that CO_2 can be as lethal as a hydrostatic pressure process but at much lower pressure, i.e., 150 bar against 3000 bar. The HP technique has indeed attracted a larger number of researchers, as can be seen from the volume of articles published and number of conferences organized (for instance, see Balny et al., 1992). The main limitation of this method is the difficulty of controlling and managing the operating pressure in such an extreme range of values, thus its widespread use in industry appears limited. Despite of this, a number of products has been already processed with HP treatment at industrial scale, mainly in Japan (Sonoike, 1997).

Results similar to Smelt's were found by Lin on *Lenconostoc dextranicum* (Lin et al., 1993). Again, the effect was clearly shown to be due to a specific interaction between CO_2 and the cell, not to high pressure itself, as the use of nitrogen at the same temperature and pressure conditions did not lead to any microorganism inactivation. This fact confirms that CO_2 exerts a peculiar effect on the microbial structure, in particular on the cell membrane, as it will be pointed out in the inactivation mechanisms section. Furthermore, it underlines that the biochemical effect of the gas can provide the major contribution toward the inactivation efficiency of the process, and that it occurs at relatively low pressure (as low as 70 bar), if compared to the pressure range of the HP process.

Another factor important in the inactivation efficiency is the cell age. Young cells were more susceptible to CO_2 treatment than mature ones. It has been shown that upon entering the stationary phase of growth (37°C, 24 h), bacteria synthesize new proteins able to protect cells against a variety of adverse conditions including high temperature, oxidative stress, high salt concentrations, and high pressure (Kashket, 1987; Mackey et al., 1995).

The influence of fat content was considered as well—*Listeria monocytogenes* were found to be more recalcitrant to CO_2 treatment when grown in a media containing fat or oil, which probably exerts a biological effect on the structure of cell walls and membrane and/or physical modification of membrane porosity (Lin et al., 1994).

In 1995, Ishikawa applied CO_2 in a semi-continuous reactor (the so-called ''micro-bubble'' method) and

Table I. Application of dense CO_2 pasteurization treatment on vegetative microbial forms in simple and complex solutions.

Solution	Treatment Regime	Inoculated Microorganism	Maximum Reduction	Temperature (°C)	Treatment Operation	Reference
Physiological saline	200 bar 2 h	*S. cerevisiae* *E. coli* *S. aureus* *A. niger*	7.5 log* 6.5 log* 5 log* 5 log*	35	Extraction vessel	Kamihira et al., 1987
Herbs	800 psi 2 h	*N.D.*	5–8 log*	45	Batch	Haas et al., 1989
Fruit juice	800 psi 16 h–30 min	*N.D.*	5 log*	22–55	Batch	Haas et al., 1989
Nutrient broth	900 psi 2 h	*E. coli* *S. aereus* *Salmonella senftenberg*	99%	Room temperature	Batch	Haas et al., 1989
Distilled water	61.2 atm 2 h	*Listeria monocytogenes*	9 log*	35	Batch	Wei et al., 1991
Foods	136.1 atm 2 h	*Salmonella*	99%	35	Batch	Wei et al., 1991
YMP (yeast malt bacto peptone extract agar)	1000–3000 psi 15–7 min	*S. cerevisiae*	7 log*	35	Batch (with magnetic stirrer)	Lin et al., 1992b
Growth media	150 bar 1 h	*E. coli*	8 log*	35	Batch	Smelt and Rijke, 1992
Nutrient medium	3000 psi 15 min	*Leuconostoc dextranicum*	9 log*	35	Batch	Lin et al., 1993
Physiological saline	100 bar 5 min	*Candida utilis* *S. cerevisiae* *Kluyveromyces fragilis*	6 log*	33	Batch	Isenschmid et al., 1995
Lactobacillus MRS 5.5% broth	210.9 kg/cm^2 14 min	*Listeria monocytogenes*	9 log*	35	Batch (with magnetic stirrer)	Lin et al., 1994
Milk	1000 psi 1 h	*Listeria monocytogenes*	3 log	45	Batch (with magnetic stirrer)	Lin et al., 1994
Distilled water	40 atm 3.5 h	*S. cerevisiae*	8 log*	40	Batch	Nakamura et al., 1994
Physiological saline	25 Mpa 15 min 30 min	*Lactobacillus brevis* *S. cerevisiae*	6 log*	35	Semi-continuous system	Ishikawa et al., 1997
Physiological saline	50 bar 20 min	*E. coli*	6 log*	35	Batch	Ballestra et al., 1996
Sterilized water	40 atm 4 h	*S. cerevisiae*	8 log*	40	Batch	Enomoto et al., 1997b
BHIB	80 bar 60 min	*S. aureus*	7 log*	25	Batch	Erkmen, 1997
Whole-skim milk	90–146 bar 2–5 h	*S. aureus*	7 log*	25	Batch	Erkmen, 1997
Growth media	60 bar 15 min	*E. coli* *L. brevis* *S. cerevisiae* *Torulopsis versatilis*	9 log*	35	Continuous flow system	Shimoda et al., 1998
Hydrophilic filter disk	55 bar 290 min	*E. coli* *S. cerevisiae* *E. faecalis*	5 log*	Room temperature	Batch	Debs-Louka et al., 1999
Growth media and biodegradable polymers	205 bar 0.6 h 4 h 4 h 4 h 0.5 h 0.6 h 1.5 h	*L. innocua* *S. aureus* *S. salford* *P. aeruginosa* *E. coli* *P. vulgaris* *L. dunnifii*	9 log* 9 log* 9 log* 8 log* 8 log* 8 log* 4 log*	34 40 40 40 34 34 40	Batch	Dillow et al., 1999a
MRS broth/phosphate buffer	2000 psi 30 min	*L. plantarum*	6 log*	30	Batch (with magnetic stirrer)	Hong et al., 1999

Table I. (continued).

Solution	Treatment Regime	Inoculated Microorganism	Maximum Reduction	Temperature (°C)	Treatment Operation	Reference
Different buffers	70 kg/cm^2 120 min	*L. plantarum*	8 log*	30	Batch with magnetic stirrer	Hong and Pyun, 1999
Kimchi vegetable (Korean food)	70 kg/cm^2 120 min	*Natural lactic acid bacteria*	40%	10	Batch with magnetic stirrer	Hong and Park, 1999
PBS buffer	10 Mpa 80 min	*E. faecium* *E. coli,* *L. casei,* *S. cerevisiae*	5–6 log*	40	Batch	Dellaglio et al., 1999
Physiological saline	60.5 bar 18 min	*E. faecalis*	8 log*	35	Batch	Erkmen, 2000a
Fruit juice-milk	60.5 bar 3–6 h 60.5 bar/24 h	*E. faecalis*	5 log*	45	Batch	Erkmen, 2000a
Nutrient broth	100 bar 50 min	*E. coli*	6 log*	35	Batch	Erkmen, 2001a
Milk	100 bar 6 h	*E. coli*	6–7 log*	35	Batch	Erkmen, 2001a
Physiological saline	60.5 bar 100 min	*Brocothirix thermosphacta*	5.5 log*	35	Batch	Erkmen, 2000b
Skinned meat	60.5 bar 150 min	*Brocothirix thermosphacta*	5 log*	45	Batch	Erkmen, 2000b
Physiological saline	60 bar 15 min	*Salmonella typhimurium*	7 log*	35	Batch	Erkmen and Karaman, 2001
PS containing BHIB	60 bar 140 min	*Salmonella typhimurium*	7 log*	25	Batch	Erkmen and Karaman, 2001
PS containing BHIB	60 bar 60 min	*Listeria monocytogenes*	6 log*	45	Batch	Erkmen, 2000c
Skim and whole milk	60 bar 16–24 h	*Listeria monocytogenes*	6 log*	45	Batch	Erkmen, 2000c
Alfalfa seeds	4000 psi 1 h	*E. coli*	92.8%	50	Batch	Mazzoni et al., 2001
Milk	3000 kpa 60–120–180 min	*N.D.*	< 2 log	50	Batch	Calvo and Balcones, 2001
Buffer solution	310.5 bar 15 min	*S. aureus* *E. coli*	7 log	42.5	Extraction vessel	Sirisee et al., 1998
Ground beef	310.5 bar 120–180 min	*E. coli* *S. aereus*	1 log 4 log	42.5	Extraction vessel	Sirisee et al., 1998
Physiological saline	74 bar 2.5 min	*B. subtilis* *Ps. aeruginosa*	7 log*	38	Semi-continuous system	Spilimbergo et al., 2002b

* = total inactivation; *N.D.* not detected.

achieved a greater efficiency than in the batch device previously used; *Bacillus brevis* and *S. cerevisiae* were studied as test microorganisms (Ishikawa et al., 1995). Accordingly, CO_2 was continuously bubbled into a pressurized cell through a porous filter (10 μm pore diameter) for a convenient time (4 min), then the CO_2 flow was stopped. The pressure was maintained at the selected level for a suitable exposure time and then gently released. Ishikawa's results were clearly obtained thanks to the improved mass-transport: In the micro-bubble apparatus, a wider interface between gas and liquid phase is assured, resulting in higher efficiency.

It was soon evident that microbial inactivation was related to CO_2 concentration. Among others, Kumagai et al. (1997) evaluated the amount of CO_2 sorbed by a microbial cell–water system by a gravimetric method, and investigated the correlation between CO_2 sorption and sterilization effect on *S. cerevisiae* in an aqueous system. The time-course for the survival ratio of the microbial cells was found to be a first-order kinetics:

$$Log\left(\frac{No}{N}\right) = k \cdot t \cdot 2,303 \qquad (1)$$

where k is the sterilization rate constant (h^{-1}).

The dependence of k on water content within the cells and on CO_2 pressure was evaluated. Water dissolves CO_2 relatively well, therefore the amount of CO_2 sorbed by the microbial cells as well as the value of k increased with increasing water content until free water appeared around the cells (critical water content). Over this critical value, the microbial cells became saturated with water and k remained almost constant. Furthermore, by increasing the pressure with an identical water content, it was possible to enhance

the value of *k*. Shimoda et al. (1998) determined the effects of the dissolved CO_2 concentration, the treatment pressure, and the rate of depressurization on inactivation, comparing a continuous-flow system and a batch one. It was found that the microbial inactivation was directly influenced by CO_2 concentration in the sample which, in turn, is influenced by CO_2 flow rate. By this continuous method, microorganisms (*E. coli*, *S. cerevisiae*, *Torulopsis versatilis*) were inactivated effectively in the range of 0.16–0.9 g/cm^3 of CO_2 density.

Such complete inactivation was attributed to cell bursting induced by explosive expansion of CO_2 previously dissolved in the cells.

Interestingly, in 1999 Debs-Louka and co-workers studied the antimicrobial effect of CO_2 on solid materials. Microbial cells of *E. coli*, *S. cerevisiae* and *Enterococcus faecalis* were inoculated onto solid hydrophilic medium and treated at room temperature (Debs-Louka et al., 1999). The effects of pressure and exposure time on the loss of viability was also addressed by a response-surface methodology. This work confirmed a linear correlation between microbial inactivation and exposure time; the authors proposed a model to predict adequately their experimental values. It was also observed that the pH end-points of the treated sample were lower by 1.5 times with respect to the control sample (6.8 pH) but they did not vary when different experimental conditions were applied.

Microbes differ in their resistance to the inhibitory effect of CO_2. This behavior can be related to the cell envelope and its permeability. For instance, Dillow et al. (1999a) achieved a complete inactivation of a wide variety of bacterial organisms (*Bacillus cereus*, *Listeria innocua*, *S. aureus*, *Salmonella salford*, *Pseudomonas aeruginosa*, *E. coli*, *Preoteus vulgaris*, *Legionella dunnifii*) at moderate temperature with a batch system. When biodegradable polymers were included in the sterilization apparatus, their results did not change. In addition, these authors confirmed (by FTIR, GPC, and DSC analyses) the absence of any chemical or physical damage in these thermally and hydrolytically labile material.

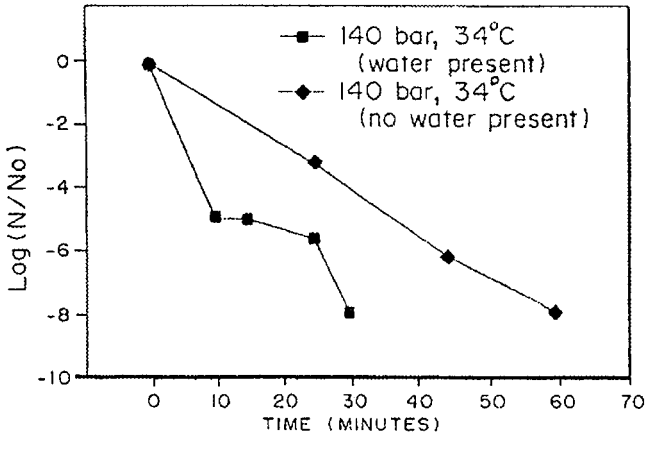

Figure 1. Comparison of sterilization kinetics in SC-CO_2 in the presence and in the absence of water at 34°C and 140 bar. Data taken from Dillow et al. (1999a).

Finally, their experimental results underlined again the fundamental role of water for the efficiency of the inactivation kinetic—dried *E. coli* exposed to SC-CO_2 could be inactivated as well, but the rate was much lower, (Fig. 1).

A *Lactobacillus* species, acid-tolerant bacterial cells typically found in dairy products, was chosen as a test microorganism by Hong (Hong et al., 1999). It was shown that microbial inactivation was mainly dependent on the transfer rate of CO_2 into cells and process effectiveness could be improved by increasing pressure and temperature. An example of the inactivation kinetic is given in Fig. 2.

The effect of dense CO_2 on microorganisms was tested not only on aqueous suspensions but also on complex liquid and solid substrates. For instance, in 1989 Haas successfully addressed the antimicrobial action of CO_2 on various foods (Haas et al., 1989). However, disruption of the texture of some products, such as strawberries, honeydew melon, and cucumber was observed, and the food samples showed a color change after treatment, especially orange juice and eggs—they became less yellow probably due to the removal of carotenoid pigments. These authors also explored to some extent the influence of extra cellular pH, discovering that a synergistic effect in the inactivation was evident when raising the temperature and lowering the pH at the same time. In particular, pH was measured as a function of pressure and it was shown that even pH values considerably below those of the nutrient broth under pressure (4.35) did not have the same anti microbial effect as CO_2 at 900 psi (Haas et al., 1989). The treatment of food samples was also considered by Wei and co-workers (1991), with good results on *Listeria* and *Salmonella* species. These authors reported that nitrogen at the same pressure, time, and temperature as for CO_2 (137 atm N_2 for

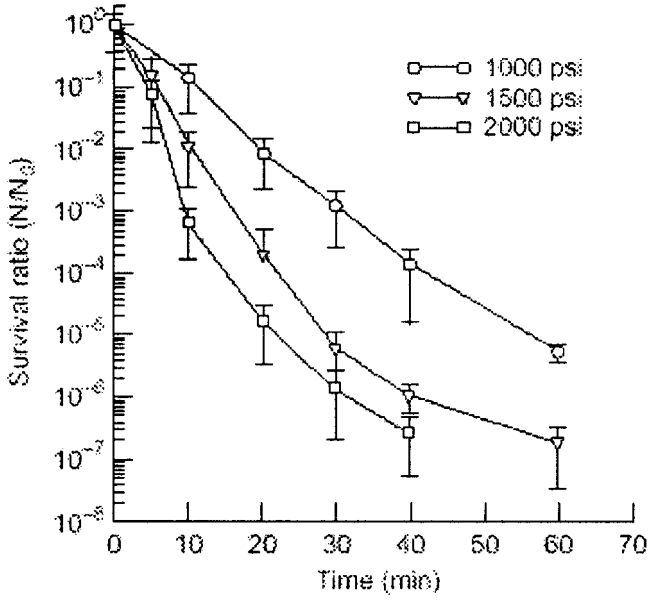

Figure 2. Inactivation of *L. plantarum* in 0.1*M* phosphate as a function of pressure and time, at 35°C. Data taken from Hong et al. (1999).

2 h at 35°C) neither reduced the microbial counts, nor affected the pH, confirming that the bacterial effect was also due to the peculiar use of CO_2 in complex systems.

The application of this technique to foodstuff has received more and more interest especially in recent years. For instance *Beach kimchi* (Chinese cabbage) was treated with high pressure CO_2 at 70 atm for 24 h (Hong and Park, 1999). A smaller lactic population than in the untreated sample was detached and further evaluation showed that sourness and overall acceptability of treated ''fermented vegetable food'' were better than those of the untreated one. The co1our, flavor, and texture were not significantly affected by the treatment. In this case dense CO_2 effectively inactivated not only airborne and exposed surface bacteria but could also penetrate porous materials to affect microbes inside the food; this result confirmed the potential of the CO_2 technique as a nonthermal process for food preservation.

Erkmen's group reported a series of studies regarding, the microbial effect of pressurized CO_2 on a range of foodborne microorganisms, some of them pathogenic and psychrotrophic, which are well known for their ability to grow on foods and are responsible of spoilage of products quite rapidly. Among others, *S. aureus*, *L. monocytogenes*, *Salmonella typhiurium*, *Brochothrix thermosphacta*, *Escherichia coli*, *E. faecalis*, and *Yersinia enferocolitica* were tested in a batch system with different solution models, such as physiological saline, broth, liquid and solid foods (Erkmen, 1997; Erkmen 2000a–2000d; Erkmen 2001a). In general, two stages were observed in the survival curves. The early one was characterized by a slow rate of reduction of microbes number, which then sharply decreased at a later stage (Fig. 3). It was found that the inactivation rate increased with increasing pressure, temperature, and exposure time and

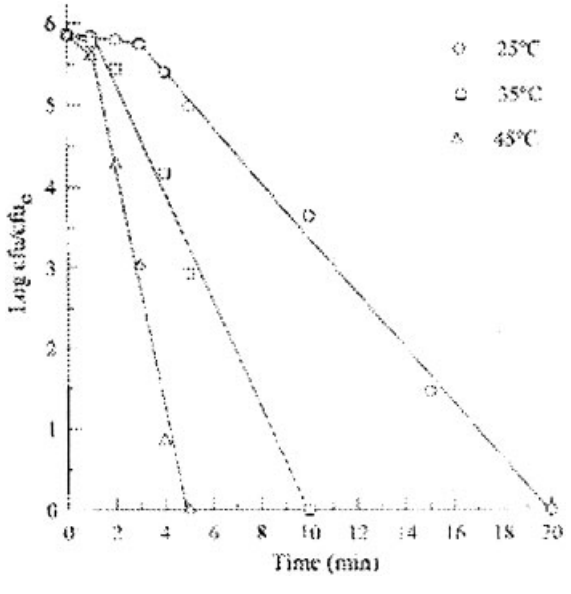

Figure 3. Antimicrobial effect of high-pressure CO_2 on *Brochothrix thermosphacta* as a function of temperature at 6.05 MPa (average pH values of 4.82). Data taken from Erkmen (2000b).

was also dependent on initial number of cells and suspending medium. Obviously, carbohydrates, fats, and other organic compounds in foods increased the resistance of bacteria to CO_2 treatment.

In relation to patients, many have been issued since 1987, mainly in Japan. They deal with the peculiar antimicrobial effect of CO_2 toward either liquid or solid materials, fruit-juice, pastry, and jelly foods at low temperature, obtained in simple apparatuses, primarily operated in batch mode (Komamiya, 1991; Kumagai et al., 1997; Muratsubaki et al., 2001; Ochiai and Nakagawa, 1993; Osajima et al., 1997; Saitou, 1992; Shimizu et al., 1995; Sugimitsu et al., 1999; Sugimoto et al., 1997; Suzuki and Sato, 1988). Interestingly, Mitsubishi Kakoki Kaisha Ltd. holds a patent on the sterilization of powdery and granular material by high-pressure CO_2 (Kobayashi et al., 1987) and Nigata Emg. Co Ltd. claimed that it is possible to sterilize dried blood without damaging its properties by means of CO_2 under specific pressure and temperature (Suzuki and Sato, 1988). In 1995 Aphios Co. (Castor and Hong, 1995) claimed that supercritical fluid treatment can be exploited for intracellular components extraction, as it caused microbial cells lysis and led to cellular materials recovery, i.e., nucleic acids (Castor and Hong, 1990) with negligible product-activity degradation. Recently, two patents (one European and one worldwide), have been granted by Praxair Technology, Inc. (Wildasin et al., 2000; 2002) regarding methods and apparatus for continuous flow reduction of microbial activity in a liquid product using pressurized CO_2. The pressure and temperature in the flow regions is maintained at a level which is sufficient to keep the CO_2 in a continuous liquid state, without freezing the liquid product. When orange juice is used, the contact time is about 3 min and the temperature is kept around 30°C. The pressurized mixture flows through a reaction zone for a sufficient time to reduce harmful microorganisms, then enters one or more expansion stages where CO_2 is depressurized and separated from the juice. Another interesting apparatus has been recently conceived by Porocrit LLC (Sims, 2001): in the first part of this continuous apparatus, a system pressure of 75–150 bar is used to achieve a dissolved CO_2 content of 5–6%. A peculiarity of the system consists of the use of a microporous polypropylene membrane contactor to carbonate the stream rapidly to saturation, which is reached in minutes or seconds depending on the flow rates considered, for both water suspensions and orange juice. In the second part of the apparatus (a holding tube), the inoculated feed is pasteurized by the CO_2 dissolved in the liquid, at a temperature that never exceeds 45°C. Dense CO_2 flow is continuously recirculated without depressurization.

As regards review articles, we quote the recently published one on the use of CO_2 in packing of fresh red meats and its effect on chemical quality changes (Jacobsen and Bertelsen, 2002).

Finally interesting studies about predictive modeling of different microorganisms inactivation are reported by

Erkmen (Erkmen, 2001b), who compared several sigmoidal models (i.e., Schute, Richards, Stannard, and Gompertz ones) to describe inactivation of *L. monocytogenes* under CO_2 pressure. It was found that simple three-parameter models were statistically sufficient to describe mathematically the experimental survival curves for *L. monocytogenes*. Again, temperature, and pressure had a significant effect on the survival ratio. The temperature dependence of the inactivation rate constant of *E. coli* was analyzed based on the Arrhenius linear square-root model. The model enabled the prediction of microbial inactivation at different CO_2 operating conditions of temperature and pressure (Erkmen, 2001c).

Kinetic studies were carried out on the high pressure CO_2 inactivation of *Salmonella typhmurium* and *L. monocytogenes*, which was found to follow a first-order reaction kinetic, with specific D value (D is the time required to decrease the microbial count by 90%) and kinetic inactivation rate k (Erkmen, 2000e; Erkmen and Karaman, 2001). The pressure dependence was expressed by the z value (defined as the pressure needed to reduce the D value by a factor of 10) determined in a wide range of pressures for two different model solutions, physiological solution (PS) and Brain Heart infusion (BHI).

Peleg (2002) showed that isobaric survival curves of *E. coli* can be fitted by a semi logarithmic relation:

$$\log S(t) = -b(P) \cdot t \cdot (n(P)) \qquad (2)$$

where $S(t)$ is the survival ratio $b(P)$ and n(P) are pressure dependent coefficients. By means of a double logistic model, pressure profile and corresponding inactivation rate could be generated. Non-isobaric survival curves can be obtained solving the differential equation:

$$d\log S(t)/dt = -b(t) \cdot n(t) \cdot -\log S(t)/b(t) \cdot ((n(t) - 1)/n(t)) \quad (3)$$

These equations enabled theoretical comparison of processes consisting of single and multiple compression–decompression cycles of equivalent time. However, as the dense-CO_2 technology has not been standardized yet, it is important to point out that the lethal effects measured in test experiments may depend quite significantly on the apparatus used. Hence, any comparison between the results of different research groups should be done carefully, taking into account both the technique applied, and the experimental conditions (i.e., initial counts, temperature, pressure, and SC-CO_2 system).

Latent Forms

Spores are the most resistant forms of bacteria. Their structure is more complex and should, in principle be less attachable by CO_2 when compared to that of vegetative cells. In fact, dormant bacterial endo-spores are highly resistant to a number of physical and chemical treatments which are normally considered germicidal (Setlow, 1995). There has been little few work addressing spore inactivation by supercritical CO_2 published in the literature. As can be determined from the listing in Table II, the literature is both scarce and conflicting.

For instance, Kamihira did not succeed in inactivating endo-spores of *Bacillus subtilis* and *Bacillus stearothermophilus* after a treatment of 2 h at 35°C at 200 bar (Kamihira

Table II. Application of dense CO_2 pasteurisation treatment on latent microbial forms in various solutions.

Solution	Treatment Regime	Microrganism Inoculated	Treatment Result (Max red.)	Temperature (°C)	HP Cell Characteristic	Reference
Physiological saline	200 atm	*B. subtilis*	0.5 log	35	Extraction vessel	Kamihira et al. 1987
	2 h	*B. stearothermophilis*	1 log			
Physiological saline	300 bar				Micro-bubble	Ishikawa et al., 1997
	60 min	*B. cereus*	6 log*	50	method	
	60 min	*B. subtilis*	6 log*	55		
	30 min	*B. megaterium*	6 log*	40		
	60 min	*B. polymyxa*	6 log*	45		
	30 min	*B. coagulans*	6 log*	40		
Sterile distilled water	58 atm	*B. megalerium*	8 log*	60	Batch	Enomoto et al., 1997a
	30 h					
MRS broth	70 kg/cm^2	*Lactobacillus spores*	6 log	30	Batch	Hong et al., 1997
	250 min					
Sterile Ringer solution	50 bar				Batch	Ballestra and Cuq, 1998
	60 min	*B. subtilis*	4 log	80		
	85 min	*Byssochlamys fulva*	90%	80		
	11 min	*Aspergillus niger conidia*	90%	50		
Growth medium	205 bar	*B. cereus*	8 log*	60	Batch	Dillow et al., 1999a
	4 h					
Physiological saline	70 bar	*B. subtilis*	7 log*	75	Batch	Spilimbergo et al., 2002b
	24h					

* = total inactivation.

et al., 1987). Only the addition of ethanol or acetic acid to SC-CO_2 made the process possible—about 50% of *B. stearothermophilus* endo-spores were killed.

On the other hand, the activity of *B. subtilis*, *B. cereus*, *Bacillus megaterium*, and *Bacillus polymyxa* spores was significantly reduced by the SC-CO_2 micro-bubble method (Ishikawa et al., 1997). The treatment at 40°C and 30 MPa for 30 min was claimed to offer a higher efficiency than a heat treatment at 100°C for 60 min. The presence of a filter in the CO_2 inlet flux, acting as a gas sparger, was reported to enhance the inactivation effect of more than 3 log, probably because the CO_2 concentration in spore suspension was increased from 50% to 80% of the saturation by the enhanced contact-surface between the liquid and the gas. Enomoto el al. (1997a) reduced the survival ratio of a sample of 10^8 bacterial spores of *B. megatlerium* in physiological solution by about 7 log with a batch treatment of 30 h at 58 bar and 60°C, and reported that a pressure around 60 bar, under the critical value, yielded optimum efficiency. According to these authors, the aggregation of spores as a result of the pressure applied during pressurization could inhibit the lethal effect of dense CO_2.

In 1998 Ballestra and Cuq (1998) reduced the survival count of *B. subtilis* spores of about 3.5 log and *Byssochlamys fulva* asco-spores of about 1 log, from initial suspensions of 10^7 and 10^5, respectively, with a batch treatment of 1 h, at an operating pressure of 50 bar and a temperature of 80°C. The rate of inactivation was pressure-dependent, at a temperature higher than a threshold value, which, in turn, depended on the test microorganism (Fig. 4). At sublethal or low-lethal temperature the antimicrobial effect of CO_2 was particularly evident, while at a higher temperature it was masked by the effect of heat. The two different slopes in the diagram suggest a double step in the inactivation kinetic, similar to the one observed for vegetative forms (Fig. 2). A first step (heat destruction of *B. subtilis* at 80°C) reflects CO_2 penetration into the cells, which occurs simultaneously with heat activation of dormant spores, cell modifications, activation of enzymes and metabolic system. The second step corresponds to inactivation of germinated spores. The disappearance of the first step at, or above 90°C could be explained by a higher rate of spore activation and easier penetration of CO_2.

In 2002 Spilimbergo et al. achieved the microbial activity reduction of *B. subtilis* spores by coupling the effect of higher temperature (75°C) and longer treatment time (24 h), when compared to bacteria species (Spilimbergo et al., 2002). The application of pressure cycles was also shown to be beneficial. In addition it was found that the effect of a mild heating (60°C) at ambient condition was not sufficient to reduce the microbial counts *B. subtilis* spores while a treatment at the same temperature but at a CO_2 pressure of 90 bar was able to inactivate them completely in 5 h (Spilimbergo, 2002).

Recently a new ''hurdle approach'' was investigated: (Spilimbergo et al., 2003): The coupled effect of CO_2 together with pulse electric field showed interesting results against spores of *B. cereus* at a particularly mild temperature (40°C).

In summary, presently, dense CO_2 alone is indeed able to promote spore inactivation, but at relatively high temperature (not less than 60°C for *B. subtilis*) and thus, is not suitable for practical applications in the food industry.

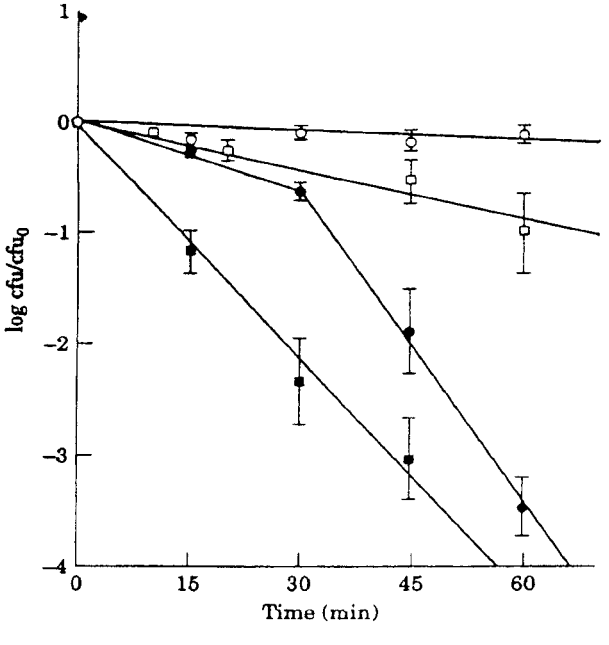

Figure 4. Influence of CO_2 under 5 MPa pressure on the thermal inactivation of *B. subtilis* spores at 80°C (round symbols) and 90°C (square symbols). Filled symbols: 5 MPa, open symbols: control. Data taken from Ballestra and Cuq (1998).

TREATMENT EQUIPMENT

A typical batch CO_2 apparatus for laboratory experiments simply consists of a CO_2 pump, a high pressure vessel, and

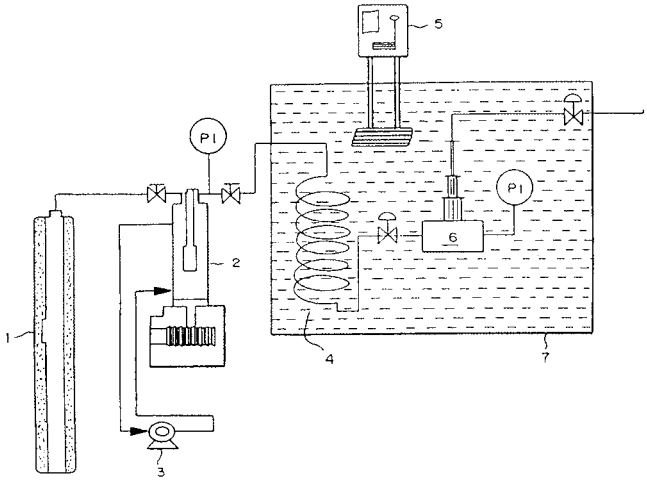

Figure 5. Schematic of batch apparatus: (1) CO_2 cylinder, (2) Syringe pump, (3) pump header heat exchanger, (4) preheating coil, (5) heater, (6) high presure cell, (7) water bath. Taken from Dillow et al., (1999b).

a discharge system to release the pressure after the run. A temperature control system is needed to set and regulate the temperature. An example of this type of apparatus is depicted in Figure 5. It includes a CO_2 cylinder (1), a syringe pump (2), a pump header heat exchanger (3), a preheating coil (4), a heater (5), a high pressure cell (6), and a water bath (7). The high pressure cell is usually constructed by stainless steel (AISI 316), with a total internal volume in the range of 10–100 mL. It should be designed in such a way that all pressurization/depressurization steps may be performed without any loss of media and contamination of the lines via back diffusion. For this reason a filter is usually utilized (with a porosity in the range of 5–10 μm); it is placed before the outlet valve to prevent any loss of material in the gas flow; the cell should also include an opening system to pour and remove the material to be treated, suitable to be hermetically sealed during the experiment. Temperature and pressure probes must be part of the system to monitor the pressure and the temperature values in the cell during the experiment. They can be connected with a programmable controller to automate the process.

The operations to run the apparatus include the following steps (see Fig. 5):

1. Introducing the material to be treated in the high pressure cell (6).
2. Switching the constant temperature control means on [water bath (7), and heater (5)] until the selected temperature has been reached.
3. Pumping the CO_2 from the cylinder (1) through the preheating coil and pump header heat exchanger (3) to the cell (6) at the desired pressure.
4. Leaving the material at the selected temperature and pressure for the selected duration of the process.
5. Depressurizing the cell and removing the treated material.

Note that, as microbial inactivation is essentially governed by penetration of CO_2 into cells, its effectiveness can be significantly increased with adequate agitation (Hong et al., 1997; Lin et al., 1994; 1992b).

MECHANISMS OF MICROBIAL INACTIVATION

Vegetative Forms

A number of hypotheses have been proposed to explain the peculiar action of CO_2 on microorganisms, but there is no definite evidence yet about the prevailing mechanism. Some can be considered as primary, while others are complementary.

In summary, dense CO_2 is claimed to cause:

- Cytoplasmatic pH decrease (acidification)
- Explosive cell rupture due to internal pressure
- Modification of cell membrane and extraction of cell wall lipids
- Inactivation of key enzymes for cell metabolism
- Extraction of intracellular substances

Bicarbonate conversion to carbonate and subsequent intracellular precipitation of salts. *Acidification* has been proposed as the main inactivation mechanism from the beginning (Kamihira et al., 1987). The fact that moisture is essential to achieve a good inactivation level has been observed by almost all the authors cited herein; they agree that CO_2 must dissolve in an aqueous solution, to form carbonic acid which, at a sufficient concentration, is dissociated into bicarbonate and hydrogen ions lowering the extracellular pH.

''Explosive rupture'' of the cells membrane was one of the first theories on the mechanism of microbial inactivation derived by Nakamura et al. 1994.(Ballestra et al., 1996). In their experiments, this team suddenly released the applied CO_2 pressure from the solution. It was thought that during this step CO_2 would have after saturation rapidly expanded through the cells so that a part of them could have been mechanically broken like a popped balloon (Ballestra et al., 1996). However, shortly these after, Hong demonstrated that it was not possible to improve the inactivation by either repeating the load and release of CO_2, or by a flash discharge of gas pressure after the process (Hong et al., 1997), thus seriously questioning the ''explosion'' hyphothesis. On the other hand Arreola et al. (1991) had already observed that a sudden decrease of pressure did not seem to play any significant role in microbial reduction.

A more realistic hypothesis was drawn by Isenschmid et al. 1994; 1995), who attributed the antimicrobial effect of CO_2 to its unique properties of lipo- and hydrophilicity. The cell membrane consists of a double layer of phospho-lipids the inner of which is lipophilic, so that the authors hypothesized that the CO_2 could easily penetrate into the membrane, lead to an increase of its fluidity and permeability, alter its characteristics, and destroy its essential domains (the so-called ''anaesthesia effect''). This hypothesis was supported by S.E.M. micrographs showing that about 25% of *S. cerevisiae* cells had intact plasma membranes, with neither protein complexes aggregation nor shrinking, while the viability was less than 2.5%. Afterwards, other micrographs presented by Dillow et al. (1999a) as well as by Hong and Pyun (1999), definitively proved that the mechanisms did not involve cell rupture due to increased internal pressure. In fact, cell walls were shown to remain largely unchanged after the treatment and the external cell shape displayed no signs of deformation. On the other hand, T.E.M. images showed a modification of cell membrane with possible leakage of cytoplasm, together with enlarged periplasmic space between the walls and the cytoplasmatic membranes (Hong and Pyun, 1999). For this reason the theorized ''anesthesia effect'' was resumed and deepened. It was suggested that CO_2 can easily diffuse through the membrane and accumulate inside the cell.Under

pressure it is likely that enough CO_2 passes through the membrane to lower the internal pH by exceeding the buffer capacity of the cell pool, so that the pH gradient and the proton motive force across the membrane collapse (Hong and Pyun, 1999). The bicarbonate formed, as well as molecular CO_2, can interfere and be fatal in the cell metabolism and on certain enzymatic and biochemical pathways. For instance, decarboxylases are known to be inhibited by a great excess of CO_2, breaking the metabolic chain (Jones and Greenfield, 1982).

In 1992 Lin et al. (1992b) had already pointed out that once the concentration of CO_2 is built up to a critical level within the cells, it is able to extract constituents to an extent that is sufficient to modify the structure of the membrane or disturb the biological system. This theory was confirmed by Hong, who investigated the physiological changes of *Lactobacillus plantarum* by high pressure CO_2 treatment (Hong and Pyun, 2001).

Another possible explanation for the peculiar bactericidial effect of CO_2 is the ''intracellular precipitation'' of carbonate Ca^+, Mg^+ from bicarbonate, between cell and cell wall, due to pressure release (Lin et al., 1993). Higher temperature favors the diffusivity of CO_2 and could possibly relax the cell wall to ease penetration (Isenschmid et al., 1995). However, some authors suggest that the inactivation process should not be operated at temperature far above the critical point of the fluid: too high values weaken the extraction power apart from enhancing food degradation (Lin et al., 1993).

Even though the question of explaining the inactivation mechanisms is not totally answered yet, it is clear that the key factor is the enhanced concentration of CO_2 in the aqueous solution, which is a function of the CO_2 pressure and temperature. This may also lead to acidification of the medium but, above all, causes the chemical modification of the lipid double-layer of the membrane, thus increasing dramatically its permeability to CO_2, which can rapidly diffuse into the cell itself. At this stage the gas can easily interact with the cytoplasm, compromise its biological balance, alter its metabolism, cause an irreversible intracellular pH decrease and modify, at least partially, its enzymatic activity. All of these factors contribute to the observed fast microbial death.

Latent Forms

Bacteria species produce spores in the stationary phase of their life cycle, only under particularly adverse conditions. Spores can survive at temperatures as high as 100°C as they are completely different from bacteria. Their envelope consists of a complex and resistant structure including wall, cortex, and coat. At temperatures typical of living cells, the lipid molecules in the two-layer membrane are in motion while the fluidity of spores membrane is much less than that of the corresponding vegetative forms. In addition the quantities of bound and free water in spores are about 65% and 74%, respectively, of the corresponding water content in the vegetative forms of bacteria, thus spores are dehydrated (Ishihara et al., 1999).

In relation to spore destruction, it is well known that they have to be activated to germinate before being inactivated. It means that spores must undergo a shock (e.g., heat treatment at 80°C for 10 min) and afterwards they can start to germinate. This step is quite fast (minutes) and determines a loss of rifrangence, an increase of the cell capacity of binding colorants and chemical agents, a loss of cortex components, the recall of water and the synthesis of new DNA, RNA, and proteins (Madigan et al., 2002).

One of the possible hypotheses for spore inactivation may be that the coupled effect of temperature (at least 60°C) and CO_2 induces a first shock in the spore structure that is able to lead to their activation. Consequently, during the long contact time of CO_2 treatment they could start their germination. At this point the coat has been destroyed, the cells recall water and the changed structure may become more sensitive to the antimicrobial effect of CO_2, so that inactivation can eventually take place.

This hypothesis has been confirmed by the fact that a higher antimicrobial efficiency can been achieved by pressure cycles (Spilimbergo et al., 2002). In this case tyndallization effect could take place as well. This phenomenon arises when spore germination occurs during the holding period between two treatments. Under such conditions, the first pressure cycles would induce spore activation, so that germination would take place during the holding time between two different cycles. In the following cycles, after spore activation and germination, inactivation would be made possible.

However, more studies are needed on spore inactivation mechanisms by CO_2 treatments.

THE POTENTIAL OF SC-CO_2 TECHNOLOGY AND FUTURE WORK

Currently many industrial sectors are considering the application of CO_2 technology in the processing of foods and biodegradable pharmaceutical and cosmetic materials; however none of the interested sectors is currently using CO_2 under pressure in their production lines. Since dense CO_2-prototype equipment is already widely available, the extensive use of CO_2 as a nonthermal pasteurization treatment is probably just a matter of few years. In view of this goal, we believe that the next future research development of CO_2 treatment should now move to deepen new issues, such as the retention of vitamins and the modifications of cell enzymes, and, in general, the effect of CO_2 processing on food, pharmaceutical, or cosmetic components.

References

Arreola AG, Balaban MO, Marshall MR, Peplow AJ, Wei CL, Cornell JA. 1991. Supercritical carbon dioxide effects on some quality attributes of single orange juice. J Food Sci 56(4):1030–1033.

Ballestra P, Cuq J-L. 1998. Influence of pressurized carbon dioxide on the thermal inactivation of bacterial and fungal spores. Lebensm-Wiss Technol 31(1):84–88.

Ballestra P, Dasilva AA, Cuq J-L. 1996. Inactivation of *Escherichia coli* by carbon dioxide under pressure. J Food Sci 61(4):829–831, 836.

Balny C, Hayashi R, Heremans K, Masson P. 1992. High pressure and biotechnology. Montrouge, France: Colloque INSERM/ John Libbey Eurotext Ltd publisher.

Blickstad E, Enfors SO, Moli G. 1981. Effect of hyperbaric carbon dioxide pressure on the microbial flora of pork stored at 4 or 140°C. J Appl Bacteriol 50:493–504.

Calvo MM, Balcones E. 2001. Inactivation of microorganisms and changes of proteins during treatment of milk with subcritical carbon dioxide. Milchwissenschaft-Milk-Science-Intl 56(7):366–369.

Castor TP, Hong GT. 1990. Supercritical fluid disruption of microbial-cells. Abstr Pap Am Chem Soc 199:N108 (CELL).

Castor TP, Hong GT. 1995. Supercritical fluid disruption of and extraction from microbial cells. Aphios Corp. Patent No. US 5380826.

Debs-Louka E, Louka N, Abraham G, Chabot V, Allaf K. 1999. Effect of compressed carbon dioxide on microbial cell viability. Appl Environ Microbiol 65(2):626–631.

Dellaglio F, Dallacasa V, Venturi A, Bertucco A, Pallado P. 1999. Effect of supercritical carbon dioxide on some bacteria and yeast of interest in the food industry. In: Ludwig H, editor. Advances in high pressure bioscience & biotechnology. Heidelberg, Germany: Springer Verlag. p 121–124.

Dempsey DJ, Thirucote RR. 1989. Sterilization of medical devices: A review. J Biomat Appl 3(3):454–523.

Dillow AK, Dehghani F, Hrkach JS, Foster NR, Langer R. 1999a. Bacterial inactivation by using near- and supercritical carbon dioxide. Proc Natl Acad Sci USA 96(18):10344–10348.

Dillow AK, Langer RS, Foster N, Hrkach JS. 1991b. Supercritical fluid sterilization method. Massachusetts Institute of Technology, Patent No. US 1999066960.

Doyle MP. 1983. Effect of carbon dioxide on toxin production by *Clostridium botulium*. Eur J Appl Microbiol Biotechnol 17(1):53–56.

Enfors SO, Molin G. 1980. Effect of high concentrations of carbon dioxide on growth rate of *Pseudomonas fragi*, *Bacillus cereus* and *Streptococcus cremoris*. J Appl Bacteriol 48(3):409–416.

Enomoto A, Nakamura K, Hakoda M, Amaya N. 1997a. Lethal effect of high-pressure carbon dioxide on a bacterial spore. J Ferment Bioeng 83(3):305–307.

Enomoto A, Nakamura K, Nagai K, Hashimoto T, Hakoda M. 1997b. Inactivation of food microorganisms by high-pressure carbon dioxide treatment with or without explosive decompression. Biosci Biotechnol Biochem 61(7):1133–1137.

Erkmen O. 1997. Antimicrobial effect of pressurized carbon dioxide on *Staphylococcus aureus* in broth and milk. Food Sci Technol-Lebensmittel-Wissenschaft & Technologie 30(8):826–829.

Erkmen O. 2000a. Antimicrobial effect of pressurised carbon dioxide on *Enterococcus faecalis* in physiological saline and foods. J Sci Food Arg 80(4):465–470.

Erkmen O. 2000b. Antimicrobial effects of pressurised carbon dioxide on *Brochothrix thermosphacta* in broth and foods. J Sci Food Arg 80(9): 1365–1370.

Erkmen O. 2000c. Effect of carbon dioxide pressure on Listeria monocytogenes in physiological saline and foods. Food Microbiol 17(6):589–596.

Erkmen O. 2000d. Inactivation of *Salmonella typhimurium* by high pressure carbon dioxide. Food Microbiol 17(2):225–232.

Erkmen O. 2000e. Predictive modeling of Listeria monocytogenes inactivation under high pressure carbon dioxide. Lebensmittel-Wissenschaft Und-Technologie 33(7):514–519.

Erkmen O. 2001a. Effects of high-pressure carbon dioxide on *Escherichia coli* in nutrient broth and milk. Int J Food Microbiol 65(1–2):131–135.

Erkmen O. 2001b. Kinetic analysis of Listeria monocytogenes inactivation by high pressure carbon dioxide. J Food Eng 47(1):7–10.

Erkmen O. 2001c. Mathematical modeling of *Escherichia coli* inactivation under high-pressure carbon dioxide. J Biosci Bioeng 92(1):39–43.

Erkmen O, Karaman H. 2001. Kinetic studies on the high pressure carbon dioxide inactivation of *Salmonella typhimurium*. J Food Eng 50(1): 25–28.

Fraser D. 1951. Bursting bacteria by release of gas pressure. Nature 167: 33–34.

Foster JW, Cowan RM, Maag TA. 1962. Rapture of bacteria by explosive decompression. J. Bacteriol 83 (330–334).

Gerard VD, Kraus J, Quirin K-W, Wohlgemuth R. 1988. Aswendung von Kohlendioxid (CO_2) unter Druck zur Bekampfung vorratsschadlicher Insekten und Milben. (use of pressurized carbon dioxide (CO_2) to combat pest insects and mites) Die Pharmaceutiche Industrie 50: 1298–1300.

Haas GJ, Prescott HE, Dudley E, Dik R, Hintlian C, Keane L. 1989. Inactivation of microorganisms by carbon dioxide under pressure. J Food Safety 9:253–265.

Hong S-I, Park W-S. 1999. High-pressure carbon dioxide effect on Kimchi fermentation. Biosci Biotechnol Biochem 63(6):1119–1121.

Hong S-I, Park W-S, Pyun Y-R. 1997. Inactivation of *Lactobacillus sp*. from kimchi by high-pressure carbon dioxide. Food Sci Technol (London) 30(7):681–685.

Hong S-I, Park W-S, Pyun Y-R. 1999. Non-thermal inactivation of *Lactobacillus plantarum* as influenced by pressure and temperature of pressurized carbon dioxide. Int J Food Sci Technol 34(2):125–130.

Hong SI, Pyun YR. 1999. Inactivation kinetics of *Lactobacillus palntarum* by high pressure carbon dioxide. J Food Sci 64(4):728–733.

Hong SI, Pyun YR. 2001. Membrane damage and enzyme inactivation of *Lactobacillus plantarum* by high pressure CO_2 treatment. Int J Food Microbiol 63(1–2):19–28.

Isenschmid A, Marison IW, Stockar UV. 1995. The influence of near-critical and supercritical CO2 on the viability and yeast cells. International meeting on Supercritical Fluids, Strasbourg (France) ISBN 2-905 267-23-8, 367-370.

Isenschmid A, Marison IW, von Stockar U. 1995. The influence of pressure and temperature of compressed CO_2 on the survival of yeast cells. J Biotechnol. 39(3): 229–237.

Ishihara Y, Saito H, Takano J. 1999. Differences in the surface membranes and water content between the vegetative cells and spores of *Bacillus subtilis*. Cell Biochem Funct 17(1):9–13.

Ishikawa H, Shimoda M, Shiratsuchi H, Osajima Y. 1995. Sterilization of microorganisms by the suprecritical carbon dioxide micro-bubble method. Biosci Biotechnol Biochem 59(10):1949–1950.

Ishikawa H, Shimoda M, Tamaya K, Yonekura A, Kawano T, Osajima Y. 1997. Inactivation of *Bacillus* spores by the supercritical carbon dioxide micro-bubble method. Biosci Biotechnol Biochem 61(6): 1022–1023.

Jacobsen M, Bertelsen G. 2002. The use of CO_2 in packaging of fresh red meats and its effect on chemical quality changes in the meat: A review. J Muscle Foods 13(2):143–168.

Jones RP, Greenfield PF. 1982. Effect of carbon dioxide on yeast and fermentation. Enz Microb Technol 4:210–223.

Kamihira M, Taniguchi M, Kobayashi T. 1987. Sterilization of micro-organisms with supercritical carbon dioxide. Agric Biol Chem 51(2): 407–412.

Kashket ER. 1987. Boienergetics of lacti acid bacteria: cytoplasmic pH and osmotolerance. FEMS Microb Rev 46:233–244.

Kauffman FL, Shank JL, Urbain WM. 1969. Irradiation with CO_2 under pressure, US 3483005.

Kobayashi T, Taniguchi M, Kamihira M, Aki T, Murakami T, Tashiro H. 1987. Sterilization of powdery or granular material. Mitsubishi Kakoki Kaisha Ltd., Patent no. JP 62074270.

Komamiya K. 1991. Sterilizing method. K. Komamiya, Patent no. JP 03000065.

Konig C, Ruffieux K, Wintermantel E, Blaser J. 1997. Autosterilization of biodegradable implants by injection molding process. J Biomed Mat Res 38(2):115–119.

Kumagai H, Hata C, Nakamura K. 1997. CO_2 sorption by microbial cells

and sterilization by high-pressure CO_2. Biosci Biotechnol Biochem 61(6):931–935.

Lin HM, Cao NJ, Chen LF. 1994. Antimicrobial effect of pressurized carbon-dioxide on listeria-monocytogenes. J Food Sci 59(3):657–659.

Lin HM, Yang Z, Chen LE. 1992a. Inactivation of *Saccharomyces cerevisiae* by supercritical and subcritical carbon dioxide. Biotechnol Prog 8(5):458–461.

Lin HM, Yang ZY, Chen LF. 1992b. Inactivation of *Saccharomyces cerevisiae* by supercritical and subcritical carbon-dioxide. Biotechnol Prog 8(5):458–461.

Lin HM, Yang Z, Chen LE. 1993. Inacticvation of *Leuconostoc dextranicum* with carbon dioxide under pressure. Chem Eng J (Lausanne) 52(1): B29–B34.

Mackey BM, Forestiere K, Isaacs N. 1995. Factors affecting the resistance of *Listeria monocytogenes* to high hydrostatic pressure. Food Biotechnol 9:1–11.

Madigan MM, Martinko John M, Parker J. 2002. Brock, biology of microorganisms, Prentice Hall PTR, Paramous.

Mazzoni AM, Sharma RR, Demirci A, Ziegler GR. 2001. Supercritical carbon dioxide treatment to inactive aerobic microorganisms on alfalfa seeds. J Food Safety 21(4):215–223.

Molin G. 1983. The resistance to carbon dioxide of some food related bacteria. Eur J Appl Microbiol Biotechnol 18(4):214–217.

Muratsubaki R, Nishida N, Takazawa Y, Shimizu T. 2001. Method and device for sterilization. Surgino Mach Ltd., Patent no. 2001259002.

Nair PD. 1995. Currently practised sterilization methods—Some inadvertent consequences. J Biomater Appl 10(2):121–135.

Nakamura K, Enomoto A, Fukushima H, Nagai K, Hakoda M. 1994. Disruption of microbial cells by flash discharge of high-pressure carbon dioxide. Biosci Biotechnol Biochem 58(7):1297–1301.

Ochiai S, Nakagawa Y. 1993. Method for high-pressure sterilizing treatment. Toppan Printing Co. Ltd., Patent No. JP 05007480.

Osajima Y, Shimoda M, Kono T, Okubo K. 1997. Apparatus for treatment with fluid under supercritical condition. Nippion Tansan KK Shimadzu, Patent No. JP 09206044.

Peleg M. 2002. Simulation of *E coli*. inactivation by carbon dioxide under pressure. J Food Sci 67(3):896–901.

Quirin KW, Stahl E, Gerard D. 1988. Dense gases fur extraction and refining. Berlin: Springer Verlag. p 218–223.

Saitou Y. 1992. Method for low-temperature sterilization of juice. Iwatani International Corp., Patent No. JP 04126056.

Setlow P. 1995. Mechanisms for prevention of damage to DNA in spores of *Bacillus* species. Ann Rev Microbiol 49:29–54.

Shibata T, Anpo K. 1983. Sterilization of laver. Koasa Shoji KK, Patent No. JP 58158161.

Shimizu M, Kimura T, Ujita G, Sato M, Oda T. 1995. Sterilization of liquid material. Kao Corp., Patent No. JP 07289220.

Shimoda M, Yamamoto Y, Cocunubo-Castellanos J, Tonoike H, Kawano T, Ishikawa H, Osajima Y. 1998. Antimicrobial effects of pressured carbon dioxide in a continuous flow system. J Food Sci 63(4):709–712.

Sims M. 2001. Method and membrane system for sterilizing and preserving liquids using carbon dioxide. Porocrit LLC., Patent No. US 6331272.

Sirisee U, Hsieh F, Huff HE. 1998. Microbial safety of supercritical carbon dioxide processes. J Food Process Pres 22(5):387–403.

Smelt JPPM, Rijke GGF. 1992. High pressure treatment as a tool for pasteurization of foods. In: Balny C, Hayashi K, Heremens K & Masson P, editors. High Pressure Biotechnology. Colloque INSERM/ John Libey Eurotext Ltd. publisher, Montrouge, France, High Press Biotechnol 224:361–364.

Sonoike K. 1997. High pressure sterilization technology. Subject for the application to food. Journal of the Japanese Society For Food Science and Technology-Nippon Shokuhin Kagaku Kogaku Kaishi 44(7): 522–530.

Spilimbergo S. 2002. A study about the effect of dense CO_2 on microorganisms. PhD thesis, Department of Chemical Engineering, University of Padova, Italy.

Spilimbergo S, Dehghani F, Bertucco A, Foster NR. 2003. Inactivation of bacteria and spores by pulse electric field and high pressure CO_2 at low temperature. Biotechnol Bioeng 1(82):118–125.

Spilimbergo S, Elvassore N, Bartucco A. 2002. Microbial inactivation by high-pressure. J Supercrit Fluids 22(1):55–63.

Sugimitsu H, Ito J, Sayato Y. 1999. Sterilization of food by ozone. J Food Hygienic Soc Japan 40(5):1369–1373.

Sugimoto T, Itagaki N, Kimura H, Wada E, Hayashi N, Takeda T, Yamamoto S. 1997. Insecticidal cereal storage and cereal storage vessel. Fujimori Kogyo KK, Patent No. JP 09163927.

Suzuki H, Sato M. 1988. Sterilization of dried blood. Nigata Eng. Co. Ltd., Patent No. JP 63119663.

Wei CI, Balaban MO, Fernando SY, Peplow AJ. 1991. Bacterial effect of high-pressure CO_2 treatment on foods spiked with listeria or salmonella. J Food Protect 4(3):189–193.

Wildasin RE, Forbes J, Robey RJ. 2000. Method and apparatus for continuous flow reduction of microbial activity in a liquid product using pressurized carbon dioxide. Praxair Technology, Inc., Patent No. US EP 979657.

Wildasin RE, Forbes J, Robey RJ, Paradis AJ. 2002. Treating liquid food and other products using carbon dioxide. Praxair Technology, Inc., Patent No. US WO 0203816.