ARTICLE IN PRESS

ELSEVIER

Available online at www.sciencedirect.com

SCIENCE DIRECT®

Journal of Biotechnology xxx (2006) xxx–xxx

Journal of BIOTECHNOLOGY

www.elsevier.com/locate/jbiotec

Effective terminal sterilization using supercritical carbon dioxide

Angela White, David Burns, Tim W. Christensen *

NovaSterilis Inc., Lansing, NY 14882, United States

Received 22 August 2005; received in revised form 23 November 2005; accepted 15 December 2005

Abstract

Gentle alternatives to existing sterilization methods are called for by rapid advances in biomedical technologies. Supercritical fluid technologies have found applications in a wide range of areas and have been explored for use in the inactivation of medical contaminants. In particular, supercritical CO_2 is appealing for sterilization due to the ease at which the supercritical state is attained, the non-reactive nature, and the ability to readily penetrate substrates. However, rapid inactivation of bacterial endospores has proven a barrier to the use of this technology for effective terminal sterilization. We report the development of a supercritical CO_2 based sterilization process capable of achieving rapid inactivation of bacterial endospores while in terminal packaging. Moreover, this process is gentle; as the morphology, ultrastructure, and protein profiles of inactivated microbes are maintained. These properties of the sterilization process suit it for possible use on a wide range of biomedical products including: materials derived from animal tissues, protein based therapies, and other sensitive medical products requiring gentle terminal sterilization.
© 2006 Elsevier B.V. All rights reserved.

Keywords: Supercritical fluid; Carbon dioxide; Spore inactivation; Peracetic acid

1. Introduction

Current methods of sterilization, which include ethylene oxide (EtO), gamma radiation, electron-beam, steam, and hydrogen peroxide plasma, have limitations with respect to their biomedical applications. These methods have been shown to alter the structure and characteristics of materials (Goldman et al., 1997; Klapperich et al., 2000; Ferreira et al., 2001; Digas et al., 2003; Willie et al., 2004), especially when applied to thermally and hydrolytically sensitive polymers. Steam sterilization, for example, is conducted at high temperatures and thus cannot be applied to thermally sensitive products, which include almost all biomaterials and drug formulations. Hydrogen peroxide plasma produces large amounts of free radicals in order to achieve sterilization (Clapp et al., 1994). These free radicals may adversely react with the chemistry of the sterilized material and degrade metal alloys (Duffy et al., 2000; Ferreira et al., 2001). In addition to the inherent restrictions of each method, several methods present environmental hazards due to the chemical or physical nature of the sterilizing agent (Osterman-

* Corresponding author. Tel.: +1 607 592 3074.
E-mail address: tim@cnymail.com (T.W. Christensen).

0168-1656/$ – see front matter © 2006 Elsevier B.V. All rights reserved.
doi:10.1016/j.jbiotec.2005.12.033

ARTICLE IN PRESS

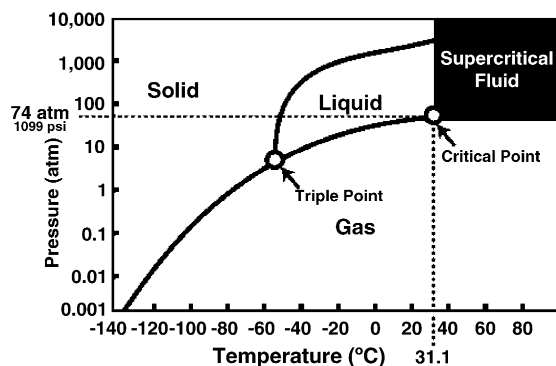

Fig. 1. Phase diagram of carbon dioxide. Carbon dioxide transitions to supercritical relatively readily at the critical points of 31.1 °C and 1099 psi.

Golkar and Bergmark, 1988; Florack and Zielhuis, 1990).

Additional alternatives are needed to fill the growing demand for the safe and efficient sterilization of increasingly sophisticated and sensitive biomedical products. A viable supercritical CO_2 sterilization process would help fill the gap in the existing sterilization methods by addressing many of their limitations. In turn, a new sterilization process would enable the continued development of novel biomedical products outside of the restrictions imposed by existing sterilization methods.

The use of supercritical CO_2 for inactivation of organisms continues to attract attention (Spilimbergo and Bertucco, 2003). Carbon dioxide has unique properties that make it an appealing medium for sterilization. At relatively low pressures and temperatures carbon dioxide transitions to a supercritical state, often referred to as a dense phase gas (Fig. 1). The properties of supercritical CO_2 lend themselves to deep penetration of substrates which has lead to uses in areas ranging from bioremediation to natural product extraction (van der Velde et al., 1992; Ge et al., 2002). The use of supercritical CO_2 as a sterilant is all the more appealing because it is non-toxic and easily removed by simple depressurization and out gassing.

Thus far supercritical CO_2 sterilization has not delivered on its promise as a potential sterilant. This is due in large part to the inability of existing methodologies to achieve industrial levels of sterilization (Spilimbergo and Bertucco, 2003). This level of sterilization for medical devices calls for a sterility assurance level of 10^{-6} (SAL10^{-6}) (A.A.I., 1995; FDA, 1993; ISO14937, 2000; Block, 2001). A SAL10^{-6} is defined as the probability of a given product being contaminated after treatment is one in a million when starting with an initial bioburden of $\geq 10^6$ colony forming units (CFUs) of a bioindicator (FDA, 1993; ISO14937, 2000). The bioindicator (BI) is a species of bacteria that is reasonably more resistant than the most resistant organism expected to contaminate a given product (FDA, 1993; ISO14937, 2000). Traditionally the BI for specific sterilization processes has been the sporular form of a given bacterial species. Bacterial spores have consistently been chosen as BIs because of their high resistance to different sterilization processes. Todate, many of those supercritical CO_2 processes that have been reported are not capable of inactivating bacterial spores (Spilimbergo and Bertucco, 2003). As such they may only be characterized as achieving high-level disinfection. Moreover, the methods that are capable of inactivating spores require that spores be in aqueous solution and often at high temperatures. Maintaining many biomedical products in aqueous solution especially at high temperatures can cause significant product deterioration. This along with the complication of removing excess moisture renders these methods problematic. Packaging also becomes a concern as aqueous sterilization is not easily compatible with terminal sterilization which must be performed on products in their final gas permeable packaging. For supercritical CO_2 sterilization to become a viable sterilization method it must consistently and rapidly achieve inactivation of bacterial endospores, be performed in a non-aqueous environment, and be able to achieve terminal sterilization.

Here we report the development of a supercritical CO_2 based sterilization apparatus and process that is capable of achieving validated SAL10^{-6} levels of terminal sterilization, at relatively low temperatures, in a short amount of time, with a minimum of moisture.

2. Materials and methods

2.1. Reagents

Ninety nine percent trifluoroacetic acid (Aldrich), 88% formic acid (Mallinckrodt), 100% ethanol

ARTICLE IN PRESS

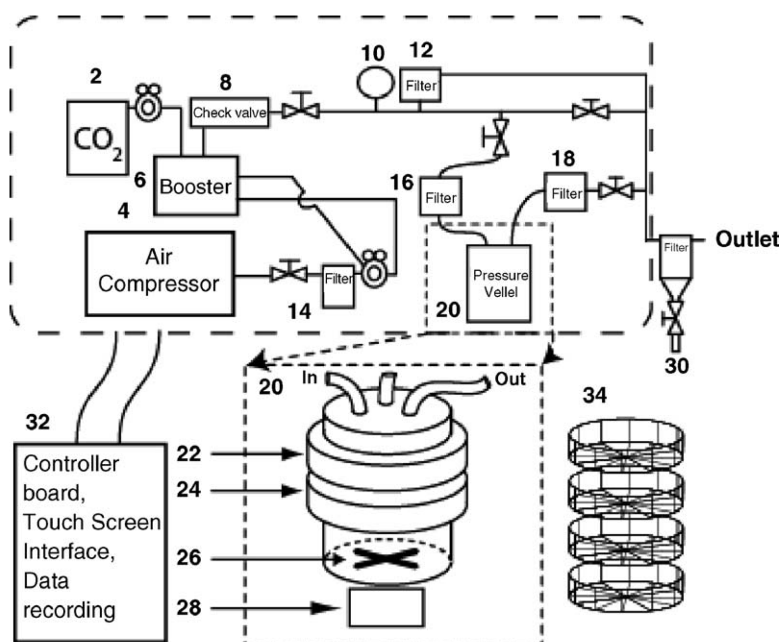

Fig. 2. Schematic diagram of supercritical CO_2 sterilization apparatus, for detailed description see Section 2. Diagram represents the essential components of both the initial 600 mL apparatus and the subsequent 20 L Nova2200 apparatus.

anhydrous (Fisher), glacial acetic acid (ACROS organics), 32% peracetic acid (Aldrich), 50% hydrogen peroxide (ACROS organics), 50% citric acid dissolved in distilled water (Mallinckrodt), succininc acid (Mallinckrodt), phosphoric acid (Sigma).

2.2. Apparatus

The apparatus, presented in Fig. 2, includes a standard compressed gas cylinder (2) containing carbon dioxide and a standard air compressor (4) used in conjunction with a CO_2 booster (6) (Haskel), check valve (8), pressure gauge (10), pressure relief (12), and a recapture filter (30). Filters (16 and 18) (0.5 μm filter) are included in the supply line input and output to exclude or retain contents. The vessel (20) is constructed of stainless steel (Parr instruments) and has a total internal volume of 600 mL or 20 L. It includes heater strips (22 and 24), and a method of internally stirring the fluid (26 and 28). Internal to the vessel is a basket 34 made of stainless steel. Control components of the system are monitored and activated by a controller board with a touch screen interface and data recording (32).

2.3. Microbiological methods

The biological indicators used were *Bacillus atrophaeus* (*B. subtilis*) (ATCC #9372, Raven biological laboratories) and *Bacillus stearothermophilus* (ATCC #7953, SGM biotech) spore strips (>10^6 colony forming units/strip) sealed in 1073B Tyvek/Mylar pouches. Spore suspensions (>10^6 CFUs/100 μL in 40% ethanol) (SGM biotech). For treatment aliquots of suspension were placed into the bottom of 140 mm × 19 mm (LXD) test tubes and tube seal in 1073B Tyvek/Mylar pouches. *B. subtilis* and *B. stearothermophilus* spore strips were cultured in test tubes containing 10 mL of nutrient broth (Difco) at 37 and 55 °C, respectively, immediately after each treatment. *Salmonella typhimurium* (ATCC #39183) was cultured overnight in 500 mL nutrient broth in shaker incubator at 37 °C and harvested by centrifugation. Serial dilutions were carried out in sterile water and vacuumed filtered onto membranes (0.22 μm, Millipore GSWG047S1) and incubated at 37 °C for *Salmonella* and *B. subtilis* or 55 °C for *B. stearothermophilus*.

2.4. TEM/SEM

Salmonella was prepared for TEM as follows: suspensions of Salmonella were rinsed in phosphate buffered saline and then centrifuged. The supernatant was removed and then the pellet was resuspended in 2.5% gluteraldehyde/0.05% tannic acid in 0.1 M sodium cacodylate at pH 7.2. The material was fixed for 2.25 h. After the primary fixation, the cells in suspension were pelleted and the gluteraldehyde removed, and 0.1 M sodium cacodylate buffer was added. The cells were then resuspended by gentle agitation. The Salmonella was rinsed 3× for 10 min. The cells were placed in a 1% osmium tetroxide solution overnight at 4 °C. The cells were rinsed 3× for 10 min in a 0.1 M sodium cacodylate buffer. The cells were dehydrated in a series of cold ethanol solutions for 10 min each, starting with a 10% ethanol solution continuing through 30%, 50%, 70% and 90% solutions. At room temperature, the cells were rinsed 2× in 100% ethanol at 10 min intervals. The cells were then rinsed in a 1:1 ethanol and acetone solution for 10 min and then 2× in 100% acetone for 10 min.

The material was infiltrated by gradually increasing the concentration of the plastic using solutions of acetone and epon-araldite. A 1:4 solution of plastic and acetone was placed on the cells. Increasing amounts of epon-araldite were added to the cells until a 4:1 solution, plastic to acetone, was achieved over a period of 24 h. This solution was removed and epon-araldite was added without the accelerator (DMP-30). This was allowed to sit on the cells overnight and then removed and a fresh solution of epon-araldite was added, this time with DMP-30 added. This remained on the cells for 4 h and then the cells were embedded in epon-araldite with DMP-30 and the blocks were polymerized at 60 °C.

Seventy nanometer sections were sectioned and then contrasted with uranyl acetate and lead citrate. The sections were viewed on a FEI Tecnai 12 electron microscope and digitally photographed using Gatan digital software.

Salmonella was prepared for SEM as follows: the cells were fixed in 2.5% gluteraldehyde/0.05% tannic acid in 0.1 M sodium cacodylate pH 7.2 for 1 h. Aliquots of cells were placed on a silica chips which has been coated with 0.1% poly-L-lysine and incubated under 100% humidity for 1 h at room temperature. The chips were then rinsed 5× for 1 min 0.1 M sodium cacodylate. The chips were then incubated for 15 min in 1% osmium tetroxide. This was followed by 3× 1 min rinses in 0.1 M sodium cacodylate. The chips were then rinsed 3× for 1 min with distilled water. Cells were dehydrated by a series of 1 min incubations in ethanol at concentrations of: 10%, 20%, 30%, etc. through 100% (3×) (2% UA in 70% ethanol; 20 min).The chips were critical point dried in a Bal Tec CPD 030 (Bal-Tec-Liechtenstein).

The chips were viewed on a Hitachi S4500 scanning electron microscope (Hitachi Instruments Inc., San Jose, CA). Digital images were collected using Princeton Gamma Tech Imix software (Princeton Gamma Tech Inc., Princeton, NJ).

2.5. Gel electrophoresis

One-dimensional SDS-Page analysis was performed by boiling pellets (10 min) of Salmonella in 3× SDS-Page loading buffer with 5% BME. Boiled samples where subjected to centrifugation and 10 μl of the supernatant loaded into wells of 4–20% gradient polyacrylamide gel (Fisher) and run at 50 V for 3 h. Gel was removed and stained by standard Commasie blue staining and digitized by scanning using a flatbed scanner.

For two-dimensional gels, Salmonella pellets were lysed in 1 mL of osmotic lysis buffer containing 10× nuclease stock and protease inhibitors stock. Four hundred microlitres each of SDS boiling buffer minus BME was added, and the samples were heated in a boiling water bath for 5 min before protein determinations were performed using BCA Assay (Smith et al., 1985) (Pierce Chemical Co., Rockford, IL). Samples were then diluted to 1.0 mg/mL in 5% BME and 50 μg loaded per gel.

Two-dimensional electrophoresis was performed according to the method of O'Farrell (1975). By Kendrik Labs Inc. (Madison, WI) as follows: isoelectric focusing was carried out in glass tubes of inner diameter 2.0 mm using 2% pH 4–8 ampholines (Gallard Schlesinger, Industries Inc., Garden City, NY) for 9600 V h. Fifty nanograms of an internal standard, tropomyosin, was added to each sample. This protein migrates as a doublet with lower polypeptide spot of MW 33 kDa and p*I* 5.2. The enclosed tube gel pH plot

ARTICLE IN PRESS

for this set of ampholines was determined with a surface pH electrode.

After equilibration for 10 min in buffer “0” (10% glycerol, 50 mM DTT, 2.3% SDS, and 0.0625 M tris pH 6.8), each tube gel was sealed to the top of a stacking gel that is on top of a 10% acrylamide slab gel (0.75 mm thick). SDS slab gel electrophoresis was carried out for about 4 h at 15 mA/gel. The slab gel was fixed in a solution of 10% acetic acid/50% methanol o/n. The following proteins (Sigma Chemical Co., ST. Loius, MO) were added as molecular weight standards to the agarose, which sealed the tube gel to the slab gel: myosin (220 kDa), phosphorylase (94 kDa), catalase (60 kDa), actin (43 kDa), carbonic anhydrase (29 kDa), and lysozyme (14 kDa). These standards appear along the basic edge of the silver stained (Oakley et al., 1980) 10% acrylamide slab gels. The gels were dried between sheets of cellophane paper with the acid edge to the left.

2.6. Calculations

Mixture analysis and interior mapping calculations and analysis were performed using Minitab 14. Microsoft Excel was used for *D* value calculations.

3. Results

In the development of a process capable of achieving SAL10^{-6} levels of sterilization inactivation of bacterial endospores is the critical measure. Using a 600 mL version of the 20 L supercritical CO_2 apparatus (Fig. 2) we were able to easily inactivate vegetative bacteria species including *E. coli* and *Salmonella typhimurium* (data not shown). However, no log reductions, as compared to controls, using dried commercially available spore preparations of *B. subtilis* or *B. stearothermophilus* endospores was observed at any time point up to 72 h. In addition, inactivation did not appear to occur even if pressure cycling was carried out greater than 30 times (3000–1500 psi) over periods of 2 h. Water has been shown to facilitate inactivation of microbes with supercritical CO_2 (Dillow et al., 1999). However, even the addition of water to humidify the interior of the vessel did not facilitate the inactivation of endospores.

Table 1
List of selected additives screened for enhancement of *B. stearothermophilus* spore inactivation in combination with supercritical CO_2

Additive	Temperature (°C)	Time (h)	Log reduction
Ethanol	60–50	3	1.2–4.0
50% Citric acid	60	2	0.03–0.62
Succinic acid	50	2	0.25–0.29
Phosphoric acid	50	2	0.18–0.25
50% H_2O_2	50	1	0.13–1.57
Formic acid	50	2	0
Acetic acid	50	2	0.12–0.85
Malonic acid	50	2	0–0.12
TFA	60	1	>6.4
5% PAA	60	1	>6.4

Time is representative of dwell at 1500 psi and the temperature noted. Log reductions were measured by serial dilutions of spore suspensions and plating to measure remaining CFUs as compared to controls. Ranges for log reductions in CFUs are reported.

3.1. Screening of Additives

Several low molecular weight, volatile additives were screened to identify additives that inactivated *B. stearothermophilus* endospores (BI) (Table 1). Of the additives assayed only trifluoroacetic acid (TFA) and peracetic acid (PAA) resulting in significant log reductions of the BI. These two closely related compounds are characterized by relatively high vapor pressures. However, while PAA is non-toxic and unstable, TFA is exceptionally stable with poorly understood toxicity. PAA has been increasingly used in medical disinfection processes and readily degrades into acetic acid and water. This degradation helps to alleviate concerns about residual toxicity (Block, 2001). There is considerable precedent for the use of PAA, while there is no history for the use of TFA in medical disinfection. As such, continued development has centered on the use of PAA as the additive of choice for supercritical CO_2 sterilization.

3.2. Mixture analysis

Aqueous PAA preparations spontaneously reach a chemical equilibrium containing acetic acid (AA) and hydrogen peroxide (HP). The primary active component is likely PAA, since HP or AA alone showed little activity as sterilants in the supercritical CO_2 process (Table 1). To determine which component in the PAA:AA:HP mixture promoted the rapid inactivation of bacterial endospores, mixture analysis was

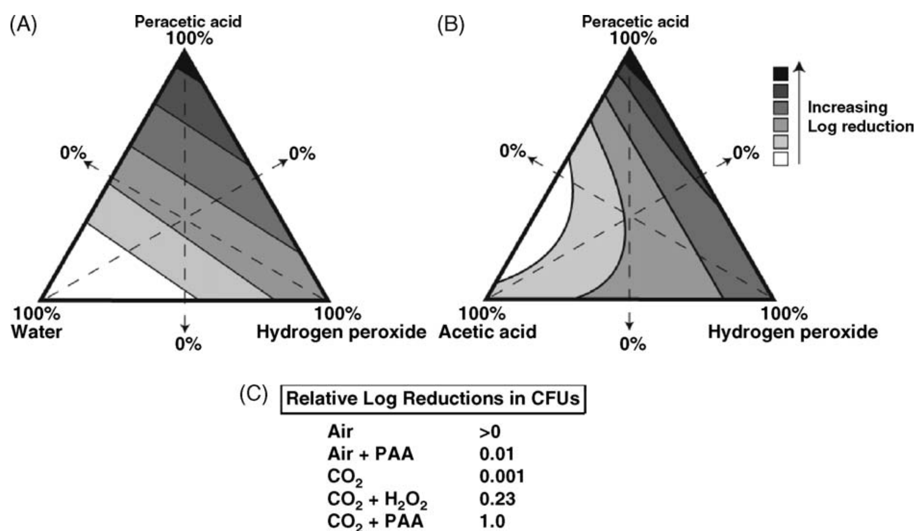

Fig. 3. Mixture analysis of PAA containing additive. (A) Contour plot of PAA vs. HP vs. water, plotting relative log reductions of CFUs for *B. stearothermophilus* spore suspensions. (B) Contour plot of PAA vs. HP vs. AA of relative log reductions. PAA is the driving force for the most significant log reductions. (C) Relative log reductions of *B. stearothermophilus* in when air is substituted for CO_2 with PAA vs. HP.

performed (Fig. 3A and B). Contour plots of inactivation of *B. stearothermophilus* endospores revealed that PAA is the driving force for inactivation while water and AA are not (Fig. 3A and B). Within the context of the mixture analysis HP appears to be responsible for some sporicidal activity. However, the greatest log reductions in CFUs are consistently realized with higher PAA concentrations.

It is a possibility that the inactivation observed when PAA is combined with supercritical CO_2 is solely a result of the PAA and has little to do with CO_2. If this were the case then it would be expected that similar inactivation would be observed if the same concentration of PAA was used in conjunction with pressurized air in the same apparatus. However, this was not observed (Fig. 3C). PAA in combination with supercritical CO_2 proved to be nearly 100× more effective than PAA with pressurized air and 5× more effective compared to HP with supercritical CO_2. This finding suggests significant synergism between PAA and supercritical CO_2 for the inactivation of endospores.

3.3. Linearity of inactivation

To determine the time required to achieve a $SAL10^{-6}$ level of sterilization requires extrapolation of the determined *D* value (time for a 1 log reduction in the BI) to a point where a 12 log reduction is predicted in the BI (12*D* value = Time to $SAL10^{-6}$) (FDA, 1993; ISO14937, 2000). Ideally a linear inactivation profile would be observed after performing survivor curve analysis over a series of time points and plotting the data in semi-log fashion (Block, 2001). In order to determine the inactivation kinetics associated with our supercritical CO_2 sterilization process we performed survivor curve analysis for time points from 15 to 60 min in our 600 mL vessel at a temperature of 35 °C with constant agitation of the fluid and 6 mL of water adsorbed a cotton ball. Because inactivation kinetics were too rapid with 0.002% PAA the level was reduced to 7.5×10^{-5}% PAA to lengthen the time required for inactivation of dry *B. subtilis* spores and allow for a higher resolution of the shape of the inactivation curve. Remaining CFUs after treatment were enumerated for time points of 15, 30, 45, and 60 min and results plotted (Fig. 4). A strong linear inactivation profile for the spores was observed, demonstrating that inactivation correlates predictably with *D* values and times required for $SAL10^{-6}$ sterilization.

3.4. Bacteriostasis

As with any microbial inactivation technology, it is important to distinguish inactivation of the given

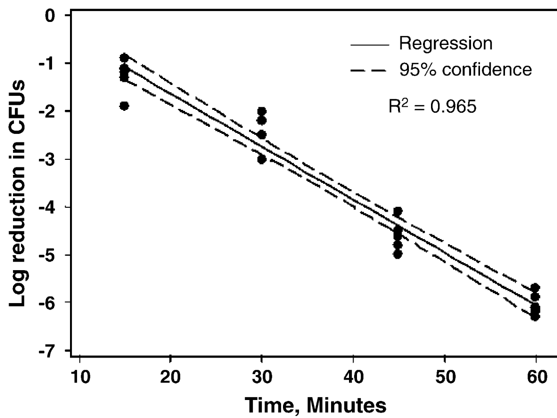

Fig. 4. Linear regression plot of log reduction vs. time for inactivation of *B. stearothermophilus* spore suspension as measured by serial dilutions and plating as compared to control.

microbe from simple growth inhibition. Such bacteriostasis might arise from a process-derived compound that is introduced into the culturing media (Berube and Oxborrow, 1991; ISO14937, 2000). If bacteriostasis were caused by an inhibitor, then inoculating a treated sample of BI with a low titer of bacteria should show no observable growth. When tubes negative for growth of the treated BI were inoculated with 10–100 CFUs of *B. subtilis* spores, bacterial growth was observed within 48 h for all tested ($n > 50$). This result indicates that treated samples of BI were inactivated, not inhibited.

3.5. Uniformity of inactivation

In addition to linearity of inactivation, uniformity of inactivation within a sterilization vessel must be measured. Those areas demonstrating the slowest rate of inactivation should be utilized for determination of *D* values as well as other testing of the sterilizer (FDA, 1993; ISO14937, 2000). Using the 20 L Nova2200 sterilization vessel (Fig. 2), 25 runs were pooled (0.002% PAA, 35 °C, 1400 psi, 30 mL water) that resulted in fractional inactivation for a set of nine *B. subtilis* spore strips sealed in Tyvek/Mylar pouches placed in the positions indicated (Fig. 5). In all of these runs the remaining space between BI-containing pouches was occupied by similar pouches containing small polypropylene tubes (1.7 mL Eppendorf). These pouches were included to simulate a full vessel in use. When the growth distribution results were plotted for each position, it was discovered that position 5a, located directly above the propeller, was significantly different from the other locations (Fig. 5). Position 5a was identified as a “hot spot” for inactivation while all other locations in the vessel had comparable inactivation profiles. This finding suggests that inactivation in the vessel is relatively uniform save for directly above the propeller at the bottom of the vessel. As a result, data associated with position 5a in subsequent testing are not included in the analysis.

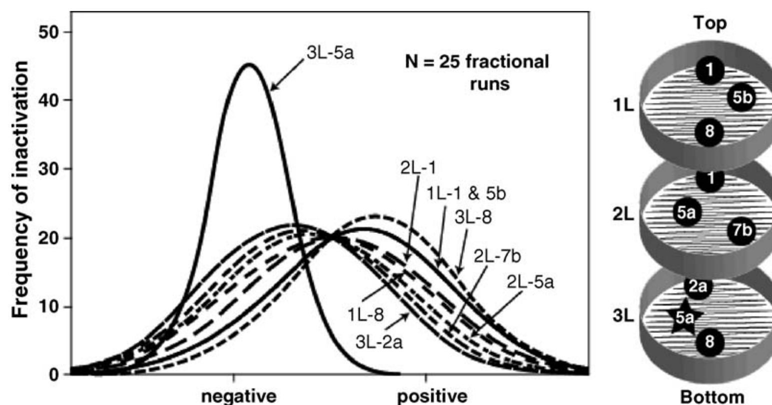

Fig. 5. Plot of the fraction of *B. subtilis* spore strips inactivated at differing locations within the 20 L sterilization chamber. Position 5a (basket diagram) directly above the impeller was identified as the “hot spot” for inactivation.

ARTICLE IN PRESS

Table 2
Results of fraction negative testing using *B. subtilis* spore strips in the Nova2200 20 L sterilization chamber

(A) Raw data from testing

Time (min)	Number of samples	Number inactivated
0	24	0
3	24	7
6	24	14
9	24	11
12	24	20
15	24	13
18	24	13
21	24	21
24	24	17
27	24	24
30	24	24

(B) Calculations using Holcomb–Spearman–Karber method and the Stumbo–Murphy–Cochran method

	min
Mean time to total kill	21.20
Variance	0.69
95% CI	1.66
Upper limit	22.86
Lower limit	19.54
D value	3.25
Upper limit	3.50
Lower limit	2.99

Conditions employed were: 1400 psi, 35 °C, 0.02% (v/v) PAA, and 0.8% (v/v) sterile distilled water.

3.6. Estimation of ‘D value’

Fraction negative analysis was performed to determine the time required for a 1 log reduction (*D* value) in the *B. subtilis* spore BI (FDA, 1993; Block, 2001). Treatments were carried out with the following parameters: 0.002% PAA, 35 °C, 1400 psi, 30 mL water and a simulated full vessel. Lots of nine BIs per run were subjected to treatments in the Nova2200 vessel starting at the time 0 when 1400 psi and 35 °C were attained and increasing in increments of 3 min. Each time point was repeated three times and the results from the three runs were pooled, with data from position 5a excluded (Table 2A). Calculations using the results were performed using the Holcomb–Spearman–Karber method and the Stumbo–Murphy–Cochran method for *D* value determination (FDA, 1993; Block, 2001). A *D* value of 3.25 min was determined, which translates to a time to achieve SAL10^{-6} sterilization using the Nova2200 vessel of 39 min (Table 2B).

3.7. Inactivated microbes remain intact

Subjecting microbes to pressure, turbulence, and supercritical solvent might be expected to impact the physical structure of microbes. To investigate this possibility, wet pellets of Salmonella harvested during log phase (>10^{12} CFUs) were subjected to 70 min treatments under the same conditions used in the determination of *D* values. Total inactivation was confirmed through serial dilutions and plating. It was found that inactivated *Salmonella* remained intact when viewed by scanning electron microcopy (SEM) (Fig. 6). This finding correlates well with previously reported data for a number of other organisms when inactivated with supercritical CO_2 (Dillow et al., 1999; Hong and Pyun, 1999). In order to visualize the interior ultrastructure of the inactivated *Salmonella,* thin section transmission electron microscopy (TEM) was performed on inactivated microbes (Fig. 7). Comparisons with untreated cells revealed little difference except that lipid bilayers

ARTICLE IN PRESS

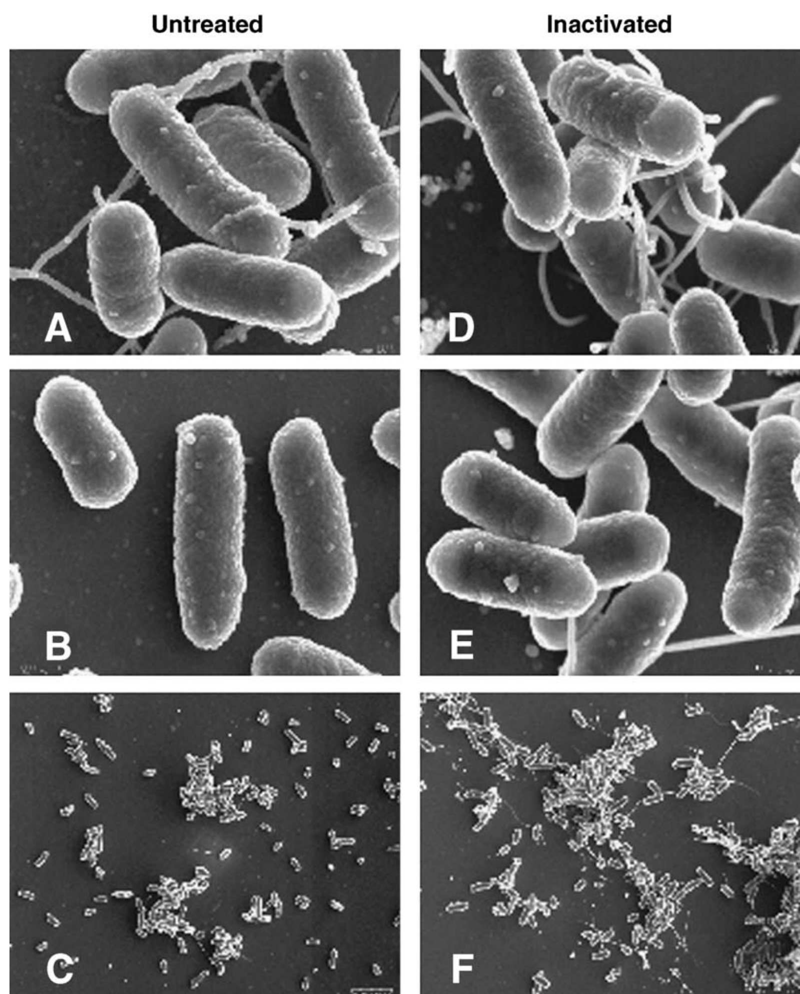

Fig. 6. SEM micrographs of Salmonella, untreated (A)–(C) and inactivated (D)–(F) by 1 h treatment in 20 L chamber at 1400 psi, 35 °C, 0.02% (v/v) PAA, and 0.8% (v/v) sterile distilled water.

in the inactivated group appear to be 'roughened' compared to the control. Furthermore, the internal structures of the inactivated cells appear less distinct in the inactivated group versus the untreated group. Similar observations have been reported for supercritical CO_2 inactivated *Lactobacillus plantarum* (Hong and Pyun, 1999).

3.8. Effects on proteins

Given the growing importance of proteins as biological therapeutics, protein profiling of inactivated *Salmonella* was performed to investigate the overall impact on proteins of the sterilization process. The same *Salmonella* pellets inactivated for SEM/TEM analysis were used to examine proteins. Both untreated and inactivated cells displayed similar banding patterns in one-dimensional SDS-Page analysis (Fig. 8A), suggesting that no wholesale degradation of proteins had occurred. A higher resolution examination of the protein profiles using two-dimensional electrophoresis also revealed no appreciable degradation of proteins (Fig. 8B and C).

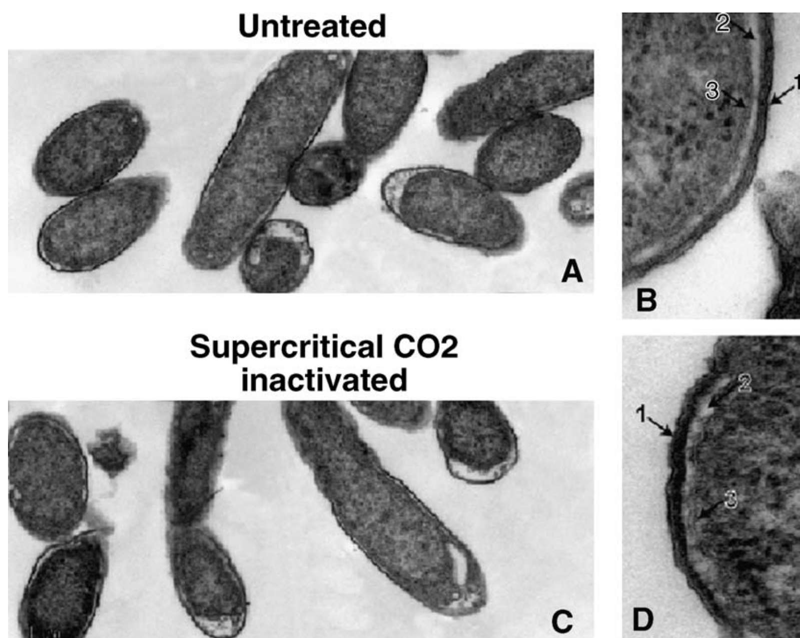

Fig. 7. Thin section TEM micrographs of Salmonella treated as in Fig. 4. Untreated (A) and (B) and inactivated (C) and (D). Higher magnifications (B) and (D) reveal little difference between the two groups as cells wall (arrows #1), periplasmic space (arrows #2) and lipid bilayers (arrows #3) are present.

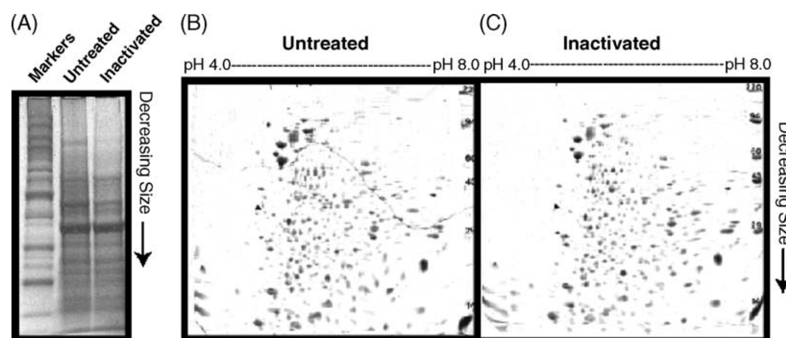

Fig. 8. Comparative protein profiles of Salmonella inactivated by supercritical CO_2 (as in Fig. 4) and untreated. (A) One-dimensional SDS-Page gel showing relative banding patterns of untreated and inactivated Salmonella. No significant differences are noted. (B) and (C) Two-dimensional SDS-Page protein analysis of untreated Salmonella (B) vs. inactivated (C). No significant differences are observed between to two groups indicating that inactivation by supercritical CO_2 process does not degrade proteins.

4. Discussion

Using a custom constructed apparatus we have demonstrated that supercritical CO_2 treatment in combination with low concentrations of the additive PAA is effective at inactivating bacterial endospores. Inactivation followed linear kinetics, making it possible to estimate SAL10^{-6} levels of sterilization by defining the *D* value for the process. Moreover, the process appears to be gentle, as morphology and protein profiles of inactivated bacteria are largely unchanged. These observations are all in the context of a terminal sterilization process that utilizes gas-permeable packaging.

Numerous theories have been proposed for the mechanism of bacterial inactivation using supercritical CO_2, including cell rupture, acidification, lipid

ARTICLE IN PRESS

modification, inactivation of essential enzymes, and/or extraction of intracellular substances (Spilimbergo and Bertucco, 2003). Of these theories, the rupture of bacterial cells has been largely ruled out since the bacteria remain intact (Dillow et al., 1999; Hong and Pyun, 1999) (this study). The remaining theories are not as easily dismissed.

Disruption of the lipid bilayer by the mass transfer of CO_2 may contribute to inactivation in what is referred to as an 'anaesthesia' effect (Isenschmid et al., 1995). According to this hypothesis, mass transfer of CO_2 increases the fluidity and permeability of the phospholipid bilayer, preventing it from reforming properly when the CO_2 is removed. It is also possible that essential enzymes are extracted and/or denatured, and that the extraction/denaturing efficiency is dependent on the mass transfer of CO_2 with water as an entrainer.

In the context of a low temperature ($\leq$40 °C) and relatively low pressure ($\leq$2000 psi) supercritical CO_2 process, two components are suggestive for a mode of action. These components include the presence of water and a method for enhancing mass transfer of CO_2 and other additives that affect cell viability (Dillow et al., 1999; Shimoda et al., 2001) (this study). Together, these factors point to the formation of carbonic acid as a key step in the ultimate inactivation of microbes. Carbonic acid, which is generated from the reaction of CO_2 with water, may be responsible for inactivation of cells through the transient acidification of the interior of the microbial cell and/or inactivation of essential enzymes. PAA is both an acid and peroxide. As an acid, PAA may have unique transport properties in supercritical CO_2 that also contribute to overall intracellular acidification. The same mass transfer enhancement may also facilitate the delivery and/or action of PAA as a sporicidal agent. This hypothesis is consistent with the synergy observed between supercritical CO_2 and PAA for inactivating bacterial endospores.

Many rapidly developing medical technologies require novel sterilization solutions. Such solutions are particularly important for emerging technologies that involve the use of biologically active large molecules such as DNA, proteins and bio-polymers. Of these areas, the increase in the use of human allograft tissue for orthopedic surgeries, cardio-vascular operations, and skin replacement, represents an area that would immediately benefit from new sterilization technologies. Ongoing safety issues surrounding contaminated tissue have resulted in negative outcomes for patients, including death in some instances (Conrad et al., 1995; CDC, 2001a, 2001b; Goodman, 2004; Kainer et al., 2004; Crawford et al., 2005). The tissue bank industry has addressed these safety issues in a number of ways with varying success (Kainer et al., 2004). Of these, aseptic processing is both expensive and prone to failure (Crawford et al., 2005), and gamma irradiation is associated with significant compromises in the biomechanical properties of tissue allografts as well as the generation of toxic lipid compounds (Rasmussen et al., 1994; Moreau et al., 2000; Akkus and Rimnac, 2001).

Supercritical CO_2 sterilization may be emerge as a more compatible option for increasing the safety of human allograft tissue. Indeed, supercritical CO_2 technologies have previously been shown to be compatible with biodegradable polymers and allograft tissue (Fages et al., 1994, 1998a, 1998b; Dillow et al., 1999). However, these earlier studies did not report procedures capable of SAL10^{-6} terminal sterilization. Here we report a process that achieves SAL10^{-6} terminal sterilization that may fill a clear and present need in tissue banking practices. Future studies will be directed at commercially viable applications of terminal sterilization with supercritical CO_2 including: tissue, biodegradable polymers, powdered drug formulations, endoscopes, composite medical devices, DNA based pharmaceuticals, and large molecule based pharmaceuticals.

References

A.A.I., 1995. Sterilization of medical devices-microbiological methods. Part 1. Estimation of population of microorganisms on products. ANSI/AAMI/ISO, 11737-1.

Akkus, O., Rimnac, C.M., 2001. Fracture resistance of gamma radiation sterilized cortical bone allografts. J. Ortho. Res. 19 (5), 927–934.

Berube, R., Oxborrow, G.S., 1991. Methods of Testing Sanitizers and Bacteriostatic Substances. Disinfection, Sterilization and Preservation, 4th ed. Lea & Febiger, Phiadelphia, PA, pp. 1058–1068.

Block, S.S., 2001. Disinfection, Sterilization and Preservation, 5th ed. Lippincott Williams & Wilkins, Phiadelphia, PA.

CDC, 2001. Septic arthritis following anterior cruciate ligament reconstruction using tendon allografts—Florida and Louisiana, 2000. Mmwr. Morbidity and Mortality Weekly Report 50 (48), 1081–1083.

CDC, 2001. Unexplained deaths following knee surgery—Minnesota, 2001. Mmwr. Morbidity and Mortality Weekly Report 50 (46), 1035–1036.

Clapp, P.A., Davies, M.J., et al., 1994. The bactericidal action of peroxides; an E.P.R. spin-trapping study. Free Radic. Res. 21 (3), 147–167.

Conrad, E.U., Gretch, D.R., et al., 1995. Transmission of the hepatitis-C virus by tissue transplantation. J. Bone Joint Surg. Am. 77, 214–224.

Crawford, C., Kainer, M., et al., 2005. Investigation of postoperative allograft-associated infections in patients who underwent musculoskeletal allograft implantation. Clin. Infect. Dis. 41 (2), 195–200.

Digas, G., Thanner, J., et al., 2003. Increase in early polyethylene wear after sterilization with ethylene oxide: radiostereometric analyses of 201 total hips. Acta Ortho. Scand. 74 (5), 531–541.

Dillow, A.K., Dehghani, F., et al., 1999. Bacterial inactivation by using near and supercritical carbon dioxide. PNAS 96 (18), 10344–10348.

Duffy, R.E., Brown, S.E., et al., 2000. An epidemic of corneal destruction caused by plasma gas sterilization. The toxic cell destruction syndrome investigative team. Arch. Ophthalmol. 118 (9), 1167–1176.

Fages, J., Marty, A., et al., 1994. Use of supercritical CO_2 for bone delipidation. Biomaterials 15 (9), 650–656.

Fages, J., Poddevin, N., et al., 1998a. Use of supercritical fluid extraction as a method of cleaning anterior cruciate ligament prostheses: in vitro and in vivo validation. Asaio J. 44 (4), 278–288.

Fages, J., Poirier, B., et al., 1998b. Viral inactivation of human bone tissue using supercritical fluid extraction. Asaio J. 44 (4), 289–293.

FDA, 1993. Guidance on Premarket Notification [510 (k)] Submissions for Sterilizers Intended for Use in Health Care Facilities. Infect Control Devices Branch, Division of General and Restorative Devices. Food and Drug Administration.

Ferreira, S.D., Dernell, W.S., et al., 2001. Effect of gas-plasma sterilization on the osteoinductive capacity of demineralized bone matrix. Clin. Ortho. 388, 233–239.

Florack, E.I., Zielhuis, G.A., 1990. Occupational ethylene oxide exposure and reproduction. Int. Arch. Occup. Environ. Health 62 (4), 273–277.

Ge, Y., Yan, H., et al., 2002. Extraction of natural Vitamin E from wheat germ by supercritical carbon dioxide. J. Agri. Food Chem. 50 (4), 685–689.

Goldman, M., Lee, M., et al., 1997. Oxidation of ultrahigh molecular weight polyethylene characterized by Fourier transform infrared spectrometry. J. Biomed. Mater. Res. 37 (1), 43–50.

Goodman, J.L., 2004. The safety and availability of blood and tissues—progress and challenges. N. Engl. J. Med.

Hong, S.I., Pyun, Y.R., 1999. Inactivation kinetics of *Lactobacillus plantarum* by high pressure carbon dioxide. J. Food Sci. 64 (4), 728–733.

Isenschmid, A., Marison, I.W., et al., 1995. The influence of pressure and temperature of compressed CO_2 on the survival of yeast cells. J. Biotechnol. 39 (3), 229–237.

ISO14937, 2000. Sterilization of health care products. General requirements for charaterization of a sterilizing agent and the development, validation and routine control of a sterilization process for medical devices.

Kainer, M.A., Linden, J.V., et al., 2004. Clostridium infections associated with musculoskeletal-tissue allografts. N. Engl. J. Med. 350 (25), 2564–2571.

Klapperich, C., Niedzwiecki, S., et al., 2000. Fluid sorption of orthopedic grade ultrahigh molecular weight polyethylene in a serum environment is affected by the surface area and sterilization method. J. Biomed. Mater. Res. 53 (1), 73–75.

Moreau, M.F., Gallois, Y., et al., 2000. Gamma irradiation of human bone allografts alters medullary lipids and releases toxic compounds for osteoblast-like cells. Biomaterials 21 (4), 369–376.

O'Farrell, P.H., 1975. High resolution two-dimensional electrophoresis of proteins. J. Biol. Chem. 250 (10), 4007–4021.

Oakley, B.R., Kirsch, D.R., et al., 1980. A simplified ultrasensitive silver stain for detecting proteins in polyacrylamide gels. Anal. Biochem. 105 (2), 361–363.

Osterman-Golkar, S., Bergmark, E., 1988. Occupational exposure to ethylene oxide relation between in vivo dose and exposure dose. Scand. J. Work Environ. Health 14 (6), 372–377.

Rasmussen, T.J., Feder, S.M., et al., 1994. The effects of 4 Mrad of gamma irradiation on the initial mechanical properties of bone-patellar tendon-bone grafts. Arthroscopy 10 (2), 188–197.

Shimoda, M., Cocunubo-Castellanos, J., et al., 2001. The influence of dissolved CO(2) concentration on the death kinetics of *Saccharomyces cerevisiae*. J. Appl. Microbiol. 91 (2), 306–311.

Smith, P.K., Krohn, R.I., et al., 1985. Measurement of protein using bicinchoninic acid. Anal. Biochem. 150 (1), 76–85.

Spilimbergo, S., Bertucco, A., 2003. Non-thermal bacterial inactivation with dense CO(2). Biotechnol. Bioeng. 84 (6), 627–638.

van der Velde, E.G., de Haan, W., et al., 1992. Supercritical fluid extraction of polychlorinated biphenyls and pesticides from soil. Comparison with other extraction methods. J. Chromatogr. 626 (1), 135–143.

Willie, B.M., Ashrafi, S., et al., 2004. Quantifying the effect of resin type and sterilization method on the degradation of ultrahigh molecular weight polyethylene after 4 years of real-time shelf aging. J. Biomed. Mater. Res. 1 (3), 477–489.