ARTICLE

BIOTECHNOLOGY and BIOENGINEERING

Sterilization of Ginseng Using a High Pressure CO_2 at Moderate Temperatures

Fariba Dehghani, Nasim Annabi, Mamata Titus, Peter Valtchev, Aldric Tumilar

School of Chemical and Biomolecular Engineering, The University of Sydney, Sydney 2006, Australia; telephone: 612-9351-4794; fax: 612-9351-2854; e-mail: fdehghani@usyd.edu.au

Received 27 April 2008; revision received 6 July 2008; accepted 15 July 2008

Published online 25 July 2008 in Wiley InterScience (www.interscience.wiley.com). DOI 10.1002/bit.22059

ABSTRACT: The aim of this study was to determine the feasibility of using high pressure CO_2 for sterilization of Ginseng powder, as an alternative method to conventional techniques such as γ-irradiation and ethylene oxide. The Ginseng sample used in this study was originally contaminated with fungi and 5×10^7 bacteria/g that was not suitable for oral use. This is the first time that high pressure CO_2 has been used for the sterilization of herbal medicine to decrease the total aerobic microbial count (TAMC) and fungi. The effect of the process duration, operating pressure, temperature, and amount of additives on the sterilization efficiency of high pressure CO_2 were investigated. The process duration was varied over 15 h; the pressure between 100 and 200 bar and the temperature between 25 and 75°C. A 2.67-log reduction of bacteria in the Ginseng sample was achieved after long treatment time of 15 h at 60°C and 100 bar, when using neat carbon dioxide. However, the addition of a small quantity of water/ethanol/H_2O_2 mixture, as low as 0.02 mL of each additive/g Ginseng powder, was sufficient for complete inactivation of fungi within 6 h at 60°C and 100 bar. At these conditions the bacterial count was decreased from 5×10^7 to 2.0×10^3 TAMC/g complying with the TGA standard for orally ingested products. A 4.3 log reduction in bacteria was achieved at 150 bar and 30°C, decreasing the TAMC in Ginseng sample to 2,000, below the allowable limit. However, fungi still remained in the sample. The complete inactivation of both bacteria and fungi was achieved within 2 h at 30°C and 170 bar using 0.1 mL of each additive/g Ginseng. Microbial inactivation at this low temperature opens an avenue for the sterilization of many thermally labile pharmaceutical and food products that may involve sensitive compounds to γ-radiation and chemically reactive antiseptic agents.

Biotechnol. Bioeng. 2009;102: 569–576.

KEYWORDS: Ginseng; sterilization; carbon dioxide; fungi inactivation; bacteria inactivation

Introduction

Sterilization is required for biomaterials, pharmaceuticals, and foodstuffs to prolong product life and minimizing health risks on consumption. The process involves the inactivation of bacteria and endospores, which may exhibit destructive effects on the product. The common sterilization methods used include autoclaving, ethylene oxide exposure, and γ-irradiation. These methods have been proven to achieve complete removal of many microorganisms including bacteria classes such as *Escherichia coli*, *Staphylococcus aureus*, and *Bacillus cereus* (Byun et al., 1998). However, these sterilization methods are often accompanied by undesirable impacts on the quality of the substrate material. Autoclaving involves exposure to high temperatures and steam degrading thermally sensitive materials. Ethylene oxide exposure is not suitable for the sterilization of food and herbal product. γ-irradiation involves exposure to high frequency radiation which may cause denaturation of important nutrients in the product, as well as changes in physical, mechanical, and optical properties of the material. Australian quarantine and inspection service considers the use of γ-irradiation for sterilization of food products as unacceptable. Hence, the most desirable objective of sterilization of sensitive materials, particularly of plant materials such as Ginseng, would be to destroy high levels of harmful microorganisms, while preserving the active compounds.

Supercritical fluid (SCF) processes that uses a fluid at above critical temperature and critical pressure, are a highly active field of research for separation and extraction, particularly of natural products (Foster et al., 2003). SCFs offer extraction on the scale of the conventional organic solvent techniques but using minimal amounts of organic modifiers thus leading the process under much gentler conditions. The technology of SCF utilizes the unique properties of these fluids to penetrate into the cellular matrix of substrate to perform sterilization. Due to their high diffusivity, the use of SCF for sterilization was pioneered by Kamihira et al. who observed that the number of living microorganisms in Koji residue decreased considerably after extraction with supercritical carbon dioxide, without any decrease in enzyme activities (Lin et al., 1992). Recent studies have shown that supercritical carbon dioxide ($SCCO_2$) treatment has been successful in the inactivation

Correspondence to: F. Dehghani

of a wide range of bacteria and yeasts at relatively low temperatures (Dillow et al., 1999; Parton et al., 2007). In addition, $SCCO_2$ has been considered for the sterilization of biomaterials, as well as for the inactivation of viruses (Fages et al., 1998).

The use of supercritical carbon dioxide is rapidly increasing in an attempt to advance the existent sterilization techniques. For instance, NovaSterilis is currently using $SCCO_2$ to inactivate pathogens from the surface of their biomedical devices (Gold, 2007). The desire to expand on the scope of the method and employ it for the sterilization of biomaterials that maybe ingested or come into close proximity with the body requires more consideration. Thus far experimentation has been performed using $SCCO_2$ in combination with an array of additives such as ethanol and hydrogen peroxide (White et al., 2006). It has been verified that many bacteria in aqueous-based samples can be completely inactivated and there is not a risk of further re-growth (Jimenez et al., 2007; White et al., 2006; Zhang et al., 2006a). The outcomes of these studies suggest the superior potential of $SCCO_2$ as a promising inactivation agent for the sterilization of food, pharmaceutical, and biological materials. However, since the vast majority of these studies have been performed upon bacteria in suspension or culture media; therefore, it cannot be assumed that the method will act similarly when used directly on biomaterials in solid state. Different operating temperatures and pressures maybe necessary and a greater issue is that carbon dioxide may extract active components of the biomaterial changing its biological activity.

Mechanism of Sterilization With $SCCO_2$

Although the actual mechanism of $SCCO_2$ sterilization is yet to be confirmed, it was suggested that inactivation is initiated by the diffusion of the fluid into the intracellular structure of the microorganism (Dillow et al., 1999; Lin et al., 1992; Spilimbergo et al., 2003, 2005). Lin et al. (1992, 1993) proposed that the inactivation process involves essentially the diffusion of carbon dioxide into, and the extraction of vital constituents such as phospholipids and hydrophobic compounds from the cell walls and membranes (Kamihira et al., 1987). This hypothesis was based on the observation that the protein level of the cell supernatant was reduced after the sterilization process, even though the cytoplasm did not leak out to the solution around the cell (Lin et al., 1992). Furthermore, the inactivated cells were not able to repair and revive within 3 weeks of extended incubation time, suggesting that the vital component necessary for this to occur could only have been extracted.

The mechanism of microbial inactivation by high pressure CO_2 has also been discussed thoroughly by Hong and Pyun (2001). It is believed that high pressure CO_2 damages the cellular membrane irreversibly, which resulted in loss of salt tolerance, release of intracellular ions, increase in proton permeability, and inactivation of some constituent enzymes. It was hypothesized that dissolution of CO_2 in water at high pressures leads to lowering the pH, which may prevent the metabolism and growth of microorganisms, and attenuate the microbial resistance to inactivation.

Ginseng Sterilization

Ginseng is a traditional Chinese medicine used primarily to improve certain psychological functions, physical performance, immune function, and conditions associated with diabetes mellitus (Castleman, 2007; Chu, 2007; Kiefer and Pantuso, 2003; Sivakumar et al., 2005). Biophenols (anti-oxidant) and ginsenosides are particularly important components in Ginseng, the former protect against cancer (Sung et al., 2005). Ginseonosides have been reported to have numerous medicinal benefits, including anti-tumor, chemoprotective, immunomodulating and anti-diabetic activities (Kiefer and Pantuso, 2003; Luo John and Luo, 2006; Quan et al., 2004; Sivakumar et al., 2005).

The cultivation of Ginseng usually takes up to 6 years and the species are submerged in wild soil for the entire period (Kwon et al., 1997). Hence the extracted Ginseng products are often contaminated with bacteria and fungi. A recent research published by the US Food and Drug Administration demonstrated that over than 78% of the samples were highly contaminated with fungi and bacteria (Tournas et al., 2005). Sterilization is therefore required to minimize the presence of these microorganisms which may cause degrading effects on the Ginseng product and/or can be harmful to human health.

It is known that γ-irradiation alters several chemical properties of Ginseng, despite the complete elimination of bacteria, yeast, moulds, and coliforms (Byun et al., 1998; Kwon et al., 1997). The research showed that γ-irradiation initiates chemical reactions, which alter Ginseng properties like acidity, pigments, degree of lipid oxidation, and color specification.

In this study, the feasibility of CO_2 with and without modifier for the sterilization of Ginseng powder was assessed. The effect of addition of modifiers such as water, ethanol, and hydrogen peroxide on the sterilization efficiency of CO_2 was examined.

Supercritical carbon dioxide was used for the extraction of active ingredients and removal of pesticides from Ginseng powder (Quan et al., 2004; Wood et al., 2006). Neat $SCCO_2$ was not efficient in extraction of active ingredients and removal of pesticides from Ginseng even at elevated temperatures and pressures (Wood et al., 2006). However, CO_2 modified with methanol and dimethyl sulfoxide were efficient in extraction of ginsenosides from Ginseng, although relatively high quantities of modifiers (>4 g modifier/g Ginseng) and consequently high operating conditions ($T > 100^{\circ}C$ and $P > 200$ bar) were required. At lower operating temperatures the extraction of active compounds was not successful. Wood et al. concluded that the dominant variables for the extraction of active compound in Ginseng by high pressure CO_2 were the

amount of modifier used and the operating temperature. It was also found that modified CO_2 was efficient in extraction of pesticides form Ginseng, while it did not affect the content of active ingredient up to 80°C (Quan et al., 2004). In this study, the sterilization of Ginseng with $SCCO_2$ was performed at the conditions below the one used in study by Wood et al. (2006) for the extraction of active ingredients in Ginseng (i.e., $T < 80$°C and $P < 200$ bar) to ensure active ingredients were preserved in the final product.

Materials and Methods

Materials

Ginseng powder extracted from White American Ginseng (Panax *quinquefolius*) was kindly donated by The Simply Ginseng Company in Bungendore, Australia. Ethanol (purity 99%) and hydrogen peroxide were purchased from Ajax. Sodium chloride peptone solution, tryptone soya agar, and sabouraud dextrose agar media were all purchased from Oxoid (Oxoid Australia Pty Ltd, Adelaide, South Australia) Distilled water was used to make the solutions. Carbon dioxide (food grade with 99.9% purity) was supplied from BOC gases.

Sterilization by High Pressure CO_2

The schematic diagram of the apparatus used for the sterilization of Ginseng powder is shown in Figure 1. A Ginseng sample (2.5 g) was loaded into the high pressure vessel (70 mL) and the required amount of additive (distilled water, hydrogen peroxide, and ethanol) was pipetted onto the sample using a disposable sterile pipettes. The vessel was then sealed, placed in a constant temperature water bath (Model No. TSB1 6876) and connected to a high pressure pump (Thar Technologies, Pittsburgh, PA, Model P50 Series). The pump was used to deliver carbon dioxide from a cylinder into the high pressure vessel. A Peristaltic pump was used to recirculate cold water (<5°C) around the head of the high pressure pump to ensure that CO_2 was in liquid phase and can be pressurized efficiently. The system was pressurized gradually using the pump at constant flow rate mode and after approaching the desired pressure, the system was then isolated for a certain time by closing the inlet valve. A pressure gauge (Swagelok, with maximum pressure range of 200 bar) was used to monitor the pressure of the vessel. At the end of each experiment, the system was depressurized slowly (i.e., 20 min) by opening the outlet valve and the sample was then collected for analysis. The high pressure vessel and all the connected fittings were sterilized using 70 wt% ethanol and dried in an oven at 80°C after each run. All the experiments were performed in sterile conditions, handling the materials with sterile tools in a laminar flow cabinet (Model No. 2CBH EN2 0060).

All experiments conducted are listed in Table I. Each experimental run was repeated at least three times and average values for microbial count were reported.

Microbiological Procedures

Preparation of Analytical Solutions and Petri Dishes

A 14.63 g/L buffered sodium chloride peptone, 65 g/L sabouraud dextrose agar media that is for culturing fungi, and 40 g/L tryptone soya agar media for culturing all aerobic bacteria were prepared and autoclaved at 121°C for 15 min. The solid media was prepared using the latter two solutions to make agar plates by adding about 20 mL of each solution into sterile Petri dishes. After the agar cooled down and solidified, the Petri dishes were kept in refrigerator. The buffered solution was stored at room temperature and used as required.

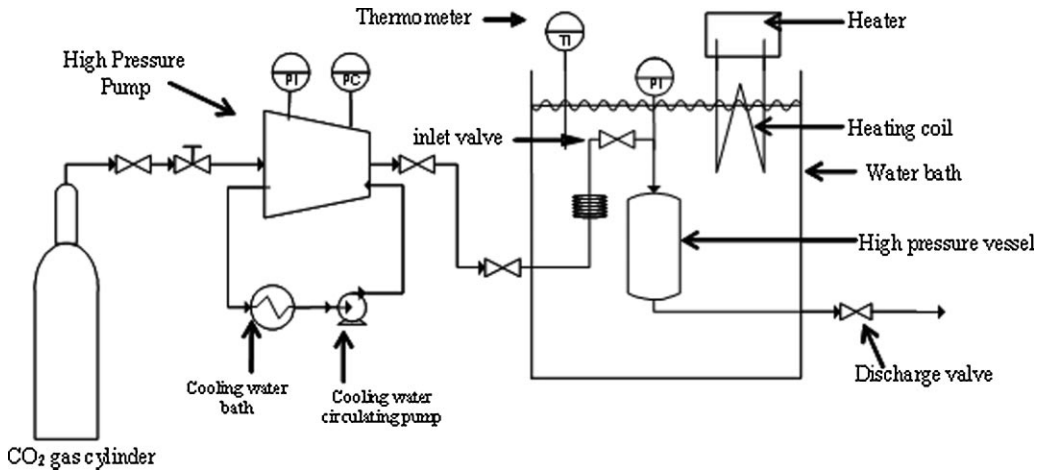

Figure 1. Schematic diagram of the sterilization process using high pressure CO_2.

Table I. Effect of temperature, pressure, and time on the TAMC reduction of Ginseng using neat carbon dioxide.

Temperature (°C)	Pressure (bar)	Time (h)	Log N_0/N
25	150	0.25	1.03
25	150	0.5	1.04
25	150	2	1.05
40	150	0.25	1.29
40	150	0.5	1.47
40	150	2	1.64
40	190	2	1.53
60	150	0.25	0.01
60	150	0.5	0.05
60	150	2	0.15
60	150	15	2.70
60	100	15	2.67

Each run was repeated at least three times.

Viable Plate Count Method for Determination TAMC and Fungi

A standard method was used for viable bacteria counting and fungi determination in solid samples (British Pharmacopoeia Online, 2007). The methods are also used by TGA accredited companies such as an AMS Laboratories Pty Ltd. to determine the total aerobic microbial count (TAMC) and detect fungi. The Ginseng powder was suspended in buffered sodium chloride peptone solution (1 g:10 mL ratio) and then diluted using sterile saline solution according to a standard serial dilution procedure. One milliliter of the diluted samples was then plated on Petri Dishes containing Tryptone Soya Agar (for TAMC) and Sabouraud Dextrose (for fungi detection). For each dilution, at least three plates were prepared and then all labeled plates were incubated at 35°C for 1–5 days. At the end of the incubation period, the number of colonies were counted. Inactivation was expressed as log N_0/N, where N_0 is the number of microorganisms contained in the sample at the initial period (control sample), and N is the number of microorganisms counted after treatment at any time t.

Validation of Sterilization Procedures

The sterilization procedures for the high pressure vessel, sample holder, and also the autoclave performance for sterilization of media and other items used for handling the materials were validated prior to conducting the experimental procedure. The vessel and sample holder were rinsed with sterilized saline solution and then viable bacterial counting was conducted as described above. No bacteria and fungi was detected after incubation confirming that the procedures used were sufficient for maintaining sterile solutions and items.

Analysis of Variance (ANOVA) Test

A single factor ANOVA was performed to determine the effect of a factor on the TAMC reduction. The effect of each factor is considered significant, when P value was less than 0.05. At each condition the experiment was repeated at least three time to have sufficient data for statistical analysis.

Results and Discussion

The Ginseng powder used in this study could not be released into the market due to the high level of bacteria (5×10^7 CFU/g) and also the presence of fungi. The acceptable levels for oral use by the Therapeutic Goods Administration in Australia (TGA) are contamination less than 10^4/g and 10^2/g for TAMC and fungi, respectively. The objective of the study was to determine the dense gas CO_2 processing variables to achieve complete inactivation of fungi and bacteria in Panax Ginseng herbal medicine in a short period of time to make the process more competitive with the current sterilization processes. The study was conducted in two stages; the preliminary stage involved experiments to assess the feasibility of using CO_2 +/− modifier to decrease microorganism level in Ginseng powders in a short period of time. In the second stage, processing conditions were determined to achieve complete inactivation of all microorganisms.

Inactivation of Microorganisms Using Neat $SCCO_2$

At the first glance neat CO_2 was used for the sterilization of Ginseng. It has been previously discussed that neat CO_2 is not efficient for complete inactivation of all microorganisms. While high pressure carbon dioxide was efficient for inactivation of viable bacteria, endospores were not completely inactivated with neat CO_2 (Hemmer et al., 2006; Setlow et al., 2002; White et al., 2006; Zhang et al., 2006a).

The result of this study in Table I confirmed that pure CO_2 was not efficient to completely inactivate microorganisms from Ginseng samples. There was not significant effect of changing the processing period from 15 min to 2 h on TAMC reduction. At 150 bar and processing time of 2 h, increasing the temperature from 25 to 60°C slightly decreased the CO_2 efficiency for TAMC reduciton. Increasing the processing time to 15 h at 60°C only decreased the TAMC 2.67 log reduction that is still beyond the limit for oral administration by TGA (e.g., TAMC decreased from 5×10^7/g to 1.10×10^5/g). A long processing time is not desirable and practical for commercial purposes. Further experimentation using additives and varying the process time, temperature, and pressure was therefore necessary to attain adequate removal of the microorganisms within a suitable period of time.

Effect of Additives on CO_2 Inactivation Efficiency

Modifiers are necessary to inactivate all of the microorganisms that present in the Ginseng sample and increase the

efficiency of the process. In this study, we assessed the feasibility of using carbon dioxide with modifiers such as water, ethanol, and hydrogen peroxide to improve the sterilization efficiency and decrease the processing time. The experiments were conducted at 100 bar and 60°C for 1 h using 1 mL of various modifiers in total volume of 70 mL CO_2. The synergistic effect of combination of modifiers was also investigated.

The results of preliminary studies demonstrated that additive at atmospheric pressure and mixture of additives with other inert gas was not efficient in decreasing total microorganism content significantly at 60°C. As shown in Table II, the addition of modifiers increased the inactivation efficiency of CO_2. The results in Figure 2 and Table II demonstrate that hydrogen peroxide was more efficient than water and ethanol in decreasing the TAMC in Ginseng powder (0.4 log reduction for $CO_2 + H_2O_2$ compared with 0.1 for $CO_2 + H_2O$). However, when equal volumes of binary mixture of these solvents were added to CO_2, a 1.19 log reduction was achieved. The synergistic effect was further observed when equal volumes of the three solvents were used, approaching 1.75 log reductions for TAMC in Ginseng powder. Utilization of the three additives was 24 times more efficient than using water + CO_2 and 13 times more effective than ethanol and 4 times more effective than hydrogen peroxide. Addition of these modifiers remarkably increased the inactivation efficiency and decreased processing time of CO_2. Within 1 h TAMC was reduced to 60% of the level achieved using neat CO_2 for 15 h (i.e., 2.7 log reduction). As shown in Figure 3, at the conditions experiments were performed, using CO_2 at 60°C and 100 bar for 1 h to sterilize 2.5 g of Ginseng powder in 70 mL volume vessel, increasing the volume of each additive from 0.05 to 1 mL had negligible effect on inactivation power of CO_2. The same order of magnitude inactivation was also achieved when 1:1, 1:3, and 3:1, 3:0 volume ratio of water/ethanol was used. It is therefore concluded that for the sample size used in this study 0.02 mL of each additive/g of Ginseng powder was sufficient to achieve 1.75 log reduction of TAMC within 1 h at 60°C.

Table II. Effect of additives on the TAMC reduction of Ginseng using carbon dioxide at 60°C and 100 bar for 1 h.

Amount of each additive (mL) H_2O/ethanol/H_2O_2	Log (N_0/N)	Synergistic effect[a]
1:0:0	0.07	—
0:1:0	0.13	—
0:0:1	0.44	—
1:1:0	0.87	4.35
0:1:1	1.02	1.78
1:0:1	1.19	2.33
1:1:1	1.75	2.73

[a]Synergistic effect was calculated by dividing the effect of mixture of all additive divided by sum of effect of each additive [log N_0/N of all additives/sum(log N_0/N)]. Each run was repeated at least three times. In each run, 2.5 g Ginseng sample was placed in the 70 mL vessel.

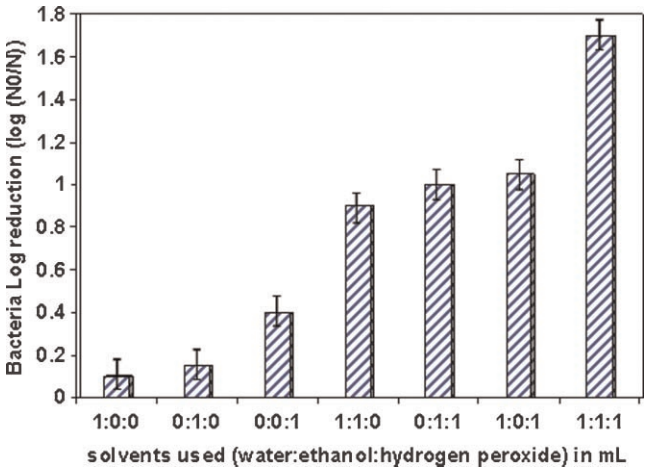

Figure 2. Effect of solvents on the inactivation of bacteria for Ginseng samples sterilized by CO_2 at 60°C, 100 bar, and 1 h. Error bars are standard errors. [Color figure can be seen in the online version of this article, available at www.interscience.wiley.com.]

As determined in other studies, the mechanism of inactivation was due to dissolving carbon dioxide in aqueous phase, then carbonic acid was formed and decreased the pH, which reduced the microorganism resistance to heat. Subsequently, CO_2 diffused into the cells and inactivated the bacteria by extraction of vital compounds, modifying the enzymatic activity and denaturation (Debs-Louka et al., 1999; Garner et al., 2006). These effects were enhanced by addition of water, ethanol, and hydrogen peroxide. Further research showed that in the presence of water, the cell walls of the viable bacteria were hydrated enabling greater permeability of CO_2 and extraction of vital compounds

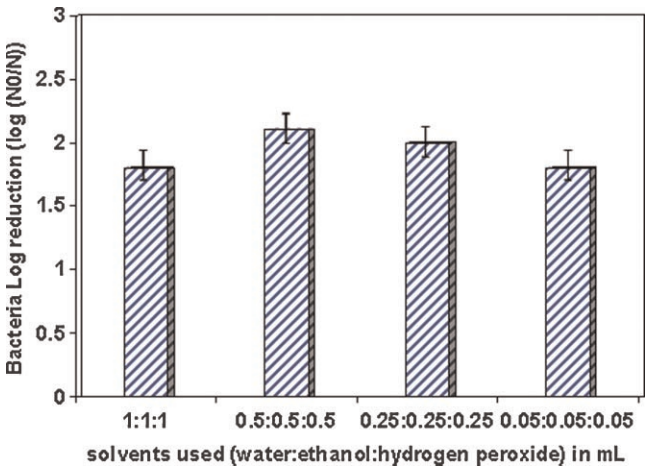

Figure 3. Effect of total amount of solvents on the inactivation of bacteria for Ginseng sample sterilized by CO_2 at 60°C, 100 bar, and 1 h. Error bars are standard errors. [Color figure can be seen in the online version of this article, available at www.interscience.wiley.com.]

that resulted in bacteria inactivation (Hong and Pyun, 1999). In systems where ethanol + CO_2 was used, ethanol modified the permeability barrier of the microorganisms, made the cells more susceptible for CO_2 and ethanol penetration and lead to bacteria inactivation (Setlow et al., 2002). In system that $H_2O_2 + CO_2$ was used, the inactivation resulted from release of dipicolinic acid (DPA) and breaking the permeability barrier, which lead to the damage to the microorganism, therefore both CO_2 and hydrogen peroxide diffused into the cell and damaged vital compounds by oxidation and hence inactivation.

Hemmer et al. (2006) found that variation of the amount of additive influences the microbial inactivation by supercritical carbon dioxide. It was hypothesized that at higher levels of added hydrogen peroxide, the operating pressure could be reduced, indicating an expected increase in bacteria inactivation.

At 60°C, 100 bar, and 1 h contact time, the synergistic effect of three solvents used was not sufficient to decrease the TAMC below the acceptable level by TGA. In addition, fungi were not completely eliminated from the system. Further modification was then required to achieve higher degree of inactivation by high pressure carbon dioxide. The effect of temperature, CO_2 pressure, and treatment time that are the major parameters on the inactivation of microorganism contaminated the Ginseng powder were then studied in further details.

Effect of Processing Time

The inactivation is generally a first order reaction and is enhanced by increasing the processing period (Garner et al., 2006). The inactivation time is a key parameter in the high pressure CO_2 process (Lin et al., 1994). Lin et al. used a process duration of 12 h to inactivate *E. coli* from stainless steel using $SCCO_2$ and distilled water.

Utilizing neat CO_2 at 60°C and 15 h processing time resulted in 2.67 log reduction of TAMC that is not desirable. A considerable reduction of TAMC in Ginseng powder samples was achieved within 1 h using equal volume of ethanol, water, and hydrogen peroxide (0.05 mL of each) using CO_2 at 60°C. The effect of processing time was then assessed on the inactivation of bacteria and fungi using these modifiers.

A complete fungi removal was achieved after 6 h using these modifiers. As shown in Figure 4 the kinetics of bacteria inactivation was a first order reaction ($\ln N = \ln N_0 - k_d t$) with $k_d = 1.9\ h^{-1}$. While 6 h was required to achieve 4.3 log reductions, it is estimated from the model that 8.5 h would be necessary for complete inactivation. The dense gas sterilization process is therefore competitive with methods such as ethylene oxide and γ-irradiation in regards to the processing time (McDonnell, 2007). Although 4.3 log reductions within 6 h decreased the TAMC in Ginseng powder below the acceptable level and completely inactivated fungi, our primary objective was to decrease the processing time within 2 h to minimize the operating cost of the process.

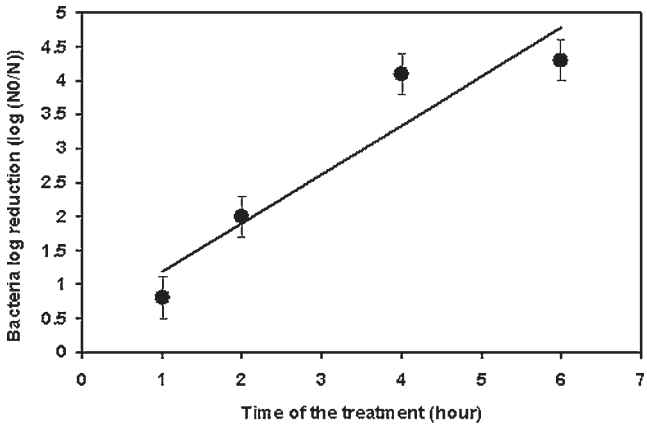

Figure 4. Effect of time of treatment on the inactivation of bacteria for Ginseng powder using CO_2 at 60°C, 150 bar, and 1:1:1 mL volume ratio of water/ethanol/H_2O_2. Error bars are standard errors.

Effect of Temperature

Bacteria inactivation is primarily controlled by diffusion. The rate of diffusion increases as the temperature is elevated thereby increasing the overall rate of microbial inactivation (Arreola et al., 1991; Garner et al., 2006; Hong and Pyun, 1999). On the other hand, Molin (1983) observed that increasing the temperature decreased microbial inactivation.

In this study, the process parameters were kept constant using CO_2 at 150 bar and 2 h processing time, using equal volume of water/ethanol/hydrogen peroxide (0.1 mL of each/g Ginseng) and the temperature was varied from 30 to 75°C. As shown in Figure 5 the inactivation efficiency was first decreased dramatically and then approached a plateau, when the temperature was increased. The data demonstrate that a lower temperature was indeed more effective for the reduction of bacteria. A 4.40 log reduction in bacteria was achieved at 30°C and 150 bar within 2 h which was greater than 99.99% inactivation. Only 2,000 TAMC/g were left in Ginseng sample, which was within the allowable limit as per the TGA regulations for oral drug administration.

The contradictory behavior observed may be due to decrease in density of CO_2 at higher temperatures, which leads to decrease of its solubility in aqueous phase (i.e., pH was decreased less) and hence decreased extraction efficiency of vital compounds. The density of carbon dioxide decreased from 0.60 to 0.51 and 0.46 g/cm^3 as the temperature increased from 60 to 70 and 75°C at 150 bar (NST 205 REFPROP database, 2007).

The fungi were not completely inactivated at all temperatures examined. As the temperature was lowered,

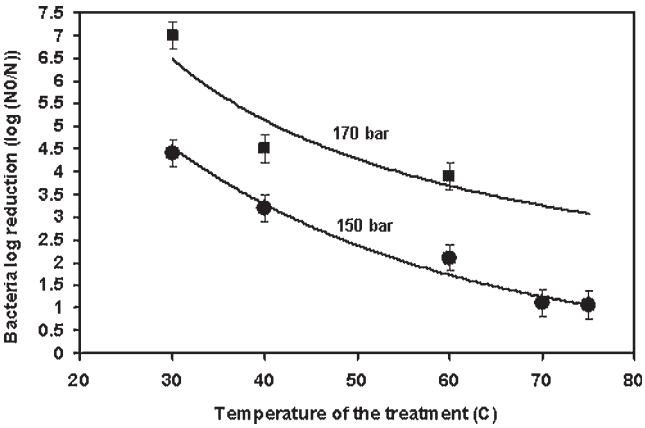

Figure 5. The effect of temperature and pressure on the inactivation of bacteria for Ginseng samples using CO_2 with water/ethanol/H_2O_2 mixture for 2 h. Error bars are standard errors.

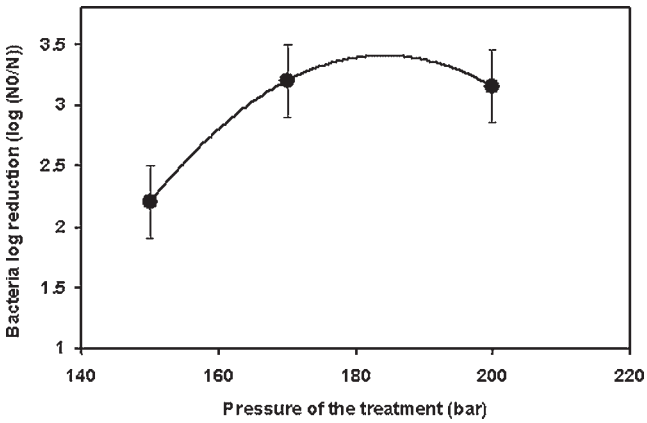

Figure 6. The effect of pressure on the inactivation of bacteria for Ginseng samples using CO_2 with water/ethanol/H_2O_2 mixture at 60°C for 2 h. Error bars are standard errors.

there appeared to be a decrease in the residual fungi in the Ginseng sample. The sterilization process was not completely effective for the inactivation of all mircroorganisms, and therefore the pressure was altered.

Effect of Pressure

Garner et al. (2006), Debs-Louka et al. (1999), and Zhang et al. (2006b) found that increasing the pressure improved the CO_2 sterilization efficiency for different types of bacteria, presumably due to enhancing the dissolution of vital compounds and further decrease in pH. The lipids in the cell membrane may be dissolved by carbon dioxide at higher pressures, which increases cellular penetration and hence microbial inactivation. In addition, high pressure increases the release of intracellular ions, disrupts glycolysis, changes membrane-bound enzymatic activities and induces membrane inactivity, which collectively aids in microbial inactivation (Arreola et al., 1991; Debs-Louka et al., 1999; Garner et al., 2006; Hong and Pyun, 1999, 2001; Hoover et al., 1989; Lin et al., 1994; Tomasula and Boswell, 1999; Werner and Hotchkiss, 2006; Zhang et al., 2006b).

By increasing the pressure from 150 to 200 bar at 60°C, the TAMC was first dramatically decreased in Ginseng powder and then approached a plateau (Fig. 6). The inactivation efficiency was increased 1.4 times when the pressure increased from 150 to 200 bar corresponding to the increase in density of CO_2 from 0.60 to 0.66 and 0.69 g/cm^3, respectively (NIST, 2007).

More interestingly, 100% inactivation was achieved for fungi at pressures above 170 bar within 2 h at 60°C using equal volume of water/ethanol/hydrogen peroxide (0.1 mL of each/g Ginseng). No fungi and bacteria were also detected in samples treated at 30°C and 170 bar. This observation indicates that a minimum of 170 bar was, therefore, necessary for the total inactivation of fungi in a period of 2 h. The results suggest that high pressure has a greater effect than temperature on the inactivation of fungi.

Decreasing the temperature at 170 bar from 60 to 40 and 30°C increased the log reduction of aerobic microbes as can be seen in Figure 5, 1.2 and 2 times, respectively, confirming that a higher solubility and density of $SCCO_2$ aided microbial death. It is the synergistic effect of a low temperature and high pressure that instigated sterilization of CO_2 as 1.6 times more bacteria were inactivated at 170 bar than at 150 bar at 30°C. If the pressure is not sufficiently high, it is possible that the TAMC may exceed the recommended limit. The best operating conditions for the range of parameters, which have been tested in this study were therefore 30°C and 170 bar using 0.1 mL of each additive/g Ginseng, where all of the microorganisms were eliminated.

Conclusions

The outcomes of this study demonstrate the potential of modified high pressure CO_2 for the complete inactivation of microorganisms in Ginseng powder with a contamination level as high as 10^7. Neat CO_2 was not effective to inactivate all microorganism within a short period of time. Addition of additives such as water/ethanol/H_2O_2 substantially enhanced the sterilization efficiency of CO_2. Processing pressure, temperature and time play a critical role in CO_2 sterilization efficiency. A processing pressure as high as 170 bar was required for complete inactivation of fungi within 2 h. Sterilization efficiency was dramatically increased by decreasing the processing temperature. A complete inactivation of both bacteria and fungi was successfully achieved within to 2 h, by decreasing the operating temperature to 30°C and increasing the pressure to 170 bar using 0.1 mL of each additive/g Ginseng.

Further optimization of the experimental conditions is certainly possible allowing for lower temperatures, additives,

pressures, and treatment period. However, the successful removal of microorganisms from Ginseng confirms the feasibility of using $SCCO_2$ at the operating conditions determined in this study for commercial sterilization of Ginseng powder. The study will be continued to measure the stability of the active ingredients in Ginseng at the optimum conditions acquired for SC sterilization and comparing the results with the conventional methods.

The authors would like to acknowledge the financial support of the University of Sydney, technical assistance of Mr. Anthony Cheung and Ms. Elizabeth Dobrinsky for performing part of the experiments and assisting in analytical procedures, and The Simply Ginseng Company for donating the Ginseng powder for this study.

References

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

British Pharmacopoeia Online. 2007. www.pharmacopoeia.co.uk

Byun M, Yook H, Kang I, Chung C, Kwon J, Choi K. 1998. Comparative effects of gamma irradiation and ozone treatment on hygienic quality of Korean red ginseng powder. Radiat Phys Chem 52(6):95–99.

Castleman M. 2007. Cancer fighter: Ginseng boosts immune function, combats stress, and fatigue, and even helps fight brest cancer. Nat Health 37(2).

Chu JHK. 2007. Siberian ginseng. Complementary and Alternative Healing University. http://alternativehealing.org/ci%20wu%20jia.htm

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.

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

Fages J, Barbier Y, Poirier B, Frayssinet P, Joffret ML, Majewski W. 1998. Viral inactivation of human bone tissue using supercritical fluid extraction. ASAIO J 44:289–293.

Foster NR, Mammucari R, Dehghani F, Barrett A, Bezanethak K, Coen E, Combes G, Meure L, Ng A, Regtop H, Tandya A. 2003. Processing pharmaceutical compounds using dense-gas technology. J Ind Eng Chem Res 42(25):6476–6493.

Garner PL, Prempah G, Williamson LN, Roberts KL, Bothun GD. 2006. Supercritical carbon dioxide-based cleaning and sterilization of *E. coli* and *S. aureus* from stainless steel substrates. 2006 AIChE Meeting. San Francisco, CA, USA (http://aiche.confex.com/aiche/2006/techprogram/P73325.HTM, accessed on 10/04/08).

Hemmer JD, Drews MJ, LaBerge M, Mathews MA. 2007. Sterilization of bacterial spores by using supercritical carbon dioxide and hydrogen peroxide. J Biomed Mater Res B 80B(2):511–518.

Hong SI, Pyun YR. 1999. Inactivation kinetics of Lactobacillus plantarum 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.

Hoover DG, Metrick C, Papineau AM, Farkas DF, Knorr D. 1989. Biological effects of high hydrostatic pressure on food microorganisms. Food Technol 43(3):99–107.

Jimenez A, Thompson GL, Matthews MA, Davis TA, Crocker K, Lyons JS, Trapotsis A. 2007. Compatibility of medical-grade polymers with dense CO_2. J Supercritic Fluids 42(3):366–372.

Kamihira M, Taniguchi M, Kobayashi T. 1987. Sterilization of microorganisms with supercritical carbon dioxide. Agric Biol Chem 51:407–412.

Kiefer D, Pantuso T. 2003. Panax ginseng. Am Fam Physician 68(8):1539–1542.

Kwon O, Kang IJ, Byun MW, Yook HS. 1997. Effects of gamma irradiation on physicochemical properties of Korean red ginseng powder. Radiat Phys Chem 49(4):483–489.

Lin H, Cao N, Chen L. 1994. Antimicrobial effect of pressurised carbon dioxide on listeria monocytogenes. J Food Sci 59(3):657–659.

Lin H, Yang Z, Chen L. 1993. Inactivation of Leuconostoc dextranicum with carbondioxide under pressure. J Chem Eng 52:B29–B34.

Lin H, Yang Z, Chen LF. 1992. Inactivation of *Saccharomyces cerevisiae* by supercritical and subcritical carbon dioxide. Biotechnol Prog 8:458–461.

Luo John Z, Luo L. 2006. American ginseng stimulates insulin production and prevents apoptosis through regulation of uncoupling protein-2 in cultured beta cells. Evid Based Complement Altern Med 3(3): 365–372.

McDonnell GE. 2007. Antisepsis, disinfection, and sterilizatiion: Types, action and resistance. Washington DC, USA: ASM Press. 361 p.

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

NIST. 2007. NIST 205 REFPROPR database. Nova Sterilis.

Parton T, Elvassore N, Bertucco A, Bertoloni G. 2007. High pressure CO_2 inactivation of food: A multi-batch reactor system for inactivation kinetic determination. J Supercritic Fluids 40(3):490–496.

Quan C, Li S, Tian S, Xu H, Lin A, Gu L. 2004. Supercritical fluid extraction and clean-up of organochlorine pesticides in ginseng. J Supercritic Fluids 31(2):149–157.

Setlow B, Loshon CA, Genest PC, Cowan AE, Setlow C, Setlow P. 2002. Mechanisms of killing spores of *Bacillus subtilis* by acid, alkali and ethanol. J Appl Microbiol 92(2):362–375.

Sivakumar G, Yu KW, Paek KY. 2005. Biosafe ginseng: A novel source for human well-being. Eng Life Sci 5(6):527–533.

Spilimbergo S, Bertucco A, Basso G, Bertoloni G. 2005. Determination of extracellular and intracellular pH of *Bacillus subtilis* suspension under CO_2 treatment. Biotechnol Bioeng 92(4):447–451.

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

Gold L. 2007. Company co-founded by Ganem wins Green Chemistry award, Chronicle Online, http://www.news.cornell.edu/stories/June07/ganem.greenchem.html, accessed on 10/04/08.

Sung H, Kang S-M, Lee M-S, Kim TG, Cho Y-K. 2005. Korean red ginseng slows depletion of CD4 T cells in human immunodeficiency virus type 1-infected patients. Clin Diagn Lab Immunol 12(4):497–501.

Tomasula PM, Boswell RT. 1999. Measurement of the solubility of carbon dioxide in milk at high pressures. J Supercritic Fluids 16(1):21–26.

Tournas V, Katsoudas E, Miracco E, 2005. Moulds, yeasts and aerobic plate counts in ginseng supplements. 11th Annual FDA Science Forum.

Werner BG, Hotchkiss JH. 2006. Continuous flow nonthermal CO_2 processing: The lethal effects of subcritical and supercritical CO_2 on total microbial populations and bacterial spores in raw milk. J Dairy Sci 89(3):872–881.

White A, Burns D, Christensen TW. 2006. Effective terminal sterilization using supercritical carbon dioxide. J Biotechnol 123(4):504–515.

Wood JA, Bernards MA, Wan W-k, Charpentier PA. 2006. Extraction of ginsenosides from North American ginseng using modified supercritical carbon dioxide. J Supercritic Fluids 39(1):40–47.

Zhang J, Burrows S, Gleason C, Matthews MA, Drews MJ, LaBerge M, An YH. 2006a. Sterilizing *Bacillus pumilus* spores using supercritical carbon dioxide. J Microbiol Methods 66(3):479–485.

Zhang J, Davis TA, Matthews MA, Drews MJ, LaBerge M, An YH. 2006b. Sterilization using high-pressure carbon dioxide. J Supercritic Fluids 38(3):354–372.