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Agriproducts Sterilization and Optimization by Using Supercritical Carbon Dioxide Fluid (SC-CO_2)

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Abstract. Sterilization via supercritical carbon dioxide (SC-CO_2) is an innovative, non-thermal bactericidal technique applicable to some thermal sensitive products. Due to the fact that supercritical CO_2 fluid shows numerous characteristics: it is inert, inexpensive, easily available, odorless, tasteless, and environmentally friendly. It is suggested that SC-CO_2 is an effective alternative for terminal sterilization of biological materials and medical devices. This study investigates the bactericidal effect of SC-CO_2 treatment for various foods to ensure microbiological safety and product quality. In this study, the Taguchi method was applied to determine optimum conditions for the SC-CO_2 process, and *Bacillus atrophaeus* spores, *Saccharomyces cerevisiae* and *Escherichia coli* (*E. coil*) were chosen as biological indicators (BI) to test the bactericidal effect. The results show that the pressure difference had no effect in sterilization ($p < 0.05$). When exposed to SC-CO_2 optimum conditions of 1200 psi at 45℃ for 32 hrs, the *Bacillus atrophaeus* spores saw 4.06 log (CFU/g) reductions, the yeast (*Saccharomyces cerevisiae*) saw 5.2 log (CFU/g) reductions and the *E. coil* saw 5.8 log (CFU/g) reductions. Also, the total plate count (TPC) in purple cabbage and cucumber saw 5.0 log reductions under the same conditions. Additionally, scanning electron microscope (SEM) images show that SC-CO_2 treatment indeed broke the structure of microbe, causing the bacteria to die.

Keywords: Supercritical carbon dioxide (SC-CO_2), sterilization, *Bacillus atrophaeus* spores, biological indicator (BI).

1. Introduction

Microbial contaminant in food has been a serious issue for a long time, especially for some thermal sensitive products [1]. Commercial sterilization methods are capable of acheiving effective and complete aseptic conditions. However, high temperature and pressure sterilization treatment may have potentially detrimental effects on the nutritional and sensory properties of the products [2]. To solve this problem, there are some non-thermal sterilization methods which can be applied to food and medicine. Some such methods involve the use of ultraviolet (UV), ethylene oxide (EtO), gamma radiation, electron-beam and hydrogen peroxide plasma, etc. [3]-[6]. However, these methods still have some limitations for industrial product applications. Although application of chemical bactericide is a more economical, rapid and effective method, a large number of pesticides create other serious problems involving: food safety, environment pollution and pesticide residue [7]. These chemical substances also have embryotoxic, teratogenic, nephrotoxic and hepatoxic properties [7]. In addition, the use of additional chemicals and irradiation can cause consumers to avoid the treated products. It is important to find a suitable non-thermal sterilization technique to ensure microbiological safety without the deterioration of product quality in the food and pharmaceutical industries. Hence, this study has developed a novel process for terminal sterilization using supercritical carbon dioxide (SC-CO_2), and also investigated the bactericidal effects of SC-CO_2 treatment on foods.

The supercritical fluid technology has already been applied to various industries, for instance with decaffeination and the extraction of a large variety of valuable fat soluble compounds from plant matrixes,

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spices and medicinal plants [8]-[11]. With the supercritical CO_2 method, it is easy to attain the supercritical state (31.1 ℃, 1072 psi), and also has a number of other benefits. For instance, it is inert, inexpensive, easily available, odourless, tasteless, highly penetrative and environment friendly [11]. Recent studies suggest that SC-CO_2 is an effective alternative for the terminal sterilization of biological materials and medical devices [12]-[15]. This study will further investigate the bactericidal effect of SC-CO_2 treatment for various foods to ensure microbiological safety and product quality.

In this study, the Taguchi method is employed to determine optimum conditions for the SC-CO_2 process. The Taguchi method is a powerful tool employed in the industry for improving productivity during research and development, so that high quality products can be produced quickly and at very low cost [16]. It helps us to optimize manufacturing processes involving multiple factors and varying levels, especially with respect to chemical engineering processes and new food product developments [16].Some reports used the Taguchi method to optimize operating conditions in food science and engineering, such as fermentation processes [17]-[19], supercritical fluid extraction [8] and agriculture product drying [20]. In this study, the Taguchi method was applied to determine the optimum conditions for the SC-CO_2 process; furthermore, *Bacillus atrophaeus* spores (*B. atrophaeus* spores), *Saccharomyces cerevisiae* (*S. cerevisiae*) and *Escherichia coli* (*E. coil*) were chosen as biological indicator (BI) to test bactericidal effect.

2. Materials and Methods

2.1. Microorganisms and Culture Conditions

The study screened *B. atrophaeus* spores (BCRC 17530), *S. cerevisiae* (BCRC 21812) and *E. coil* (BCRC 10675) purchased from the Culture Collection and Research Center (CCRC) in Taiwan for the sterilization test. The biomass of the test sample contained about 10^8 CFU/g. Among the tested strains obtained included a lyophilized powder in a glass ampoule sealed under vacuum. Bacteriological analyses were carried out in triplicate on 10g raw dried samples that were blended with 90 mL of sterile water as described in the China National Standard (CNS 10890 N6186, 1991) method [21]. Pour plates were prepared from 10-fold dilutions in trypticase soy agar (Difco™, Sparks, MD, USA) for the *B. atrophaeus* counts, with yeast mold agar (DifcoTM, Sparks, MD, USA) for the yeast (*S. cerevisiae*) counts, and plate count agar (PCA, DifcoTM) for the *E. coil* counts. Counts were made after incubation at 35 ℃ for 48 hrs.

2.2. Experimental design

The schematic representation of the SC-CO_2 sterilization process and optimizations experiment designs according to the Taguchi method is shown in Fig. 1. *B. atrophaeus* spores, *S. cerevisiae* and *E. coil* were chosen as the biological indicator (BI) to test bactericidal effect. The log reduction of BI was calculated by using the following equation:

$$\log(N_{reduction}) = \log(N_{raw}) - \log(N_{sterilized}) \text{ (Log CFU/g)} \quad (1)$$

2.3. Description of the Apparatus

A batch-operated supercritical carbon dioxide sterilization system is shown in Fig. 2. Ten grams of lyophilized powder were loaded into a 100 mL sterilization vessel. Supercritical fluid extractions were conducted at 1200-3500 psi and 40-50 ℃, during a 16-32 hr. period in a static mode.

2.4. Taguchi method

The Taguchi method is mainly used to achieve a high bactericidal effect for the SC-CO_2 and effectively reduce the number of experimental trials. The control factors shown in Table 1 include different kinds and levels of treatment time, temperature, pressure, and valve opening degree during the SC-CO_2 sterilization process. $L_9(3^4)$ orthogonal arrays were selected for the experiments and there were 9 experimental runs with 4 factors (columns) and 3 levels (rows). The signal-to-noise ratio (S/N ratio, η) was calculated from the experimental data via a loss function, which created a transformation function of the repetitive data to another value and was used as a measure of the variation present in the experiment [16],[17]. The loss function depends on the criteria for the quality characteristic being optimized, and a high S/N ratio value is used as an indicator of optimal conditions [16]. The objective in this study was to find the optimum SC-CO_2 sterilization conditions to enhance the bactericidal effect for BI. Here, the bactericidal effect of BI was treated as having larger-the-better performance characteristics. For the loss function for the S/N ratio (η_{ij}), larger-the-better can be expressed as:

$$\eta_{ij} = -10\log\left(\frac{1}{n}\sum_{k=1}^{n}\frac{1}{y_{ijk}^2}\right) \text{ (db)} \quad (2)$$

The optimal level of the process parameters were identified and could be computed based on the selected levels of factors for forecasting the optimal performance of the S/N ratio (η_f). The estimated S/N ratio (η_f) equation can be expressed as [20]:

$$\eta_f = \overline{\eta} + \sum_{i=1}^{\alpha} (\eta_i - \overline{\eta}) \text{ (db)} \quad (3)$$

Here, $\overline{\eta}$ is the total mean of the S/N ratio, and η_i is the S/N ratio for choosing the optimal procedure parameters and its level, and α is the number of the process parameters that significantly affected the optimal conditions in the SC-CO_2 sterilization process. For the confirm experiment, the study purchased cabbage (*Brassica oleracea* L.) and cucumber (*Cucumis sativus* L.) from a local market in Pingtung City in southern Taiwan as a test sample to test the practicability with optimal conditions for the SC-CO_2 sterilization process.

Fresh ginger (Zingiber officinalis Roscoe) was purchased

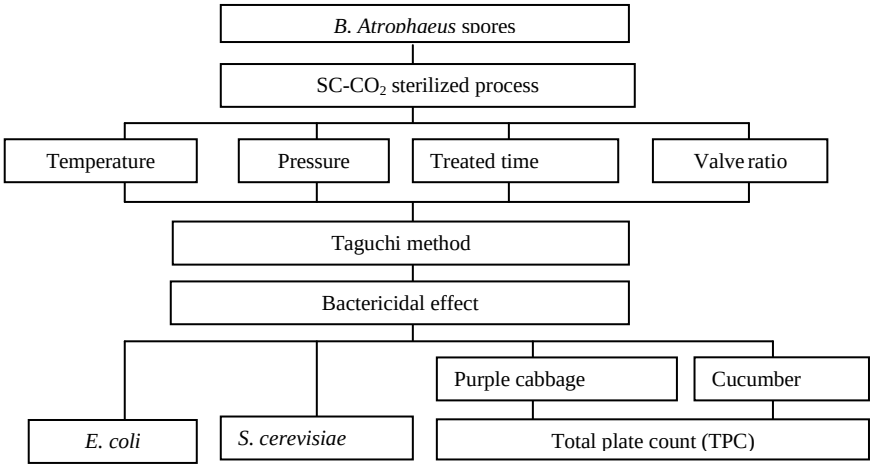

Fig. 1: Schematic representation of experiment design on SC-CO_2 sterilization process

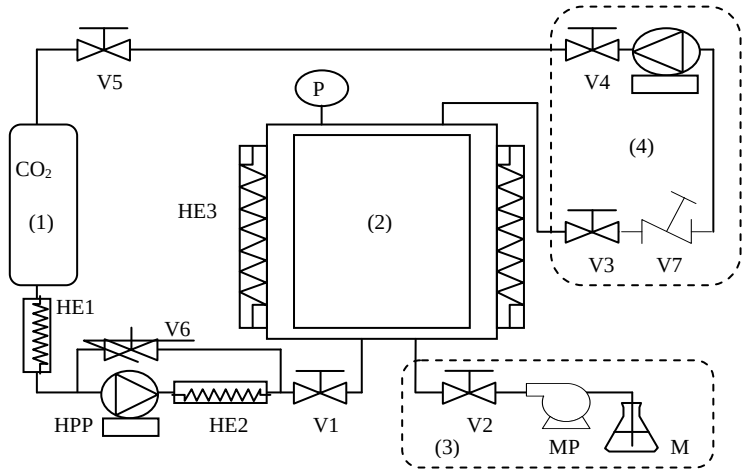

Fig. 2: Scheme of the SC-CO_2 sterilization equipment: (1) CO_2 tank, (2) sterilization vessel, (3) co-solvent injector system, (4) CO_2 recycle collector, (HE1-3) heat exchangers, (HPP) high pressure pump, (M) modified solvent, (MP) modifier pump, (P) pressure gauges, (V1-5) exhaust valve, (V6) back pressure valve, (V7) needle valve.

Table 1. The control factors and levels of in the SC-CO_2 sterilization process

Factors	A	B	C	D
	Temperature (℃)	Treatment time (hrs)	Pressure (psi)	Valve ratio (%)
Level 1	40	16	1200	30
Level 2	45	24	2000	60
Level 3	50	32	3000	100

2.5. Statistical analysis

Analysis of variance (ANOVA) was used to investigate the significance of the influence and confidence of the processing parameters on the performance [22], [23]. A regression analysis of the experimental data was obtained using SAS® 8.2 (Statistical analysis system, Cary, NC, USA). Duncan's multiple-range test was used to compare the difference between the mean at a probability level < 0.05.

2.6. Microstructure of the Samples

The microstructures of bacteria in the SC-CO_2 sterilization process were observed using a scanning electron microscope (SEM, Hitachi S-4300, Tokyo, Japan). Lyophilized powder was coated with a gold layer using a vacuum sputter-coater, and photographed at an accelerator potential of 10 kV. The microstructures of bacteria examined included the surface and cross-section.

3. Results and Discussion

3.1. Optimal conditions for the SC-CO_2 sterilization process

The S/N ratio was calculated from the average experiment data using a loss function for the higher-the better performance Eq. (2) and their ANOVA analysis, as shown in Fig. 3 and Table 2. The results show that the pressure difference had no effect for sterilization ($p < 0.05$). The optimal conditions are A2 (45℃), B3 (Treated 32 hrs), C1 (1200 psi), and D2 (60% valve ratio); the estimated S/N ratio (η_f) using Eq. (3) is 12.21 (db) and the S/N ratio from the confirmation experiment is 12.15 (db).

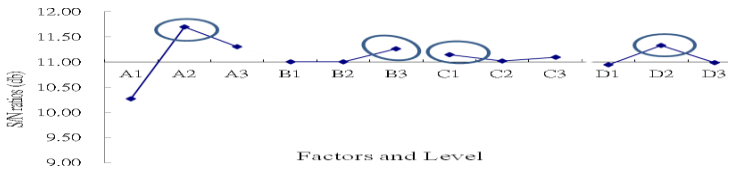

Fig. 3: The results of the L_9 (3^4) orthogonal array experiments for different operating conditions in the SC-CO_2 sterilization process and its signal-to-noise ratio (S/N ratio) in *B. atrophaeus* spores: (A1-3) temperature (℃), (B1-3) treatment time (hrs), (C1-3) pressure (psi), (D1-3) valve ratio (%)

Table 2. Analysis of variance (ANOVA) of factors affecting bactericidal effect

Factor	Sum of squares	DOF	Variance	*F*-ratio	Confidence (%)	Significant
A	1.3125	2	0.66	1018.28	100.00	**
B	0.0453	2	0.02	35.18	100.00	**
C	0.0175	2	0.01	13.56	99.97	*
D	0.0542	2	0.03	42.07	100.00	**
Error	0.5112	18	0.03			

** : Significant at least at the 1% level, $p < 0.01$

* : Significant at least at the 5% level, $p < 0.05$

3.2. SEM micrographs

Fig. 4 shows the microstructures of *B. atrophaeus* spores which were observed (1000X) during untreated and inactivated SC-CO_2 sterilization process, respectively. The samples were kept from original tissues as shown in Fig. 4(a). SC-CO_2 sterilization would lead to shrinkage of tissue formation and to death as shown in Fig. 4(b).

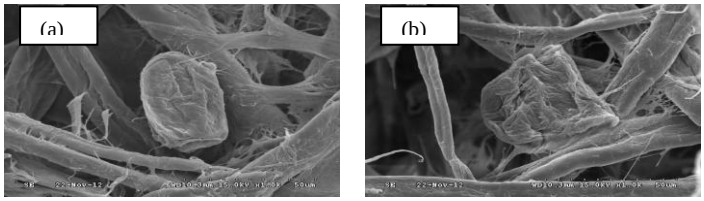

Fig. 4: SEM micrographs of *B. atrophaeus* spores: (a) untreated (1000 ×), (b) inactivated by SC-CO_2 sterilization process (1000 ×)

3.3. Practical application

The cabbage and cucumber were used with optimal conditions to test the practicability of the SC-CO_2 sterilization process. Table 2 shows that under the SC-CO_2 optimum conditions of 1200 psi at 45℃ for 32 hrs, the *Bacillus atrophaeus* spores expereinced 4.06 log (CFU/g) reductions, yeast (*Saccharomyces*

cerevisiae) experienced 5.2 log (CFU/g) reductions and *E. coil* experienced 5.8 log (CFU/g) reductions. Furthermore, the total plate count (TPC) of in the purple cabbage and cucumber experienced 5.0 log reductions in the same conditions. This means that SC-CO_2 does indeed have a good bactericidal effect on purple cabbage and cucumber.

Table 2. TPC in sterilization of purple cabbage and cucumber under different treatment conditions.

	Biomass (Log CFU/g)			
	Control	Hot air 120 ℃ sterilization	Hot air 45 ℃ sterilization	SC-CO_2 sterilization
B. atrophaeus spores	$6.02^{a} \pm 0.12$	$0.53^{c} \pm 0.12$	$5.53^{b} \pm 0.23$	$0.18^{c} \pm 0.02$
S. cerevisiae	$6.23^{a} \pm 0.12$	ND[d]	$5.52^{b} \pm 0.42$	$1.02^{c} \pm 0.02$
E. coil	$5.82^{a} \pm 0.12$	ND[d]	$5.02^{b} \pm 0.13$	$0.02^{c} \pm 0.01$
Practical application	Total plate count (TPC) (Log CFU/g)			
Purple cabbage	$5.12^{a} \pm 0.12$	$0.18^{c} \pm 0.02$	$4.82^{b} \pm 0.14$	ND[d]
Cucumber	$5.28^{a} \pm 0.18$	$0.23^{c} \pm 0.05$	$4.95^{b} \pm 0.26$	ND[d]

[abcd] Means with the same rows in a column do not significantly differ from each other (Duncan's multiple-range test, $p < 0.05$). Individual bars represent data as the mean of 3 replicates ± the standard deviation.
ND: not detected.

4. Conclusions

The results show that the pressure difference had no effect in sterilization ($p < 0.05$). The optimal conditions are A2 (45 ℃), B3 (Treated 32 hrs), C1 (1200 psi), and D2 (60% valve ratio) on Taguchi method. When exposed to SC-CO_2 optimum conditions of 1200 psi at 45 ℃ for 32 hrs, the *Bacillus atrophaeus* spores saw 4.06 log (CFU/g) reductions, the yeast (*Saccharomyces cerevisiae*) saw 5.2 log (CFU/g) reductions and the *E. coil* saw 5.8 log (CFU/g) reductions. Also, the total plate count (TPC) in purple cabbage and cucumber saw 5.0 log reductions under the same conditions. The SC-CO_2 based sterilization apparatus and process is indeed capable of achieving validated microbe biomass induced 4 log (CFU/g) levels of terminal sterilization.

5. Acknowledgements

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