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Research Article

CO_2-assisted high pressure processing on inactivation of *Escherichia coli* and *Staphylococcus aureus*

Liang Zhao[a,b,c], Xiao Qin[b,c], Yongtao Wang[a,b,c], Jiangang Ling[d], Weile Shi[b,c], Sicheng Pang[b,c], Xiaojun Liao[a,b,c,*]

[a] *Beijing Advanced Innovation Center for Food Nutrition and Human Health, College of Food Science and Nutritional Engineering, China Agricultural University, Beijing 100083, China*
[b] *Beijing Key Laboratory for Food Nonthermal Processing, National Engineering Research Center for Fruit & Vegetable Processing, Beijing 100083, China*
[c] *Key Laboratory of Fruit & Vegetable Processing, Ministry of Agriculture, Beijing 100083, China*
[d] *Institute of Agricultural Products Processing, Ningbo Academy of Agricultural Science, Ningbo 315040, China*

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ABSTRACT

CO_2-assisted high pressure processing was termed as CO_2-HPP, and the inactivation of *Escherichia coli* and *Staphylococcus aureus* in liquid samples were treated by CO_2-HPP in this study. When CO_2 made up 20% of the total volume, it exhibited assisted inactivation of *E. coli* and *S. aureus* in phosphate buffered saline (PBS, pH 7.0) under HPP. Twenty percent CO_2-300 MPa/3 min induced 1.0 and 2.5 more log units reduction of *E. coli* and *S. aureus* than 300 MPa/3 min, respectively. Both mid-exponential and stationary phase cells of *E. coli* and *S. aureus* showed higher sensitivity to CO_2-HPP than HPP. However, the two microbes showed different inactivation behavior in real food of cucumber juice (pH 6.6) and apple juice (pH 4.3), and CO_2-HPP was more suitable for *S. aureus* than *E. coli* in the two juices. As compared with HPP, CO_2-HPP showed more severe damage on morphology and intracellular structure of *E. coli* and *S. aureus* cells through transmission electron microscopy (TEM), scanning electron microscopy (SEM), and flow cytometer microscopy (FCM), including appearance of collapsed cells, cell disruption, and more holes on cell membrane, membrane permeability, and strength of cytoplasm aggregation, which were probably due to the penetration, explosion, and acidification of CO_2 under HPP. The CO_2-assisted inactivation of bacteria subject to HPP has the potential application in food industry, especially for liquid food.

1. Introduction

Carbon dioxide (CO_2) is ubiquitous on the Earth, and its utilization is being highly considered nowadays. Due to the antimicrobial activity and natural characteristic [1], this gas is usually used in food industry in the way of high pressure carbon dioxide (HPCD), modified atmosphere packaging (MAP), and carbonation, which are efficient in the inactivation or inhibition of the growth of microorganisms. HPCD is a non-thermal processing that affects microorganisms and enzymes through molecular effects of CO_2 held under pressure below 50 MPa [20,59]. Under HPCD, food is contact with either (pressurized) sub- or supercritical CO_2 (Tc = 31.1 °C, Pc = 7.38 MPa) for a certain amount of time, which has the unique ability to diffuse through solids like a gas, and dissolve materials like a liquid. It has the ability of killing bacteria, yeast and fungi at moderate pressure and low temperature [2–4]. However, due to the hurdle of stable equipment and large investment cost, HPCD preservation technique has not yet been implemented on a large scale by the food industry until now [4,5].

MAP is mainly based on substitution of the surrounding atmosphere of the products with gas that has the potential of extending the shelf-life of foods [6], [7]. The main gases used in MAP are CO_2, O_2, and N_2. Due to the ability of inhibition of microbial growth and reduction of the respiration rate of fresh products, CO_2 has been widely used [6,8]. However, nearly all MAP is applied in solid products, such as fresh or fresh-cut fruits and vegetables, meat and meat produces [6,9], and it requires large amount of gas and high barrier packaging film.

Carbonation is achieved by keeping liquid samples in gas cylinder at low pressure (usually lower than 5 MPa) for a period of time, to get specific concentration [10–12]. Due to acidification of CO_2, it has the ability of microbial inhibition, or even inactivation when synergistic with other techniques. However, it usually needs a long pre-treatment time (> 20 min) to get specific concentration, which is not suitable for commercialization.

Similar to HPCD, high pressure processing (HPP) is also a non-

* Corresponding author at: College of Food Science & Nutritional Engineering, China Agricultural University, No.17, Qinghua East Road, Haidian District, Beijing 100083, China.
E-mail address: liaoxjun@hotmail.com (X. Liao).

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thermal processing, which achieves pasteurization and preserving more quality attributes of the foods than traditional thermal treatment under 100–1000 MPa at normal temperature [13]. The pressure is produced by a hydraulic pump or a piston and is isostatically transmitted inside the pressure vessel to the food product instantaneously and uniformly [14]. Up to date, it is the most successful commercialized non-thermal technique in food industry, which has already been used in fruit and vegetable, egg, meat, dairy, seafood, alcoholic beverages products, and so on [14–16]. However, HPP has limitation on inactivation of Gram-positive microorganisms, spore, and utilization in low-acid food [17]. Hence, many researchers are working on HPP plus other techniques to tackle the issue, in which MAP and carbonation are found [18–24]. Al-Nehlawi et al. used $V_{CO2}/V_{sausage}$ volume of 4:1 for MAP combined with 350 MPa/10 min to achieved 3 extra log units reduction of *Leuconostoc carnosum* [21]. Rode et al. reported that combined carbonation (0.1 MPa/24 h/1 °C) and MAP (71.3 ± 0.7% CO_2 and 0.15 ± 0.1% O_2) with 400 MPa or 600 MPa for 2 min achieved 1.3 extra log reduction of *Listeria innocua* ATCC 33090 in fish soup [19]. However, these two combined methods of MAP + HPP and carbonation + HPP are hard to be commercialized, since the compression ratio of large amount of CO_2 achieved by MAP need to be considered under HPP, and such a long treatment time was needed by carbonation.

Considering the efficiency of inactivation and possibility of commercialization, we propose a method of CO_2 assisted HPP (CO_2-HPP), which inflated liquid samples with little amount of CO_2 and combined it with HPP. To our knowledge, no articles have reported the assisted inactivation effect of a small amount of CO_2 combined with HPP.

E. coli and *S. aureus* are representative of Gram-positive and Gram-negative bacteria, respectively, which are quite common food-borne bacteria [25–27]. Some strains of *E. coli* and *S. aureus* are pressure-resistant [27–30]. Malone et al. found that 500 MPa only achieved 0.6-3.4 log units reduction of 17 different strains of *E. coli*, and *E. coli* O157:H7 EC-88 strain showed highest pressure resistance [31]. Baptista et al. achieved different reduction of *S. aureus* ATCC 6538, 2153 MA and 2065 MA, in the range of 3.36 to 6.31 log units, with an initial concentration of 10^9 CFU/mL using 600 MPa/15 min. The existence of HPP-resistance microorganisms requires enhanced technique to improving inactivation effect of HPP [32].

The objective of this study was to investigate how CO_2-HPP play the role on inactivation of *E. coli* and *S. aureus* in liquid system, including PBS, cucumber juice, and apple juice. And bacterial morphology, structure and membrane permeability after CO_2-HPP were analyzed and used to speculate the inactivation mechanism.

2. Material and methods

2.1. Experimental design

Several factors were studied on inactivation of *E. coli* and *S. aureus* by CO_2 + HPP, including volume ratios (0, 5, 10, 20, and 30%), HPP conditions (300, 400 and 500 MPa for 1 or 3 min), growth phase (mid-exponential and stationary phase), and suspended matrix (PBS, cucumber juice, and apple juice). All analyses were carried out with triplicate for each treatment.

2.2. Preparation of microbial matrix

PBS (0.01 M and pH 7.0) was prepared by sodium dihydrogen phosphate and disodium phosphate dodecahydrate (Beijing Aobox Biotechnology Co., Ltd, Beijing, China).

Cucumber and apple in this study were supplied by Shandong Top Agricultural Products Co., Ltd (Shandong, China). After washing in tap water and slicing, sliced cucumbers and apples in 0.2% L-ascorbic acid were squeezed into juice by a GT6G7 screw juicer (Light Industry Machinery Factory, Zhejiang, China). Raw juices were filtered through 4 layers of gauzes individually and were sterilized by 121 °C/15 min for consequent microbial suspension. The pH of cucumber juice and apple juice were 6.6 and 3.8, respectively.

2.3. Microbial preparation

Escherichia coli O157:H7 NCTC 12900 (National Culture Type Collection, Colindale, London, United Kingdom) in this study was a detoxification type, a well characterized *Stx* negative strain. *Staphylococcus aureus* CGMCC 1.0128 was from China General Microbiological Culture Collection Center (CGMCC, Beijing, China). The stock cultures were maintained in tryptic soy broth (TSB, Beijing Aobox Biotechnology Co., Ltd., Beijing, China) with 25% glycerol at −80 °C. Prior to use, *E. coli* and *S. aureus* were activated by streaking onto tryptic soy agar (TSA, Beijing Aobox Biotechnology Co., Ltd., Beijing, China) plate and incubated at 37 °C for 24 h. And then one colony was picked and inoculated in TSB for 12 h at 37 °C with shaking at 180 rpm until it was re-cultured with the concentration of 1% in 1.5 L TSB and rotated at 37 °C 180 rpm for 2.5 h to get the mid-exponential phase cells, and 5 and 5.5 h to get stationary phase of *E. coli* and *S. aureus*, respectively. The microbial solutions were centrifuged with high speed refrigerated centrifuge GIII (Hitachi, Japan) at 8340 × *g* for 10 min at 4 °C and pellets were washed with PBS under the same centrifuge condition and then re-suspended in 1.5 L sterilized PBS, cucumber juice or apple juice. The final concentration of *E. coli* and *S. aureus* was around 10^8 CFU/mL. The prepared microbial solutions were stored at 4 °C no more than 12 h before use. Except comparison of growth phase, mid-exponential phase cells were used in all the studies.

2.4. Gas inflation

A self-designed CO_2 inflation device comprised gas inflation box, vacuum pump, and gas cylinder. The gas inflation box (400 mm × 300 mm × 300 mm) was made of 25 mm-thick acrylic plate, and all doors of the box were sealed with magnet and fixed with rubber cushion at the inner circle. Two gloves were fixed tightly inside the two front doors which allowed hands in. In the inflation box, a maximum vacuum pressure of infinite close to 0.1 MPa can be achieved by a V-i280SV vacuum pump (Zhejiang Value Mechanical & Electrical Products Co., Ltd., Zhejiang, China) with the pumping rate of 14.4 m^3/h. Purity of CO_2 was 99.9%, which was purchased from Beijing Qianxi Gas Selling Center (Beijing, China). The inside of inflation box was exposed to UV light for 20 min before each inflation process. All inflation procedures were conducted at room temperature (25 ± 2 °C).

Before gas inflation, serial samples with 100, 95, 90, 80, and 70% to the bottle volume were filled into 80 mL-polyethylene terephthalate (PET) bottles, and the corresponding headspace was 0, 5, 10, 20, and 30% of the bottle volume.

The bottled samples were aseptically transferred into the inflation box, and the vacuum pump was turned on with all the doors of inflation box being closed. After approximately 45 s, the vacuum pressure inside box reached up to 0.1 MPa, and the samples were de-aerated by keeping the vacuum pressure for 2.5 min. Then, the vacuum pump was closed, the pressure-relief valve fixed CO_2 cylinder was turned on and CO_2 went a hose connecting CO_2 cylinder with the inflation box till the pressure gauge was adjusted to 0.1 MPa. When the inside pressure of the box came back to the atmospheric pressure, the inflation step still continued for 20 s before turning off the pressure-relief valve. Finally, the PET bottles were capped in the inflation box as quickly as possible. And then, the bottled samples were kept in fridge (4 °C) until further HPP treatment.

2.5. HPP treatment

HPP was performed by Model FPG7100 high pressure food processor (Stansted Fluid Power, Essex, UK) with 2 L pressure chamber, one heating system, one low pressure pump, two pressure intensifiers,