The Journal of Supercritical Fluids xxx (xxxx) xxx–xxx

Contents lists available at ScienceDirect

The Journal of Supercritical Fluids

journal homepage: www.elsevier.com/locate/supflu

Non-thermal microbial inactivation by using supercritical carbon dioxide: Synergic effect of process parameters

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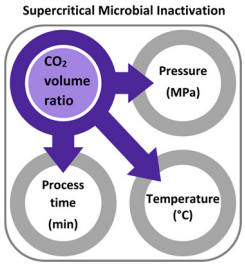
GRAPHICAL ABSTRACT



ARTICLE INFO

Keywords:
Supercritical fluid
Lactobacillus casei
Apple juice
Emerging technology

ABSTRACT

In this study, we evaluated the influence of CO_2 volume ratio (%) and its synergic effect with the pressure (MPa), temperature (°C) and process time (min) on the inactivation of *Lactobacillus casei* cells inoculated in apple juice (10^7 CFU/mL). The effects of process variables on the logarithmic reduction of *L. casei* cell counts were estimated by a two-level Plackett–Burman factorial design. A Box–Behnken design was subsequently employed to evaluate the interaction between the different factors on the supercritical microbial inactivation. The results showed that the process intensification led to a higher logarithmic reduction. Operating at 15 MPa, 55 °C, 30 min and a 70% CO_2 volume ratio, the process was able to reduce *L. casei* cell counts by more than 6 log cycles. In addition, this work reports the validation of an experimental apparatus assembled by our research group for food processing by employing supercritical carbon dioxide.

1. Introduction

The growing demand for food products that promote health and well-being has pushed the production sector to develop new, non-thermal stabilization processes. Conventional heat treatment used for the inactivation of microorganisms in food products has been associated with losses of the nutritional and sensorial quality of these products. The modern consumer searches for products with high sensoriality while also investigating the processing conditions and their functionality for health promotion through consumption [1,2].

In this scenario, supercritical CO_2 technology represents an interesting alternative for the development of non-thermal processes for microbial inactivation. CO_2 has significant advantages such as being nontoxic, non-flammable, low-cost, and environmentally safe; therefore, it is recommended for the development of environmentally friendly processes [3]. However, the main features that make supercritical CO_2 suitable to meet the new trends of product development are related to its critical point. CO_2 presents critical properties at 31 °C and

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https://doi.org/10.1016/j.supflu.2018.05.013
Received 27 March 2018; Received in revised form 11 May 2018; Accepted 14 May 2018

7.38 MPa, conditions that are relatively easily achieved; this feature makes it attractive for the industrial processing of heat sensitive products, such as vitamins, flavors, natural pigments and antioxidant compounds [4]. Furthermore, its potential as an antimicrobial agent is related to the changes it promotes in microbial cellular metabolism when in the supercritical phase; pressure and temperature adjustments may alter the solubility and diffusivity properties in the medium it is dispersed, thus enhancing its effect on inactivation due to better permeation into the microbial cell [5].

Supercritical technology as a microbial inactivation process has been applied in food products in recent years. Many beverages, such as apple juice [6], orange juice [7], strawberry juice [8], coconut water [9], milk [10], whey-grape juice [2], and others were processed by using supercritical CO_2. However, studies exploring the effects of CO_2 volume ratio and its synergic effects on others process parameters, such as pressure, temperature and process time, remain scarce. In this context, the aim of this study was to evaluate the effects and interactions of the supercritical process variables such as CO_2 volume ratio, pressure, temperature and time on the inactivation of *Lactobacillus casei* cells inoculated in clarified apple juice. *L. casei* is known by its action as a probiotic microorganism that promotes health and well-being to the human organism [11,12]. However, we selected this microorganism as a model system for supercritical inactivation because lactic acid bacteria are known as microorganisms capable of deteriorating fruit juices and are, therefore, associated with technological problems in the industrialization of these products. In addition, this work presents the results for the validation of a homemade experimental apparatus for food processing by employing supercritical carbon dioxide assembled by our research group.

2. Material and methods

2.1. Apple juice

Commercial samples of clarified apple juice (Fischer S/A Agroindústria, Videira, Brazil) free of preservatives were used as a model system in this study. The experiments were performed after the soluble solids content (SSC) was adjusted to 12°Brix. The SSC was adjusted in all experiments using a digital refractometer model PAL-1 (Atago Brasil Ltda., Ribeirão Preto, Brazil). For experimental validation of the microbial inactivation apparatus assembled by our research group, apple juice samples were incubated for 1 day at 30 °C in order to increase the total natural microbial flora count. After the incubation period, the samples presented approximately 1×10^3 colony-forming units (CFU)/mL. The microbial analyses of apple juice were performed using the plate count method as described in the "2.2.2 Microbiological analysis" section. Regarding the experiment to study the effects of all supercritical process conditions on the microbial inactivation of *L. casei*, the juice was subjected to thermal treatment at 105 °C for 10 min to inactivate any other potential contaminants.

2.2. Assembly of the microbial inactivation equipment and operating procedures

Supercritical treatments of microbial inactivation were performed in a batch homemade equipment assembled by our research group in LASEFI (Laboratory of Supercritical Technology: Extraction, Fractionation, and Identification of Vegetal Extracts) at the Department of Food Engineering (DEA)/School of Food Engineering (FEA), University of Campinas (UNICAMP)/Brazil. Fig. 1 presents the flow diagram of the experimental apparatus. The reactor (vessel) consisted of a 630 mL stainless steel cylinder (height: 240 mm, inner diameter: 67.85 mm). The equipment can operate at pressures up to 21 MPa and at a temperature range of 20–90 °C. The steps for equipment operation are as follows: (i) The samples are loaded into the reactor, (ii) The CO_2 contained inside the reservoir (approximately 6 MPa) is cooled to −4 °C with a thermostatic bath (Marconi, MA-184, Piracicaba, Brazil), (iii) The CO_2 is pressurized with a pneumatic pump (Maximator, M-111 L, Nordhausen, Germany), (iv) The system sample + pressurized CO_2 are heated through an isobaric path using a secondary thermostated bath (Marconi, MA-126, Piracicaba, Brazil), (v) The temperature and pressure are measured using thermocouples, and pressure gauges are installed according to Fig. 1, and (vi) After the process time, the system sample + CO_2 is expanded at the micrometering valve, which allows the separation of the CO_2 and the sample.

2.2.1. Validating the microbial inactivation equipment

After assembly of the equipment by the LASEFI researchers, the kinetic results of microbial inactivation of the mesophilic microorganisms obtained from the homemade equipment were compared with the kinetic results obtained by Gasperi, Aprea, Biasioli, Carlin, Endrizzi, Pirretti and Spilimbergo [6]. The authors studied the microbial inactivation by using supercritical carbon dioxide on microorganisms initially present in the apple juice and performed kinetic analysis at 1, 5, 10 and 15 min, keeping the system at 36 °C, 10 MPa and a 67% CO_2 volume ratio. Therefore, we reproduced these experimental conditions aiming to validate the homemade equipment. The apple juice samples before supercritical processing presented a total microbial count of approximately 5×10^2 CFU/mL. They determined the total microbial survival before and after each treatment by standard plating techniques. A pH analysis was performed using a digital pH-meter model Tecnal TEC-5 (Tecnal, Piracicaba, Brazil).

2.2.2. Microbiological analysis

We quantified aerobic, mesophilic, heterotrophic bacteria (AMHB) following the method described by Ryser and Schuman [13]. The enumeration of AMHB was carried out by the pour plate method in plate count agar (PCA, Kasvi®, SP, Brazil) and incubated at 37 °C for 48 h. The results obtained were expressed as a survival %, N_F / N_0 (%), where N_F represents the number of colonies in the treated apple juice samples and N_0 is the number of colonies in the untreated samples [6]. Microbiological analyses were performed twice for each supercritical processing, which was carried out in three replicates.

2.3. Bacterial strain, inoculum preparation and inoculation in apple juice

A strain of *Lactobacillus casei* (ATCC® 334™) isolated from dairy products was used in the supercritical inactivation experiments. An aliquot of the strain, frozen at 80 °C, was transferred to Man, Rogosa and Sharpe (MRS) broth (Merck KGeA, Darmstadt, Germany) and incubated at 35 °C ± 1 °C/48 h. Then, the cells present in the suspension were centrifuged for concentration of the cell mass and the cell pellet was washed three times with sterile, 0.1% peptone water to prepare the inoculum used to inoculate the samples. The concentration of the inoculum (cells in suspension) was adjusted to 10^9 CFU/mL.

Pasteurized apple juice was inoculated with the previously prepared cell suspension. The final concentration of the *L. casei* cells was 10^7 CFU/mL of apple juice, which was later confirmed by plate counts on MRS agar (Merck KGeA, Darmstadt, Germany) incubated at 35 °C ± 1 °C/48 h.

2.4. Microbial inactivation experiments by using supercritical carbon dioxide

2.4.1. Plackett–Burman design

A Plackett–Burman design was applied for the screening of significant factors affecting the supercritical microbial inactivation. Twelve trials were performed in order to evaluate the significance of four variables grouped as supercritical process parameters (pressure, temperature, process time and CO_2 volume ratio). Plackett–Burman designs are effective screening methods that can lead to the identification of factors involved in supercritical microbial inactivation [14].