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Microbial inactivation of *E. coli* cells by a combined PEF–HPCD treatment in a continuous flow system

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ABSTRACT

A laboratory scale continuous flow unit was set up and used to study the effect of pulsed electric fields (PEF) pre-treatments on microbial inactivation by high pressure carbon dioxide (HPCD) processing with the aim of investigating the synergistic effect of the combined treatment. McIlvaine buffer solution inoculated with *Escherichia coli* cells ATCC26 was pre-treated with PEF (25 °C) at different field strength (E = 6–12 kV/cm) and energy input (W_T = 10–40 J/mL) and then processed with HPCD (25 °C) at pressures of 8.0, 14.0 and 20.0 MPa and holding times of 4, 7 and 11 min.

Results showed that treating the microbial suspension only with PEF, the inactivation level slightly increased with increasing the field strength and energy input with no significant effect of the pressure applied. The maximum inactivation level obtained was 2.25 Log-cycles at 12 kV/cm and 40 J/mL. When the bacterial cells were treated only with HPCD, the inactivation level was almost independent on the pressure of CO_2, and gradually increased with increasing the holding time up to a maximum value of 2.41 Log-cycles. The combination of PEF and HPCD treatment resulted in a marked increase of the microbial inactivation with increasing the field strength, energy input, holding time and operative pressure. A clear synergistic effect was evident when holding time was longer than 4 min, regardless the intensity of the PEF treatment applied.

Industrial relevance: Consumers demand for fresh and natural products forces food manufacturers to investigate milder preservation processes and stimulate the current trend to use hurdle technologies. Pulsed electric field (PEF) and high pressure carbon dioxide (HPCD) are emerging non-thermal technologies which have antimicrobial capabilities when applied alone or in combination with other physicochemical hurdles. The present work demonstrated, for the first time, the feasibility of combined PEF-HCPD process based on the coupling of a PEF pre-treatment stage to HPCD treatment in a continuous flow unit. The results support the view that the combined process is able to induce substantial microbial inactivation at mild treatment conditions and at room temperature suggesting the idea that this process could be applied to foods with thermosensitive components.

1. Introduction

The current trend of the food industry is the individuation of mild preservation processes able to respond to consumer demand for minimally processed foods. To this extent, hurdle technologies have been developed as a new concept for the production of safe and stable foods of high quality using less severe processing conditions. They are based on the combination of existing and/or novel preservation factors, applied in series or in parallel, providing additional or synergistic effects that no microorganism in the food should be able to overcome (Leinster & Gorris, 1995).

Studies on non-thermal inactivation technologies that offer alternatives to conventional thermal treatments also contributed to the development of hurdle technology in the last few years (Raso, Pagán, & Condón, 2005).

Pulsed electric fields (PEF) and high pressure carbon dioxide (HPCD) technologies are two of the most promising non-thermal processing methods which have gained increasing interest in the last two decades as they can provide cold pasteurization of liquid foods with a minimum impact on their nutritional and organoleptic properties (Ferrentino, Plaza, Ramirez-Rodrigues, Ferrari, & Balaban, 2009; Garcia-Gonzalez et al., 2007; Mosqueda-Melgar, Elez-Martinez, Raybaudi-Massilia, & Martín-Belloso, 2008; Odriozola-Serrano, Aguiló-Aguayo, Soliva-Fortuny, & Martín-Belloso, 2013).

The PEF process involves the application of a high intensity electric field (10–50 kV/cm) as a sequence of pulses of short duration (1–10 μs) to a liquid food which is placed between or passed through two electrodes of a treatment chamber (Pataro, Senatore, Donsì, & Ferrari, 2011).

The exact mechanisms underlying the interactions between electric pulses and cell membranes, as well as the dynamics of the pores

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formation are not yet fully understood. However, it is widely accepted that the cell membrane plays a significant role in amplifying the applied electric field, as the conductivity of the intact membrane is several orders of magnitude lower than the conductivities of the extra cellular medium and cell cytoplasm (Barsotti, Merle, & Cheftel, 1999). Hence, when the biological cells are exposed to an external electric field, the trans-membrane potential increases as a result of the charging process at the membrane interfaces. If a critical value of the field strength is exceeded, a critical trans-membrane potential can be induced (typically 0.7–1.0 V for most cell membranes) that leads to the formation of either reversible or irreversible pores in the membrane (electroporation) (Zimmermann, 1986) depending on the electric field strength (E) and energy input levels (W_T) applied. In general, with increasing the intensity of these parameters the degree of membrane permeabilization is enhanced (Heinz, Alvarez, Angersbach, & Knorr, 2002). The formation of reversible pores in the cell membranes leads to the loss of the selective permeability (Wouters & Smelt, 1997) as well as to the occurrence of sublethal injuries (Pagán & Mañas, 2006) that would be responsible of the final microbial inactivation observed after simultaneous or successive exposure to other stresses. On the other hand, if irreversible pores form, the permanent rupture of the cell membrane takes place giving rise to the microbial inactivation.

In the HPCD technique, the liquid food is intimately in contact with either sub- or supercritical (i.e., pressurized, generally <20 MPa) CO_2 for a certain time in a batch, semi-batch or continuous manner (Garcia-Gonzalez et al., 2007). The pressure and temperature of CO_2 and treatment time have been reported, among others, as the main process parameters determining microbial lethality (Garcia-Gonzalez et al., 2007; Liao, Hu, Liao, Chen, & Wu, 2007).

The microbial inactivation mechanism of HPCD is, however, not yet fully elucidated, although several theories have been proposed in recent years (Damar & Balaban, 2006; Garcia-Gonzalez et al., 2007). According to Garcia-Gonzalez et al. (2007), the steps of the inactivation hypothesized can be summarized as follows: (1) solubilization of the pressurized CO_2 in the external liquid phase decreasing the extracellular pH, (2) diffusion of CO_2 through the cell membrane, (3) penetration of CO_2 in the microbial cell and consequent decrease of the intracellular pH, (4) inactivation of the key enzymes and inhibition of cell metabolism due to pH, (5) inhibitory effect of the molecular CO_2 and HCO_3^- on cell metabolism, (6) disordering of the intracellular electrolyte balance, and (7) removal of vital constituents from the cells and cell membranes. Most of these steps will not occur consecutively, but rather take place simultaneously in a very complex and interrelated manner (Garcia-Gonzalez et al., 2007). However, on the basis of the available literature data, it may be concluded that the relative importance of each of these steps in the lethal actions of HPCD process cannot be exactly defined as it may change with the equipment, microbial strain and treatment conditions applied.

For both PEF and HPCD treatments the ability to inactivate vegetative microbial forms has been widely demonstrated (Garcia-Gonzalez et al., 2007; Mosqueda-Melgar et al., 2008). However, in some cases, it is necessary to apply intense process conditions (high field strengths and energy inputs for PEF; high pressure, treatment time and temperature for HPCD), to obtain substantial microbial inactivation ensuring food safety and stability, in particular if the treatment is carried out in batch equipments (Ballestra & Cuq, 1998; Pataro et al., 2011). Therefore, in order to obtain the required lethality with less severe processing conditions, hurdle technology concept has been applied. Additive or synergistic effects have been observed by combining either PEF or HPCD with traditional or novel preservation factors such as moderate heating, pH, antimicrobials, high hydrostatic pressure, pulsed light, and ultrasound (Alvarez & Heinz, 2007; Caminiti et al., 2011; Li, Zhao, Wu, Zhang, & Liao, 2012; Liao, Zhang, Hu, & Liao, 2010; Mosqueda-Melgar, Raybaudi-Massilia, & Martín-Belloso, 2012; Ortuño, Martínez-Pastor, Mulet, & Benedito, 2013; Wang, Pan, Xie, Yang, & Lin, 2010).

Interestingly, the combination of a PEF pretreatment with the HPCD process can be suggested since the electroporation of the cell membrane could enhance the diffusion of CO_2 through the cell membrane during the following HPCD treatment in a way that microbial inactivation can be attained at lower processing conditions. However, to our knowledge, only very few studies have been performed to investigate the advantages of coupling PEF with HPCD treatment for the inactivation of *Saccharomyces cerevisiae* (Pataro, Ferrentino, Ricciardi, & Ferrari, 2010), *E. coli*, *Staphylococcus aureus* bacteria and *Bacillus cereus* spores (Spilimbergo, Dehghani, Bertucco, & Foster, 2002) and in none of them the processing of the liquid matrix was carried out in a continuous PEF–HPCD system.

The objective of this study was the design and setup of a continuous flow PE-F-HPCD unit with the aim of investigating the effectiveness of the combined treatment to inactivate microorganisms. In the experimental campaign PEF processing parameters, namely field strength and energy input, and HPCD processing variables, namely pressure and treatment time, have been changed in a wide range to evaluate the synergistic effect of the two stresses on the microbial inactivation of *E. coli* cells at room temperature.

2. Material and methods

2.1. Microorganisms and treatment medium

Cells of *E. coli* ATCC26 from a broth subculture were inoculated in 1 L of sterile Tryptic Soya Broth (TSB) (OXOID, Milan, Italy) and incubated at 37 °C for 18 h, without shaking, in order to obtain microbial cells in the early stationary phase. The time to reach the early stationary growth phase was determined from the growth curves (data not shown) and confirmed, before each experiment, measuring the optical density (OD) of the culture broth at 600 nm with a UV–Vis spectrophotometer (Model V530, Jasco Europe, Cremella (LC), Italy).

Bacterial cells were harvested from the mother broth culture by centrifugation (Beckman JAvant 25, Beckman Coulter, Germany) at 6000 rpm for 10 min at 4 °C and re-suspended in 1 L of sterile McIlvaine buffer solution to a final concentration of about $5{\cdot}10^8$ cfu/mL.

The pH of the solution (pH meter, LAB-TEST II, Pbi International, Milan, Italy) was 3.8 and its electrical conductivity (σ) (Conductivity meter HI 9033, Hanna Instrument, Milan, Italy) was adjusted to 2 mS/cm at 25 °C by adding distilled water.

2.2. Experimental apparatus

PEF–HPCD inactivation experiments were carried out in a bench-scale continuous flow unit available in the laboratory of Prodal Scarl at the University of Salerno (Fig. 1). The unit, specifically designed, allows the conduction of microbial inactivation experiments by applying a single PEF treatment, a single HPCD treatment or a PEF pretreatment followed by a HPCD treatment.

It consists of a peristaltic pump (model PU-2080, Jasco Europe, Cremella (LC), Italy) used to transfer the microbial suspension through the system at controlled flow rate ranging from 0.001 to 20 mL/min. The product before entering the PEF treatment chamber flows through a stainless steel coiled tube (3.9 mm inner diameter, 1.2 mm thickness, 0.5 m length) immersed into a water heating bath (Thermo Haake DC 10, Henco srl, Italy) to reach the set temperature of in the range between 20 to 60 °C. The treatment zone consists of two co-linear treatment chambers, hydraulically connected in series, with an inner diameter of 3.9 mm and a gap between the electrodes of 5 mm. For the PEF treatment a high voltage pulse generator (Diversified Technology Inc., Bedford, WA, USA) designed to provide both monopolar and bipolar square wave pulses was used. The applied voltage (0–25 kV/cm), pulse width (τ = 1–10 μs), and pulse repetition rate (1–1000 Hz) can be set independently being only limited by the average power of 25 kW. Two T-thermocouples were used to measure the product