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Inactivation of *Listeria monocytogenes* using supercritical carbon dioxide in a high-pressure variable-volume reactor

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ABSTRACT

This work evaluates the inactivation of *Listeria monocytogenes* through the use of supercritical carbon dioxide. For this purpose, the experimental design methodology was employed as a tool to assess the effects of temperature, depressurization rate, pressure cycling and the mass ratio of cell suspension to CO_2. It was observed that the depressurization rate and mass of cell suspension to CO_2 ratio were statistically significant and inactivation kinetics was verified to be of first-order. It was also observed that the inactivation increased with increasing depressurization rates in the range of 6–12 MPa min^{-1}, keeping nearly constant above the upper value. The decimal reduction times ranged from 15.38 to 20.41 min. Results obtained in this work may be quite useful to the food industry committed to microorganism inactivation using the innovative supercritical carbon dioxide technology.

1. Introduction

Listeria monocytogenes is a gram-positive bacillus, anaerobic facultative, oxidase negative, catalase positive, with motility at 25 °C by means of peritrichous flagella. It is easily found into the environment, growing at temperatures ranging from 4 to 45 °C (Jawetz, Melrick, & Adelberg, 1998). According to Gerner-Smidt (2007) and Tham, Ericsson, Loncaveric, Unnerstad, and Danielsson-Than (2000) the outbreaks of listeriosis are associated with consumption of ready-to-use foods, maintained under refrigeration and consumed without heating such as milk and derivates, meat and derivates, seafood and vegetables in general. In this sense, it is essential the evaluation of new procedures to preserve theses foods since the consumers require high-quality foods, which should preserve they nutritional and sensory attributes increasing the shelf-life.

Among these new technologies for food preservation, supercritical carbon dioxide (SC-CO_2) processing is being used to inactivate enzymes and pathogens microorganisms, resulting in a minimal degradation of thermo-labile nutrients of foods, preserving sensory and nutritional characteristics, also increasing shelf-life (Choi et al., 2008; Norton & Sun, 2008). In SC-CO_2 treatment, food comes in contact with pressurized CO_2 for a certain period of time in a batch, semi-batch or continuous apparatus. It is well-known that SC-CO_2 has the ability to diffuse through solids and dissolve materials resulting in a bactericidal action.

According to Spilimbergo and Bertucco (2003) the biocidal effects of SC-CO_2 on pathogens bacteria present in foods are due to (1) explosion of wall cell as a function of the internal pressure, (2) modification of cell membrane and extraction of lipids from the cell wall, (3) inactivation of enzymes essential for microbial metabolism and, (4) extraction of intracellular compounds. Patterson (2005) stated that the cell membrane is more affected by high pressure treatment, which leads to modifications on organization of lipid–peptide complex, breaking the membrane structure composed of two layers of phosphatide acids. When the membrane is re-organized, alterations of protein functions that are responsible to control the ions permeability are verified, leading to loss of membrane functionality.

The use of SC-CO_2 to inactivate microorganisms, including *L. monocytogenes*, is well reported in literature either in the free form or directly on food (Choi, Bae, Kim, Kim, & Rhee, 2009; Erkmen, 2000, 2001a, 2001b; Erkmen & Karaman, 2001; Ferrentino, Balaban, Ferrari, & Poletto, 2010; Furukawa et al., 2009; Karaman & Erkmen, 2001; Yagiz et al., 2009; Zhang et al., 2006). Some authors stated that fast depressurization could burst cells and/or enhance mass transfer across cell membranes (Syed et al., 2012; Zhang et al., 2006). In addition, the pressure cycling operation, which involves repeated release and compression of CO_2, also can enhance deactivation due to increasing cell rupture and mass transfer (Zhang et al., 2006).

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However, the repeated procedures of pressurization/depressurization present undesirable results in non-thermal food processing using SC-CO_2. At each pressurization/depressurization cycle, CO_2 is released from the reactor, which may promote the extraction of free water and soluble compounds (vitamins, pigments, volatile compounds) altering the texture, sensorial and nutritional characteristics of the foods. In a previous work, Silva et al. (2013) evaluated the SC-CO_2 inactivation *Escherichia coli* using a high-pressure variable-volume reactor, which enables to apply cycles of pressurization/depressurization without CO_2 releasing. The authors verified that the number of pressure cycling and system pressure showed a significant influence on inactivation, indicating that the increase in the number of pressure cycling and system pressure improve inactivation efficiency. However, to the best of our knowledge this procedure has not been employed to inactivate other microorganisms.

In this context, the main objective of this work was to evaluate the effects of temperature (33–41 °C), depressurization rate (1–10 MPa min^{-1}), pressure cycling (1–7 cycles) and mass of cell suspension to CO_2 ratio (1:0.1–1:1.5) on SC-CO_2 inactivation of *L. monocytogenes*.

2. Experimental

2.1. Inoculum preparation

The microorganism *L. monocytogenes* ATCC 7644 was subcultured in 50 mL of LB broth (triptone 10.0 g/L; yeast extract 5.0 g/L; NaCl 5.0 g/L; pH 7.5) at 36 °C for 24 h. The cultures used in all experiments were freshly prepared by the same procedure. The initial number of *L. monocytogenes* was around 10^{10} colony forming units (CFU) per mL.

2.2. Experimental apparatus and procedure

Inactivation experiments were conducted employing the static-synthetic method in a high-pressure variable-volume view cell, schematically presented by Silva et al. (2013). Briefly, the experimental set-up consists of a variable-volume view cell, with a maximum internal volume of 27 mL, with two sapphire windows for visual observation, an absolute pressure transducer (Smar LD 301), with a precision of ~0.03 MPa, a portable programmer (Smar, HT 201) for the pressure data acquisition and a syringe pump (ISCO 260D). The inactivation cell contains a movable piston, which permits the pressure control inside the cell.

Initially, an amount of cell suspension was loaded into the inactivation cell with help of sterile syringe. The charge of CO_2 was performed with the help of the syringe pump (resulting accuracy of 0.005 g in CO_2 loadings). The mass of cell suspension and CO_2 were based on the experimental design. Then, the cell content was kept at continuous agitation with the help of a magnetic stirrer and a Teflon-coated stirring bar. A metallic jacket surrounds the cell and water from a thermostatic bath was used as heating/cooling fluid, which flows through the jacket, so that the cell was kept at temperature defined in the experimental design. The pressure system was increased at pressurization rate of 10 MPa min^{-1} using CO_2 as pressurizing fluid from its vapor pressure at room (laboratory) temperature (~5 MPa) up to 8 MPa, which was considered the initial working pressure. At this point the system was hold for a short period (~1 min) to allow system stabilization, and then the pressure was increased until 20 MPa and, again the system was hold for a certain short period to allow system stabilization. After this procedure, the system pressure was decreased down to 8 MPa at the depressurization rate pre-established in the experimental design. This procedure was repeated according to the number of pressure cycles determined by the experimental design so that the treatment time for all runs was always 2 h.

The effects of temperature, depressurization rate, pressure cycling and mass of cell suspension to CO_2 ratio were evaluated by means of a central composite design (CCD). Based on the analysis of effects of first CCD a second experimental design was conceived to evaluate the effects of rate of depressurization, pressure cycling and mass ratio of cell suspension to CO_2. The levels of each independent variable of the two CCD are presented at Table 1. All results were analyzed using Statistica® 7.0 (Statsoft Inc., Tulsa, OK, USA) considering a significance level of 95% ($p < 0.05$).

Based on the analysis of the second CCD additional experiments were carried out to better elucidate the effects of depressurization rate and treatment time. As mentioned, in the first set of experiments the treatment time was 2 h, mass of cell suspension to CO_2 ratio (1:0.2), temperature (33 °C), and the pressure cycling (1 cycle) evaluating the depressurization rate at 60, 80, 120, 160, and 200 bar min^{-1}. In the second set of experiments, it was evaluated the influence of treatment time (5, 10, 15, 20, 40, 60, 80, 100 and 120 min) on the survival of *L. monocytogenes* for three different rate of depressurization (10, 12 and 16 MPa min^{-1}). Other variables were maintained at the same level of first set of experiments.

After each treatment, the number of survival was determined according to the methodology described previously, where an aliquot of treated sample was cultivated at 37 °C for 24 h on LB agar plates (triptone 10,0 g/L; yeast extract 5.0 g/L; NaCl 5.0 g/L; agar 15 g/L).

3. Results and discussion

Results concerning the number of survivals after the SC-CO_2 inactivation of *L. monocytogenes* obtained in the first CCD are presented at Table 2. The microbial counts ranged from 11,000 to 565,000 CFU mL^{-1} (runs 16 and 1, respectively), corresponding to log-reduction of 5.96 and 4.24, respectively. To better evaluate the influence of each independent variable on number of survival after treatment, data of Table 2 were used to calculate the effects of each factor, which are presented in Fig. 1, in the form of Pareto chart, considering a significance level of 95% ($p < 0.05$).

From Fig. 1 it can be seen that system temperature was not significant, being maintained constant at 33 °C for other experimental conditions, which is very close to critical temperature of CO_2 (31 °C). The mass of cell suspension to CO_2 ratio was statistically significant, indicating that the increase of CO_2 proportion in the system favors the microorganism inactivation. The increase of CO_2 concentration enables a better diffusion through the cell membrane facilitating the cell burst during the depressurization, when occurs the expansion of CO_2, leading to improved inactivation efficiency (Erkmen & Karaman, 2001). However, similar results

Table 1
Level of independent variables investigated in the first and second central composite design (CCD) to inactivate *L. monocytogenes* using SC-CO_2.

Variables	Levels		
	−1	0	1
First CCD (2^4)			
Temperature (°C)	33	37	41
Rate of depressurization (R − MPa min^{-1})	1.0	5.5	10.0
Pressure cycles	1	4	7
Mass of cell suspension to CO_2 ratio (CS:CO_2 − wt%)	1:0.1	1:0.8	1:1.5
Second CCD (2^3)			
Rate of depressurization (R − MPa min^{-1})	1.0	5.5	10.0
Pressure cycles	1	4	7
Mass of cell suspension to CO_2 ratio (CS:CO_2 − wt%)	1:0.2	1:0.5	1:0.8

Table 2
Matrix of the experimental results obtained in the first CCD to evaluate the inactivation of *L. monocytogenes* using SC-CO_2.

Run	Temperature (°C)	*R* (MPa min^{-1})	Pressure cycles	CS:CO_2 (wt%)	Survivals (CFU mL^{-1})	Log-reduction
1	−1(33)	−1(1)	−1(1)	−1(1:0.1)	565,000	4.24
2	1(41)	−1(1)	−1(1)	−1(1:0.1)	500,000	4.31
3	−1(33)	1(10)	−1(1)	−1(1:0.1)	190,000	4.73
4	1(41)	1(10)	−1(1)	−1(1:0.1)	530,000	4.28
5	−1(33)	−1(1)	1(7)	−1(1:0.1)	410,000	4.39
6	1(41)	−1(1)	1(7)	−1(1:0.1)	500,000	4.31
7	−1(33)	1(10)	1(7)	−1(1:0.1)	321,000	4.50
8	1(41)	1(10)	1(7)	−1(1:0.1)	120,000	4.93
9	−1(33)	−1(1)	−1(1)	1(1:1.5)	90,000	5.05
10	1(41)	−1(1)	−1(1)	1(1:1.5)	80,000	5.1
11	−1(33)	1(10)	−1(1)	1(1:1.5)	60,000	5.23
12	1(41)	1(10)	−1(1)	1(1:1.5)	30,000	5.53
13	−1(33)	−1(1)	1(7)	1(1:1.5)	15,000	5.83
14	1(41)	−1(1)	1(7)	1(1:1.5)	43,000	5.37
15	−1(33)	1(10)	1(7)	1(1:1.5)	34,000	5.47
16	1(41)	1(10)	1(7)	1(1:1.5)	11,000	5.96
17	0(37)	0(5.5)	0(4)	0(1:0.8)	61,000	5.22
18	0(37)	0(5.5)	0(4)	0(1:0.8)	63,000	5.21
19	0(37)	0(5.5)	0(4)	0(1:0.8)	72,000	5.15

Initial microbial count was always 1×10^{10} CFU mL^{-1} for all runs.

were obtained at runs 9–15 and at the central point of CCD (runs 17–19), indicating that mass of cell suspension to CO_2 ratio lower than 1:1.5 also can be effective inactivate *L. monocytogenes*. In addition, maintaining the mass of cell suspension to CO_2 ratio to values around 1:1.5 can influence the analysis of effects of other variables, since the magnitude effect of this variable was about three times higher than depressurization rate and pressure cycling, which were not significant within the evaluated range, although presented the tendency to influence the inactivation.

Based on these aspects, a second CCD was conceived to evaluate the effects of pressure cycling and depressurization rate, which were maintained within the same range of first CCD, whereas the mass of cell suspension to CO_2 ratio was evaluated in a narrower range (1:0.2–1:0.8). Results obtained are presented in Table 3, where it can be observed a considerable reduction in the number of survival in comparison with data presented in Table 2, confirming that the strategy adopted was effective. The best results in terms of microbial count were obtained at runs 2 and 3, which were 8.7×10^3 and 2.5×10^3 CFU mL^{-1}, respectively. In these runs it is clearly verified the influence of CO_2 on the inactivation, since

Table 3
Matrix of the experimental results obtained in the first CCD to evaluate the inactivation of *L. monocytogenes* using SC-CO_2.

Runs	CS:CO_2 (wt%)	*R* (MPa min^{-1})	Pressure cycling	Survivals (CFU mL^{-1})	Log-reduction
1	−1(1:0.2)	−1(1)	−1(1)	220,000	4.66
2	1(1:0.8)	−1(1)	−1(1)	44,250	5.36
3	−1(1:0.2)	1(10)	−1(1)	8700	6.07
4	1(1:0.8)	1(10)	−1(1)	2500	6.61
5	−1(1:0.2)	−1(1)	1(7)	440,000	4.36
6	1(1:0.8)	−1(1)	1(7)	180,000	4.75
7	−1(1:0.2)	1(10)	1(7)	61,000	5.22
8	1(1:0.8)	1(10)	1(7)	10,000	6.00
9	0(1:0.5)	0(5.5)	0(4)	190,000	4.73
10	0(1:0.5)	0(5.5)	0(4)	141,500	4.85
11	0(1:0.5)	0(5.5)	0(4)	170,000	4.77

Initial microbial count was always 1×10^{10} CFU mL^{-1} for all runs.

increasing the mass of cell suspension to CO_2 ratio from 1:0.2 (run 2) to 1:0.8 (run 3) led to a decrease in the microbial count.

In the same way, data of Table 3 regarding microbial count were used to calculate the effects of each variable on response, which are shown in Fig. 2 in the form of Pareto chart. Depressurization rate and mass of cell suspension to CO_2 ratio were statistically significant, whereas pressure cycling did not influence the inactivation within the range evaluated in this work. Result obtained in the analysis of effects corroborate with the fact that increasing the concentration of CO_2 inside the cell associated with a fast depressurization rate results in cell burst, being advantageous for the inactivation process.

From Fig. 2 it is evident that increasing the depressurization rate positively affects SC-CO_2 inactivation of *L. monocytogenes*. In order to better evaluate the effect of depressurization rate on the inactivation, six additional experiments were carried out ranging from 60 to 200 bar min^{-1} with other variables maintained at conditions specified in run 3 of Table 3. Results obtained are presented in Fig. 3, where it can be seen a linear decrease in the number of survival for depressurization rate ranging from 6 to 12 MPa min^{-1}. For rates above this value, although yet occurs reduction in the number of survival, a less pronounced effect on the inactivation was verified. Silva et al. (2013) also verified a pronounced effect of depressurization rate on the inactivation of *E. coli* and, in the same way that Monks, Tiggamann, Mazutti, Oliveira, and Valduga (2012) attributed this result to the fact that CO_2 is first solubilized inside the microbial

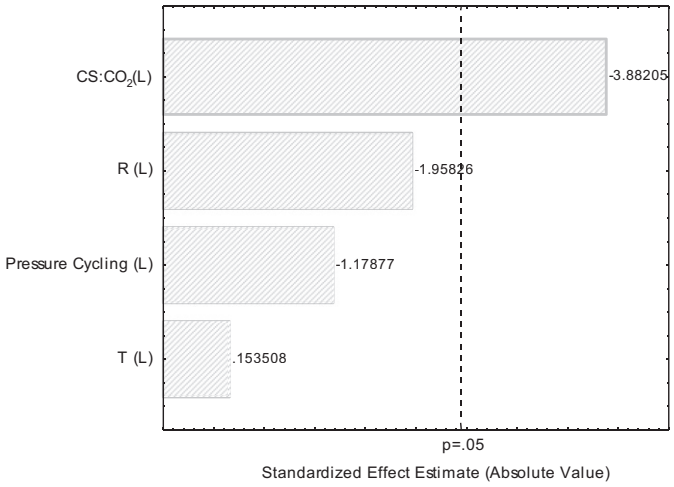

Fig. 1. Pareto chart showing the effects of independent variables on microbial survivals after the SC-CO_2 inactivation of *L. monocytogenes* obtained in the first CCD.

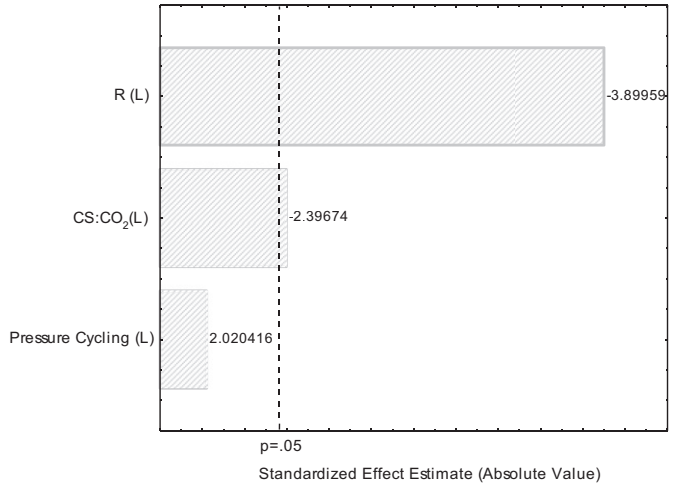

Fig. 2. Pareto chart showing the effects of independent variables on microbial survivals after the SC-CO_2 inactivation of *L. monocytogenes* obtained in the second CCD.

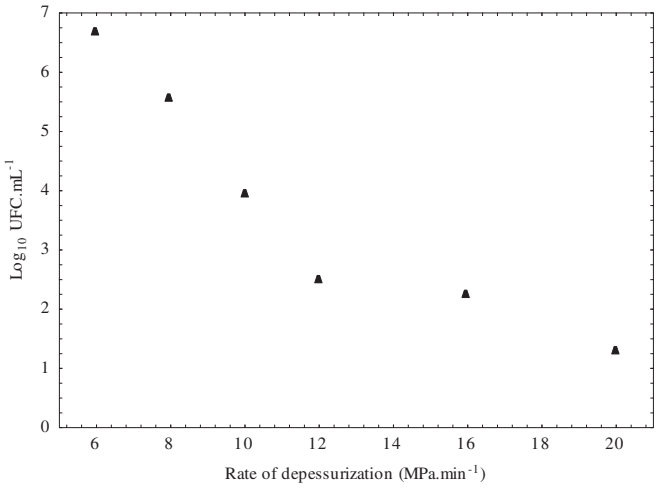

Fig. 3. Influence of depressurization rate on the microbial survival after the SC-CO_2 inactivation of *L. monocytogenes* carried out experimental condition of run 3 of the second CCD.

cell and the fast depressurization leads to abrupt expansion of CO_2, causing cell disruption then leading to inactivation.

To evaluate the kinetic of SC-CO_2 inactivation of *L. monocytogenes*, three additional experiments were performed at depressurization rates of 10, 12 and 16 MPa min^{-1} for time ranging from 5 to 120 min with other variables maintained at conditions specified in run 3 of Table 3. Results obtained are presented in Fig. 4, where it can be noticed a more pronounced reduction of the survivors after 60 min of treatment. In addition, the inactivation after 120 min was more effective with the increase of depressurization rate, hence corroborating the results presented above. The increase of depressurization rate clearly decreases the value of the normalized microbial count (N/N_0), indicating that it affects the inactivation.

Toward better evaluating the effects of depressurization rate, it was assumed that the kinetics of the SC-CO_2 inactivation of *L. monocytogenes* follow a first order. By determining the inactivation constant from data of Fig. 4, the decimal reduction time (*D*-value) for each experimental condition was calculated. *D*-value is related with the required time to reduce 90% of the initial number

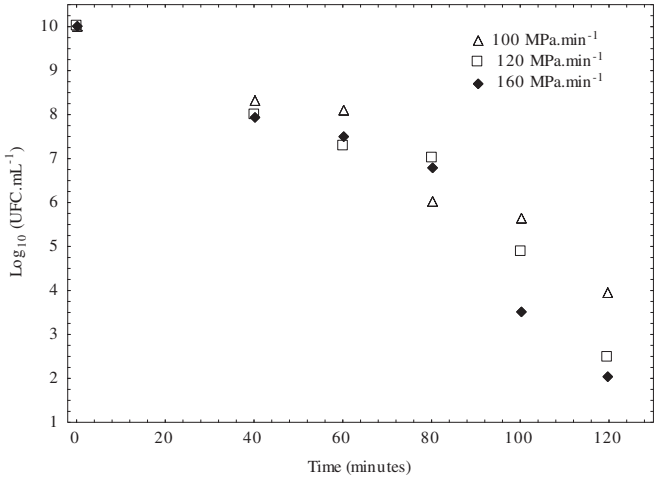

Fig. 4. Influence of depressurization rate on the kinetic evaluation of the microbial survival after the SC-CO_2 inactivation of *L. monocytogenes* carried out experimental condition of run 3 of the second CCD.

of microorganisms. In this work, the *D*-values obtained were 15.38, 17.24 and 20.41 min for depressurization rates of 10, 12 and 16 MPa min^{-1}, respectively.

The *D*-values obtained in this study are greater than those reported for *E. coli* (Silva et al., 2013) and *Salmonella typhimurium* (Erkmen & Karaman, 2001), both gram-negative bacteria. According to Erkmen and Karaman (2001), gram-positive bacteria as *L. monocytogenes* are more resistant to high-pressure treatment than gram-negative ones, because they present thicker wall cellular, presenting thickness ranging from 20 to 80 nm, whereas gram-negative bacteria presenting a maximum value of 20 nm. The influence of wall cell thickness is easily verified in the first 60 min of treatment (Fig. 4), where the inactivation is less pronounced than for greater times. This occurs because the time required for CO_2 to diffuse through the cell wall and solubilize the internal compounds is greater than for gram-negative bacteria, as *E. coli*.

4. Conclusions

The use of SC-CO_2 showed to be effective to inactivate *L. monocytogenes*. Depressurization rate and mass of cell suspension to CO_2 ratio were the most significant variables on the process. The best experimental condition to inactivate *L. monocytogenes* was mass of cell suspension to CO_2 ratio of 1:0.2, depressurization rate of 10 MPa min^{-1}, 1 pressure cycling, at 33 °C for 2 h leading to 6.1 log-reduction. Results obtained in this work are useful to support the development of a platform for an effective non-thermal sterilization of foods in pilot/industrial scale.

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