Effect of pressure, depressurization rate and pressure cycling on the inactivation of *Escherichia coli* by supercritical carbon dioxide

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Abstract

This work is focused on the use of supercritical carbon dioxide to inactivate the pathogenic *Escherichia coli*. For this purpose, the experimental design methodology was employed to evaluate the effects of pressure (8–28 MPa), depressurization rate (1–11 MPa min⁻¹) and pressure cycling (1–5 cycles) on the microorganism inactivation using the static-synthetic method in a variable-volume reactor. The number of pressure cycles and system pressure showed a significant influence on *E. coli* inactivation with supercritical CO₂, indicating that the increase in the number of pressure cycles and system pressure improve inactivation efficiency. Microbial inactivation followed first order reaction kinetics, where the rates increased with increasing pressure from 8 to 16 MPa. The decimal reduction times (D) ranged from 1.03 to 5.35 min. The pressure dependence of the *E. coli* specific inactivation rates can be described by the z value, which was found to be 11.4. The results reported here may be useful to provide an effective non-thermal sterilization of foods in pilot/industrial scale.

1. Introduction

High pressure carbon dioxide (HPCD) is a non-thermal food preservation technology. It reduces the microbial numbers in liquid foods by several orders of magnitude. In HPCD treatment, food comes in contact with pressurized CO₂ for a certain amount of time in a batch, semi-batch or continuous apparatus. Pressurized CO₂ has the ability to diffuse through solids and dissolve materials resulting in a bactericidal action. The mechanisms by which pressurized CO₂ inactivates microorganism have been extensively studied in recent years (Ferrentino, Balaban, Ferrari, & Poletto, 2010; Karaman & Erkmen, 2001; Zhang et al., 2006). The knowledge of the exact inactivation mechanisms could help in the interpretation of the survival kinetics curves identifying the parameters that are the forcing factors causing inactivation (Ferrentino et al., 2010).

The biocidal effects of HPCD have been quantified in various species of bacteria and have been summarized by Garcia-Gonzalez et al. (2007), Spilimbergo and Bertucco (2003) and Zhang et al. (2006). Using CO₂ as a sterilant agent has several potential benefits since it is not flammable, non-toxic, inert in most situations and hence it does not react with polymers, presents low critical temperature (31.1 °C), which is only slightly above room temperature, so thermal degradation is not a problem when a process is operated around the critical temperature; at supercritical state CO₂ has low viscosity and zero surface tension, so it can quickly penetrate complex structures and porous materials (Furukawa et al., 2009). Finally, CO₂ is inexpensive and readily available, which makes switching to CO₂-based sterilization economically feasible (Zhang et al., 2006).

The presence of *Escherichia coli* in foods such as meat, fish and milk is an indicator of fecal contamination, causing outbreaks of diarrhea, gastroenteritis and hemolytic uremic syndrome (Karaman & Erkmen, 2001). Studies regarding the effects of compressed CO₂ on *E. coli* have been reported (Ballestra, Dasilva, & Cuq, 1996; Erkmen, 2001a; Gunes, Blum, & Hotchkiss, 2006; Karaman & Erkmen, 2001; Kim, Rhee, Kim, & Kim, 2007). Karaman and Erkmen (2001) evaluated the inactivation kinetics of *E. coli* by high pressure carbon dioxide and they verified that the inactivation rates increased with increasing pressure from 2.5 to 10 MPa, temperature from 20 to 48 °C and exposure time. Kim et al. (2007) used supercritical carbon dioxide to inactivate the pathogenic *E. coli* O157:H7 and generic *E. coli*. For both strains it was verified a reduction of 8 log orders in the microbial count within 15–30 min, in treatment range of 8–15 MPa and 35–45 °C. In addition, it was reported the deformation and collapse of the treated cells with supercritical CO₂.
All the above-mentioned studies reported the effects of pressure, temperature, physiological state, growth medium and exposure time on the inactivation kinetics of E. coli. However, the authors did not evaluate the effect of depressurization rate and number of pressure cycles. Some authors stated that a fast depressurization rate could burst cells and/or enhance mass transfer across cell membranes (Syed et al., 2012; Zhang et al., 2006). It was verified that the cells were mechanically ruptured by the fast expansion of CO₂ within cells during flash pressure discharge (Foster, Cowan, & Maag, 1962; Fraser, 1951). Pressure cycling involves repeated release and compression of CO₂. The pressure cycling operation can enhance deactivation due to increasing cell rupture and mass transfer (Zhang et al., 2006). Fraser (1951) achieved more than 90% cell rupture after two pressure cycles, at least a 15% increase from a single pressurization step. Dillow, Dehghani, Hrkach, Foster, and Langer (1999) observed significant increase in log-reduction with pressure cycling. For example, with a treatment at 34 °C, 20.5 MPa and total treatment time of 0.6 h, the degree of inactivation jumped from 3-log with three pressure cycles to 9-log with six pressure cycles.

However, the repeated procedures of pressurization/depressurization present undesirable results in non-thermal food processing using supercritical carbon dioxide. To each cycle of pressurization/depressurization reported in the previous works the CO₂ is released from the reactor. This promotes the extraction of free water and soluble compounds (vitamins, pigments, volatile compounds) altering the texture, sensorial and nutritional characteristics of the foods. An alternative to overcome this problem is to apply cycles of pressurization/depressurization without the releasing of the CO₂. This procedure is widely used to determine the high-pressure phase equilibria by static-synthetic method using a high-pressure variable-volume reactor (Corazza, Cardozo, Antunes, & Dariva, 2003a, 2003b; Lanza et al., 2005).

In this context, the main objective of this work is to evaluate the effects of pressure (8–28 MPa), depressurization rate (1–11 MPa min⁻¹) and pressure cycling (1–5 cycles) on the inactivation of E. coli through the use of supercritical carbon dioxide using the static-synthetic method in a variable-volume reactor. Firstly, it was applied the experimental design methodology to evaluate the effects of the independent variables and then it was investigated the influence of pressure on the inactivation kinetics.

2. Experimental

2.1. Inoculum preparation

The microorganism E. coli ATCC 25922 was sub-cultured in 100 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 final number of E. coli ranged from 8.0 × 10⁷ to 2.0 × 10⁸ 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, shown in Fig. 1. 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, 10 mL of the inoculum was loaded into the inactivation cell. The charge of a fixed amount, 6 g, of CO₂ was performed with the help of the syringe pump (resulting accuracy of ±0.005 g in CO₂ loadings). With known values of pressure and temperature in the syringe pump reservoir, solvent density was estimated using the HBT (P-V-T) correlation for compressed liquids (Reid, Prausnitz, & Poling, 1987), making possible to estimate the mass of solvent charged into the reaction vessel. 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 oil from a thermostatic bath was used as heating/cooling fluid, which flows through the jacket, so that the cell was kept at 36 °C. The pressure system was increased using CO₂ as pressurizing fluid from its vapor pressure at room temperature (~5 MPa) up to the pressure established in the experimental design at pressurization rate of 10 MPa min⁻¹. At this point the system was hold for a certain short period to allow system stabilization, and then the pressure was decreased down to CO₂ vapor pressure 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.

Fig. 1. Schematic diagram of the experimental apparatus. A – gas reservoir; B – syringe pump; C – equilibrium cell; D – sapphire windows; E – magnetic stirrer; F – light source; G – pressure transducer; H – energy source; I – pressure indicator; J – feeding valve; K – relief valve; L, M – sphere valves; N – pressure valve; O – relief valve; P – check valve; Q – thermocouple; R – temperature indicator.
After completion of the experiment, the number of survivor microorganisms was determined according to the methodology defined previously. The effects of pressure, depressurization rate, and pressure cycles were evaluated by means of two central composite designs (CCD). Table 1 presents the ranges evaluated in each CCD.

### 3. Results and discussion

#### 3.1. Evaluation of the inactivation of *E. coli* using supercritical CO2

Table 2 presents the results obtained in the first CCD to evaluate the effects of pressure, depressurization rate, and pressure cycle on inactivation of *E. coli*. It can be noticed from this table that the number of survivors varied greatly after the high-pressure treatment, with observed values of log-reduction of 5.0 (run 1) until full inactivation (runs 2, 6, 8–11), with 8 log-reduction. In addition, Table 2 reports the total treatment time of each run, which varied from 28 to 240 min.

Results shown in Table 2 were used to compute the main effects of independent variables on the inactivation of *E. coli*, which are presented in Fig. 2 in terms of Pareto chart. In this work it was adopted the Pareto chart to display the effect of process variables on *E. coli* inactivation by supercritical CO2 because this tool can be straightforwardly obtained from statistical treatment of experimental data from any available commercial software (e.g., Software Statistica® 6.0 (Statsoft Inc.) used here to assist the design and the statistical analysis), and is a simple, elegant and practical manner, very used in statistical analysis, to provide information on main and cross interaction effects (variables), whether they are positive or negative and also the magnitude of the effect. Given a confidence level (in our case, the commonly used 95%, or *p* < 0.05) it is easy to see from Fig. 2 that pressure cycles and depressurization rate were the significant variables (*p* < 0.15) on the microorganism inactivation, while system pressure did not present a significant effect in the range evaluated. The significant effect of curvature indicates that it is necessary to evaluate the effects of independent variables in a broader range than that studied, inserting axial points in the CCD or carrying out a new CCD.

From the effect analysis it can be seen that as high is the depressurization rate more efficient is *E. coli* inactivation. This may be related to the fact that CO2 is first solubilized inside the microbial cell and the fast depressurization leads to abrupt expansion of CO2, causing the disruption of the cells then leading to inactivation. Monks, Tiggamann, Mazutti, Oliveira, and Valduga (2011) verified that the sudden expansion of the CO2 is more effective for disruption of *Sporidiobolus salmonicolor* cells to recovery carotenoids. Fast pressurization and depressurization process is believed to enhance the transfer of CO2 into and out of the cells thus provoking the extraction of materials (Zhang et al., 2006). Regarding the number of pressure cycles, it is clear that as high is the number, more efficient is the inactivation. However, both variables pressure cycles and depressurization rate carry implicitly the effect of another parameter that is the processing time. As can be seen in Table 2, for processing times longer than 120 min, there was complete inactivation of *E. coli*.

Thus, it seems difficult to verify if the inactivation was caused by the manipulation of independent variables or also due to the exposure time. In this sense, a second CCD was carried out fixing the exposure time at 160 min and changing the levels of independent variables, taking into account the analysis of the effects observed from the execution of the first CCD. In this second design, system pressure was evaluated in the range of 8–18 MPa, depressurization rate from 1 to 11 MPa min–1 and pressure cycles from 2 to 4 cycles. Although the analysis of the effects of the first CCD showed that the levels of pressure cycles and depressurization rate should be increased, their levels were decreased to enable the evaluation of inactivation in a total time of 160 min.

Table 3 presents the results obtained in the second CCD, where it can be observed that even for milder conditions of the CCD almost complete elimination of *E. coli* was noted after 160 min of treatment. Results of this table were used to compute the main effects of independent variables on the inactivation of *E. coli*, which are presented in Fig. 3 in terms of Pareto chart. The pressure cycles and pressure were the significant variables (*p* < 0.15) on the inactivation whereas the effects of depressurization rate and curvature were not significant in the range studied.

In the second CCD the number of cycles also presented significant influence on the inactivation of *E. coli* with supercritical CO2, indicating that the increase in the number of pressure cycles

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Table 1: Range of the independent variables investigated in the two CCD.

<table>
<thead>
<tr>
<th>Levels</th>
<th>–1</th>
<th>0</th>
<th>1</th>
</tr>
</thead>
<tbody>
<tr>
<td>1st CCD</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Pressure (MPa)</td>
<td>8.0</td>
<td>18.0</td>
<td>28.0</td>
</tr>
<tr>
<td>Depressurization rate (K) (MPa min⁻¹)</td>
<td>1.0</td>
<td>6.0</td>
<td>11.0</td>
</tr>
<tr>
<td>Pressure cycles</td>
<td>1</td>
<td>3</td>
<td>5</td>
</tr>
<tr>
<td>2nd CCD</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Pressure (MPa)</td>
<td>8.0</td>
<td>13.0</td>
<td>18.0</td>
</tr>
<tr>
<td>Depressurization rate (K) (MPa min⁻¹)</td>
<td>1.0</td>
<td>3.5</td>
<td>6.0</td>
</tr>
<tr>
<td>Pressure cycles</td>
<td>2</td>
<td>3</td>
<td>4</td>
</tr>
</tbody>
</table>

Table 2: Matrix of the experimental results obtained in the first CCD to evaluate the inactivation of *E. coli* with supercritical CO2.

<table>
<thead>
<tr>
<th>Run</th>
<th>Pressure cycles R (MPa min⁻¹)</th>
<th>Pressure (MPa)</th>
<th>Log-reduction</th>
<th>Time (min)</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>–1 (1)</td>
<td>–1 (1)</td>
<td>–1 (8)</td>
<td>5.0</td>
</tr>
<tr>
<td>2</td>
<td>1 (5)</td>
<td>–1 (1)</td>
<td>–1 (8)</td>
<td>8.0</td>
</tr>
<tr>
<td>3</td>
<td>–1 (1)</td>
<td>1 (11)</td>
<td>–1 (8)</td>
<td>5.4</td>
</tr>
<tr>
<td>4</td>
<td>1 (5)</td>
<td>1 (11)</td>
<td>–1 (6)</td>
<td>6.1</td>
</tr>
<tr>
<td>5</td>
<td>–1 (1)</td>
<td>–1 (1)</td>
<td>1 (28)</td>
<td>5.0</td>
</tr>
<tr>
<td>6</td>
<td>1 (5)</td>
<td>–1 (1)</td>
<td>1 (28)</td>
<td>8.0</td>
</tr>
<tr>
<td>7</td>
<td>–1 (1)</td>
<td>1 (11)</td>
<td>1 (28)</td>
<td>7.0</td>
</tr>
<tr>
<td>8</td>
<td>1 (5)</td>
<td>1 (11)</td>
<td>1 (28)</td>
<td>8.0</td>
</tr>
<tr>
<td>9</td>
<td>0 (3)</td>
<td>0 (6)</td>
<td>0 (18)</td>
<td>8.0</td>
</tr>
<tr>
<td>10</td>
<td>0 (3)</td>
<td>0 (6)</td>
<td>0 (18)</td>
<td>8.0</td>
</tr>
<tr>
<td>11</td>
<td>0 (3)</td>
<td>0 (6)</td>
<td>0 (18)</td>
<td>8.0</td>
</tr>
</tbody>
</table>

R = depressurization rate.
improves the inactivation efficiency, thus confirming previous results. It is believed that the effect of pressurization/depressurization process stresses the microorganism, accelerating its inactivation rate. Spilimbergo, Elvassore, and Bertucco (2002) already verified that the application of pressure cycles was beneficial to bacteria inactivation.

Regarding system pressure, it was found that, within the range evaluated, its increment improves the efficiency of microorganism inactivation. The depressurization rate did not present influence in the studied range, apparently opposing the results obtained previously. This result could be due to the high compressibility observed for CO2 within pressure range investigated at the experiment temperature (36 °C), where small variations in pressure may cause large changes in solvent density. In the first CCD the pressure range evaluated was from 8 to 25 MPa, while in the second CCD upper value was lower, 16 MPa. Due to solubilization (solvent) power and static pressure itself, it is plausible to believe that the ability of CO2 to cross the cellular membrane and dissolve into it may be higher at 25 MPa than at 16 MPa. As the amount of CO2 inside of the cells is quite susceptible to inactivation with supercritical CO2. The increase of pressure clearly decreases the value of the normalized microbial count (N/N0), indicating that it affects the inactivation (data not shown). The effect of pressure on the inactivation of E. coli with supercritical CO2 was assumed to follow a first order kinetics. Thus, if N is the number of microorganisms, its variation with time can be expressed by:

\[
\frac{dN}{dt} = -k \cdot N
\]  

Integration of Eq. (1) results:

\[
\frac{N}{N_0} = \exp(-k \cdot t)
\]  

where N and N0 are the microbial count obtained at time t and t = 0, respectively, and k is an inactivation constant.

The value of inactivation constant depends on the type of microorganism, medium, pressure and temperature. The inactivation constant k is determined from linearization of Eq. (2), as:

\[
\ln \left(\frac{N}{N_0}\right) = -k \cdot t
\]  

Thus, by plotting a graph of ln(N/N0) versus time gives a straight line with intercept in zero and the slope represents the inactivation constant. Fig. 4 presents the experimental and predicted data by the first order model to determine the inactivation constant for the pressure range of 8—16 MPa. As can be seen from the results, the inactivation of E. coli with supercritical CO2 follows a first order kinetics, as is confirmed by the good agreement between experimental and predicted data. The correlation coefficient, R², was higher than 0.9600 in all situations.

In the calculations involving heat and/or pressurized fluid treatments of foods it is employed the concept of decimal reduction time (D), which can be defined as the required time to reduce 90%
of the initial number of microorganisms. It is assumed that this value is independent on the initial count of microorganisms, but it is dependent on temperature, pressure, type of microorganism and culture medium or food that is growing. Arranging Eq. (3) gives:

\[
D = \ln\left(\frac{0.1 \cdot N_0}{N_0}\right) / -k = \frac{\ln(0.1)}{-k}
\]

(4)

Table 4 presents the results obtained regarding the inactivation constant and the decimal reduction time. As can be seen, the inactivation constant increases with pressure, indicating that high pressures accelerate the rate of inactivation. Moreover, the decimal reduction time decreases with increasing pressure, as expected. These results show that a relatively short processing time is needed to obtain one log reduction in microbial count. For example, at 16 MPa it is needed 1.03 min to reduce one log in the count, while at 8 MPa 5.35 min is required. From the data of time of decimal reduction it is possible to make predictions of the processing time, for given the initial count and the degree of sterility required. Karaman and Erkmen (2001) reported that the inactivation of E. coli follows a first order kinetics. The inactivation constant and decimal reduction times ranged from 0.0848 to 0.4771 min⁻¹ and from 4.90 to 27.46 min, respectively. D-values obtained in this study were lower than those verified for Salmonella typhimurium, which is also a gram-negative bacteria (Erkmen & Karaman, 2001). Erkmen (2001b) verified that gram-positive bacteria, such as Listeria monocytogenes, are more resistant to pressure treatment compared to gram-negative ones.

Analyzing the values of the inactivation constant as a function of pressure, a linear behavior within the range studied with a correlation coefficient (R²) of 0.9671 is observed, as follows:

\[
k = 0.0219 \cdot P - 1.4396
\]

(5)

where \( k \) is given in min⁻¹ and \( P \) is the system pressure (MPa).

The above equation is valid within the pressure range evaluated in this study and its applicability can be found in predicting the inactivation constant and, consequently, predict the time of decimal reduction at different pressures than those experimentally studied in this work.

Another parameter frequently used to represent the inactivation of microorganisms is the \( z \) parameter, which expresses the variation in the rate of cell death with pressure and temperature, representing an increase of pressure and temperature required to reduce 90% of the time of decimal reduction. A relationship between the time of cell death and the temperature or pressure is presented in the following equation, where the time of cell death or decimal reduction time is plotted on a graph as a function of temperature or pressure, resulting in a linear behavior:

\[
\log(D) = \frac{1}{z} \left( P - P \text{ref} \right) + \log(D \text{ref})
\]

(6)

where \( P \) and \( P \text{ref} \) represent the pressure of treatment and reference pressure (both in MPa), respectively, \( D \) and \( D \text{ref} \) are the time of decimal reduction at the system pressure and at the reference pressure (both in minutes), respectively. In this work, it was adopted the reference pressure as 12 MPa, which corresponds to the mean value between the minimum and maximum pressure. \( D \text{ref} \) was taken from Table 4 at 12 MPa. In this sense, the parameter \( z \) was determined by linearization, as illustrated in Fig. 5. The value of \( z \) obtained in this study was 11.4, what indicates that to reduce 90% in the time of decimal reduction it is necessary to increase 11.4 MPa the pressure of the system. Karaman and Erkmen (2001) verified that the \( z \) parameter ranged from 39.2 to 89.9 bar in the temperature range of 25—40 °C. At 40 °C, \( z \) value obtained by Karaman and Erkmen (2001) (9.0 ± 1.5) is close to that obtained in this study (11.4).

4. Conclusions

This work evaluated the inactivation of E. coli using supercritical CO₂. The experimental design methodology was used as a tool to determine the effects of pressure cycles, depressurization rate and system pressure on the microorganism inactivation. The number of pressure cycles and pressure presented a significant influence on the inactivation of E. coli with supercritical CO₂, indicating that the increase in the number of pressure cycles and system pressure improves the inactivation efficiency. Microbial inactivation followed a first order reaction kinetics, where the rates increased with increasing pressure from 8 to 16 MPa. The decimal reduction times \( (D) \) ranged from 1.03 to 5.35 min. The pressure dependence of the E. coli specific inactivation rates could be described by the \( z \) value, which was found to be 11.4 MPa. Results reported here can be useful to develop a platform for an effective non-thermal sterilization of foods in pilot/industrial scale.

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References


