Inactivation of *Escherichia coli* (ATCC 4157) in Diluted Apple Cider by Dense-Phase Carbon Dioxide

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**ABSTRACT**

Dense-phase carbon dioxide (CO2) treatments in a continuous flow through system were applied to apple cider to inactivate *Escherichia coli* (ATCC 4157). A response surface design with factors of the CO2/product ratio (0, 70, and 140 g/kg), temperature (25, 35, and 45°C), and pressure (6.9, 27.6, and 48.3 MPa) were used. *E. coli* was very sensitive to dense CO2 treatment, with a more than 6-log reduction in treatments containing 70 and 140 g/kg CO2, irrespective of temperature and pressure. The CO2/product ratio was the most important factor affecting inactivation rate of *E. coli*. No effect of temperature and pressure was detected because of high sensitivity of the cells to dense CO2. Dense CO2 could be an alternative pasteurization treatment for apple cider. Further studies dealing with the organoleptic quality of the product are needed.

There has been an increasing interest in nonthermal food preservation techniques because of growing consumer demand for more natural food products (16). Nonthermal processes offer better retention of natural flavor and nutrients in treated foods compared with traditional thermal processes (16).

Use of carbon dioxide (CO2) under pressure is a promising nonthermal process. CO2 is nontoxic, inexpensive, incombustible, and environmentally and physiologically safe. CO2 at ambient pressure has been known for its inhibitory effect on microbial growth for a long time (9). The inactivating power of CO2 above ambient pressure has been shown in several studies (1, 5, 8, 11, 13, 18, 19). The state of CO2 can have significant influences on antimicrobial activity. CO2 exists in its supercritical state above 31°C and 7.34 MPa (21). Supercritical CO2 has properties of both liquid and gas, with altered viscosity, diffusivity, and solubility resulting in improved dissolving power (21). The effectiveness of CO2 depends on its concentration and its state (gas, liquid, or supercritical), which, in turn, relies on pressure and temperature (11). The main inactivation mechanisms of dense CO2 on microbial cells have been attributed to a cytoplasmic pH decrease, explosive cell rupture, modification of the cell membrane, inactivation of key enzymes, and extraction of intracellular substances (19).

High-density CO2 in fruit juice and milk has shown promising results in its ability to inactivate microorganisms (1, 20). Although some adverse effect of high-pressure CO2 on the organoleptic quality of milk has been reported (20), there is a lack of research in this area for fruit juices.

Unpasteurized fresh apple juice has been identified as vehicle for *Escherichia coli* O157:H7 infection resulting in hospitalizations (3, 10). The National Advisory Committee on Microbiological Criteria for Foods has recommended that fruit juice production should include treatments capable of producing a 5-log unit reduction in *E. coli* O157:H7 (14). Thermal pasteurization of juices results in loss of fresh flavor and nutrients. Interests in nonthermal pasteurization of juices have increased, and irradiation, UV light, high hydrostatic pressure, and dense-phase (i.e., liquid CO2 above and below critical temperature and pressures) CO2 have been investigated in several fruit juice products (1, 4, 15, 23).

Fruit juices are often spoiled by fermentative yeasts, such as *Saccharomyces cerevisiae*, because of their ability to grow at low pH. Antimicrobial activity of pressurized CO2 toward yeasts including *S. cerevisiae* has been reported (5, 12, 18). An alternative pasteurization method for juices should not only increase shelf life of the product through inactivation of spoilage organisms but also inactivate the potential pathogens in the product.

The objective of this work was to investigate the effects of dense-phase CO2 treatment on *E. coli* (ATCC 4157) in apple cider. Influences of process parameters (CO2 concentration, temperature, and pressure) were studied in a continuous dense-phase CO2 pilot plant.

**MATERIALS AND METHODS**

**Apple cider.** Pasteurized apple cider was obtained from Cornell Orchards and Cornell Dairy Store and contained no additives. It was diluted to 8% Brix and boiled for 3 min to eliminate background microorganisms. The pH of the juice was 3.8.

**Microorganism and inoculation.** Nonpathogenic *E. coli* ATCC 4157 was inoculated into cider and incubated at 37°C with shaking for 24 h. This procedure was repeated twice to select surviving cells. The surviving organisms were inoculated onto nutrient agar (Difco, Becton Dickinson, Franklin Lakes, N.J.) and incubated at 37°C for 24 h. The organism was harvested from the plates, and a concentrated culture of about 2 × 108 CFU/ml was
prepared in phosphate buffer with the use of McFarland Equivalence Turbidity standards. Boiled and cooled cider was inoculated with the concentrated culture to approximately $5 \times 10^6$ CFU/ml before processing with dense CO$_2$. *E. coli* in the cider was confirmed with Gram staining and morphological examination.

**Dense-phase CO$_2$ treatment.** A dense-phase CO$_2$ pilot plant–sized processing unit (Praxair, Inc., Danbury, Conn.; Fig. 1) was used to treat apple cider. This flowthrough system uses a diaphragm pump (Fig. 1A) to feed cider from an inlet sample tank (Fig. 1B) through a holding tube (Fig. 1C) where the cider meets and statically mixes with an inlet line of pressurized CO$_2$. A bottom-siphon liquid CO$_2$ pressurized tank (Fig. 1D) is fed through at regulated pressures at or above 4.1 MPa. The flow rate was measured with a CO$_2$ flowmeter installed on the machine. Carbonated cider then enters a temperature-controlled treatment holding tube (600 ml volume; Fig. 1E) that is pressurized to preset levels. An air-driven pump (Fig. 1F) moves the cider through the treatment loop. After the treatment loop, the treated cider passes through two successive temperature-controlled pressure release areas (Fig. 1G) that serve to prevent the product from freezing on a pressure drop and allow for evacuation of CO$_2$ as gas from the product.

**Enumeration of viable cells.** For each treatment, 25 ml of samples before and after the treatment were taken, and appropriate dilutions in 0.1% peptone water were pour-plated in duplicate immediately following the treatment. Duplicate samples were collected in 50-ml sterile plastic sample tubes directly from the pilot plant unit for each treatment. Nutrient agar (Difco, Becton Dickinson) was used as the medium for pour plates. The plates were incubated at 32°C for 2 days, and numbers (CFU) were counted. The result was expressed as a log reduction ($\log[N/N_0]$), where $N$ is viable cells in the treated product and $N_0$ is viable cells before the treatment. Samples from the experiment were also stored at 6°C, and the viable cells were enumerated after 1 week of storage.

**Experimental design and statistical analysis.** A central composite response surface design with $\alpha = 1$ was used. The response variable was a log reduction of *E. coli* in diluted cider. The factors with their coded and actual levels are shown in Table 1. Treatment at the design center point (35°C, 27.6 MPa, 70 g CO$_2$/kg product) was replicated six times. One replication occurred for the other points in the design matrix. The design was created and analyzed with Minitab (Release 12.2, Minitab Inc., State College, Pa.), and a full quadratic model was fitted to the data.

### RESULTS

The equation describing the response surface was

$$\log(N/N_0) = 1.83 - 0.088T - 0.1395C_02 - 0.012P + 0.0009T^2 + 0.0007C_02^2 - 0.0002P^2 + 0.0004TP,$$

where $T$ is temperature and $P$ is pressure. The equation had an $R^2 = 99.6\%$. The regression equation and the linear and quadratic terms in the equation were all significant ($P = 0.000$). Interactions among the treatment factors were not significant; all the coefficients for interaction terms had insignificant contributions to the model ($P > 0.10$). Table 2 illustrates the coefficients and their corresponding $P$ values.

### TABLE 2. Regression coefficients with their standard deviation and $P$ values for the response surface equation

<table>
<thead>
<tr>
<th>Term</th>
<th>Coefficient</th>
<th>SD</th>
<th>$P$</th>
</tr>
</thead>
<tbody>
<tr>
<td>Constant</td>
<td>1.8291</td>
<td>1.77950</td>
<td>0.328</td>
</tr>
<tr>
<td>$T$</td>
<td>$-0.0880$</td>
<td>$0.10645$</td>
<td>0.428</td>
</tr>
<tr>
<td>$C_02$</td>
<td>$-0.1395$</td>
<td>$0.00647$</td>
<td>0.000</td>
</tr>
<tr>
<td>$P$</td>
<td>$-0.0122$</td>
<td>$0.02508$</td>
<td>0.637</td>
</tr>
<tr>
<td>$T^2$</td>
<td>0.0009</td>
<td>0.00150</td>
<td>0.576</td>
</tr>
<tr>
<td>$C_02^2$</td>
<td>0.0007</td>
<td>0.0003</td>
<td>0.000</td>
</tr>
<tr>
<td>$P^2$</td>
<td>$-0.0002$</td>
<td>0.00035</td>
<td>0.637</td>
</tr>
<tr>
<td>$T \times P$</td>
<td>0.0004</td>
<td>0.00043</td>
<td>0.392</td>
</tr>
<tr>
<td>$T \times C_02$</td>
<td>$-0.0000$</td>
<td>0.00013</td>
<td>0.751</td>
</tr>
<tr>
<td>$C_02 \times P$</td>
<td>$-0.0000$</td>
<td>0.00006</td>
<td>0.879</td>
</tr>
</tbody>
</table>

$^a$ $T$, temperature in °C; $C_02$, CO$_2$/product ratio in grams per kilogram; $P$, pressure in MPa.
of the full quadratic model fitted to the data. As illustrated in Table 2, linear and quadratic coefficient of CO₂ concentration were the most important factors that explained the variability in the data. Some of the interaction terms had coefficients of 0 and thus did not appear in the equation.

CO₂/product ratio was the most effective factor affecting cell viability in the treated cider (Figs. 2 and 3). Both 70 and 140 g CO₂/kg product were very effective in inactivating cells, irrespective of the temperature and pressure levels. A more than 6-log reduction in population of E. coli was achieved with treatments containing 70 and 140 g CO₂/kg product. There was no difference between the 70 and 140 g/kg CO₂/product ratio because both levels decreased the cell population to undetectable levels. Treatments without CO₂, irrespective of temperature and pressure level, resulted in no significant loss of cell viability (Figs. 2 and 3). The sensitivity of E. coli to CO₂ was such (at gaseous, liquid, and supercritical state) that the effects of pressure and temperature, if any, were masked.

The experiment covered wide temperature (25 to 45°C) and pressure ranges (6.9 to 48.3 MPa). Although increasing temperature and pressure tended to increase inactivation (Figs. 2 through 4), the differences were not significant (P > 0.10). No temperature and pressure effects were detected in treatments conducted without CO₂ (0 g/kg) or with CO₂ at both 70 and 140 g/kg.

The survival of E. coli in diluted apple cider from the experiment were also determined after 1 week of storage at 6°C (data not shown). No effect of storage on the number of E. coli in samples was detected.

The pH and total soluble solid content of the cider were not affected by any of the treatments. The pH and total soluble solid content of treated ciders were the same as those of untreated cider, which were 3.8 and 8%, respectively. No visual changes in the cider were detected after the treatments.

**DISCUSSION**

The fitted response surface equation explained the variation in the data well. We kept some of the insignificant coefficients (for temperature, pressure, and interaction) in the model because these factors have been shown to have significant effects on cell inactivation in other high-pressure CO₂ treatments (2, 18). The reason they became insignificant in the analysis of our data could possibly be because the CO₂ effect dominated any possible temperature and pressure effect. If lower levels of CO₂ were used, the temperature and pressure effect might have been more apparent because it has been shown that both temperature and pressure have significant effects on microbial inactivation by CO₂ in static systems (2, 6).

The treatment conditions covered three states of CO₂: gas (35°C and 6.9 MPa; 45°C and 6.9 MPa), liquid (25°C and 6.9 MPa; 25°C and 27.6 MPa; 25°C and 48.3 MPa), and supercritical (35°C and 27.6 MPa; 45°C and 27.6 MPa; 45°C and 48.3 MPa). We found that CO₂ effectively inactivated E. coli at all tested conditions. This was in agreement with the work of Kamihira et al. (11), in which they reported that CO₂ at these three states effectively inhibited E. coli.

The minimum CO₂/product ratio that could be obtained in our equipment was 70 g/kg. It was apparent from the data that this minimum ratio of CO₂ in the product was sufficient to decrease the initial population of E. coli in diluted cider (about 5 × 10⁶ CFU/ml) to an undetectable level. The response surface tended to flatten between 70 and 140 g/kg CO₂ (Figs. 2 and 3), because at both levels, the population dropped to an undetectable level from about 10⁶ CFU/ml. Theoretically, the inactivation level could have been more than 6 log units because of the initial and undetectable populations after treatment. This could influence the shape of the response surface. Thus, this flattened part of the response surface could be a function of the inability to detect very low numbers (CFU). Further investi-
gation with a higher initial inoculation level in the juice medium is necessary to clarify this situation. Sensitivity of *E. coli* to high-pressure CO$_2$ has been shown by others (5). With a static batch processing system, these authors found that *E. coli* was the most sensitive organism tested and that inactivation started at 3.5 MPa. Treatment at 5.5 MPa for 60 min resulted in a 3.6-log reduction in *E. coli* and a 1.5- and 0.2-log reduction in *S. cerevisiae* and *Enterococcus faecalis*, respectively. However, they did not report the process temperature.

Ballestra et al. (2) used pressurized gaseous CO$_2$ in a batch system to treat *E. coli* in Ringer solution. They tested pressures between ambient, 1.2, 2.5, and 5 MPa and temperatures 25, 35, and 45°C. They found that the inactivation rate increased with increased pressure and temperature. These authors obtained up to a 4-log reduction after 14 min of exposure to CO$_2$ at 45°C and 5 MPa or after 80 min of exposure to CO$_2$ at 2.5 MPa at the same temperature. Erkmen obtained greater reduction of *E. coli* in broth, but relatively lower inactivation in milk in a batch treatment of up to 6 h (6). We had greater inactivation (a 6-log reduction) in a shorter time in diluted cider, possibly because of higher pressure and liquid CO$_2$ mixing in a flowthrough system in our experiment.

Shimoda et al. (18) obtained a 5.7-log reduction of *E. coli* in a nonfood environment with a continuous CO$_2$ flow system at 6 MPa and 35°C. The CO$_2$ and product flow rates in their experiment were 1 and 20 kg/h, respectively. Thus, the ratio of CO$_2$ to product was 50 g/kg product, which was slightly lower than our minimum CO$_2$ level (70 g/kg product). Our 6-log reduction result is in agreement with their result.

The treatment loop in our equipment was 600 ml at a flow rate of 120 ml/min, and resident time was 5 min. Pressure was reduced in two stages in the pressure reduction zone of the equipment: first to 4 to 5 MPa and then to 1.3 to 2 MPa. Thus, especially at high process pressure ranges (27.5 and 48.3 MPa), an important mechanical cell rupture from sudden release of pressure might be expected. Shimoda et al. (18) also suggested that the rapid pressure drop contributed to inactivation.

In general, our continuous system showed greater inactivation of *E. coli* than batch treatment, as illustrated by others (18). These authors also found that continuous treatment resulted in more effective inactivation than batch treatment. They attributed the inactivation mechanism to enzyme inaction and cell bursting in batch and continuous treatment, respectively. Ballestra et al. (2) also reported that the inactivation of cells in their experiment (batch CO$_2$ treatment) was mainly due to enzyme inactivation, although they also showed evidence of deformation of cell walls in treated cells. More mechanical damage to cell walls in continuous flowthrough systems than in batch systems can be explained by more rapid pressure reduction in the former than in the latter.

The response of *E. coli* O157:H7 to CO$_2$ at ambient atmosphere depends on the product. For example, CO$_2$ at ambient pressure had inhibitory effects on *E. coli* O157:H7 in apple products (7, 24), whereas no effect was observed in some minimally processed fresh produce (9, 17). No study on the influence of high-pressure CO$_2$ on pathogenic *E. coli* O157:H7 has been found in the literature, although research on other pathogens has been reported (22). We were unable to use pathogenic *E. coli* O157:H7 in our experiment because of the location of the equipment. Although high-pressure CO$_2$ treatment needs to be tested on the pathogen to validate the process for safety assurance, we expect that the pathogen would also be susceptible to and can be eliminated from cider by high-pressure CO$_2$ treatment on the basis of our results with nonpathogenic *E. coli*.

Our data show that dense-phase CO$_2$, when used in a continuous flow system, can effectively inactivate *E. coli* in apple cider and could be a nonthermal alternative method to achieve a 5-log reduction in pathogenic microorganisms. The nonpathogenic strain of *E. coli* (ATCC 4157) was found to be very sensitive to dense-phase CO$_2$. A more than 6-log reduction of *E. coli* was achieved by relatively moderate levels of CO$_2$, temperature, and pressure. This process has the potential to be an alternative to thermal pasteurization. However, further studies are needed to validate the effectiveness of the process with pathogenic *E. coli* O157:H7 as the test organism. In addition, shelf life studies of cider are needed to assess spoilage organisms and organoleptic quality and to propose an optimum process.

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