Inactivation of yeasts in grape juice using a continuous dense phase carbon dioxide processing system

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Abstract: The effects of dense phase CO\textsubscript{2} processing parameters, including temperature (25 and 35 °C), CO\textsubscript{2} concentration (0, 85 and 170 g kg\textsuperscript{-1}) and pressure (6.9, 27.6 and 48.3 MPa), on yeast survival and sensory properties of grape juice were investigated. The dense phase CO\textsubscript{2} process resulted in more than a 6 log reduction in yeast population. As the CO\textsubscript{2} to juice concentration, temperature and pressure increased, the inactivation rate increased. CO\textsubscript{2} in the supercritical state was more effective in inactivating yeast than in the subcritical state. The process did not cause detectable flavor degradation. Dense phase CO\textsubscript{2} processing can be an effective non-thermal alternative process for pasteurization of grape juice.

Keywords: dense carbon dioxide; grape juice; supercritical carbon dioxide; yeasts

INTRODUCTION

Non-thermal food preservation techniques have been of interest recently owing to consumer demand for fresh-like food products. Thermal pasteurization is the traditional and most common method to prevent microbial spoilage of highly acidic juices. However, it can cause degradation of flavor, nutrient, color and texture.

Dense phase CO\textsubscript{2} is a promising non-thermal process to preserve food.\textsuperscript{1} CO\textsubscript{2} at ambient pressure can inhibit microorganisms but, when it is applied at elevated pressures, it can effectively inactivate a number of microorganisms. The physical state of CO\textsubscript{2} can have a significant influence on antimicrobial activity\textsuperscript{1} but to our knowledge detailed studies of this effect have not been reported. CO\textsubscript{2} exists in its supercritical state above 31 °C and 7.34 MPa.\textsuperscript{2} Below this critical temperature–pressure combination, CO\textsubscript{2} exists in the subcritical liquid or gaseous state depending on the specific temperature–pressure combination.\textsuperscript{2} Supercritical CO\textsubscript{2} has properties of both liquid and gaseous CO\textsubscript{2} with altered viscosity, diffusivity and solubility, resulting in improved dissolving power.\textsuperscript{2} Theories explaining the inactivating mechanism of CO\textsubscript{2} involve a cytoplasmic pH decrease, explosive cell rupture, modification of a cell's membrane, inactivation of key enzymes and extraction of intracellular substances.\textsuperscript{1} Dense phase processing showed promising results in inactivation of microorganisms in orange juice and milk.\textsuperscript{1,4} In addition, dense CO\textsubscript{2} processes effectively inactivated yeasts in several studies.\textsuperscript{5–11} Grape juice is usually preserved through thermal pasteurization and addition of chemical preservatives (eg SO\textsubscript{2}) for both juice products and wine. However, these methods can cause undesirable effects on product quality.\textsuperscript{12} In wine making, heat treatment of the wine or the must has detrimental effects on the quality of the final product.\textsuperscript{12} For this reason SO\textsubscript{2} addition is the most common method to control microbial spoilage, although sterile filtration and sorbic acid addition may also be used.\textsuperscript{12} However, SO\textsubscript{2} can be harmful for asthmatics and, at high concentration, flavor is adversely affected.\textsuperscript{12} Therefore, alternative preservation methods for grape juices and wines are of interest. Removal of iron and other metals from grape juice to inhibit yeast growth has been a subject of interest recently.\textsuperscript{13} However, alteration of mineral composition may not be desirable from the nutritional point of view in non-fermented grape juice products.

Although major volatile products of yeast metabolism are ethanol and CO\textsubscript{2}, they contribute very little to wine flavor.\textsuperscript{14} Organic acids, higher alcohols and esters are the major volatiles produced during fermentation.\textsuperscript{14} These compounds may be undesirable at high concentration and, therefore, fermentation has to be carefully controlled for production of good quality wines. Kloeckera apiculata, Candida stellata, Saccharomyces cerevisiae and Brettanomyces intermedia
are among the common spoilage yeasts in wine and grape juice. These organisms in must can interfere with fermentation and cause off-flavor in wine. Therefore, the must and wine can be sterilized prior to and after fermentation to control the flavor compounds in wine. New non-thermal methods of sterilization are desired to keep natural flavors in the product.

The objective of this research was to investigate the effects of dense phase CO2 on microorganisms that may be present in grape juice. The influence of process parameters including temperature, pressure and concentration of CO2 on yeast inactivation was studied.

**MATERIALS AND METHODS**

**Grape juice**

Grape juice concentrate (83% Brix) was kindly donated by Professor Thomas Henick-Kling and obtained from the Cornell Enology Laboratory at NYS Agricultural Experiment Station (Geneva, NY, USA) and kept frozen at −20°C until used. It was diluted to 20% Brix and filter-sterilized using a 0.22-µm vacuum-driven disposable filtration system (Millipore, Bedford, MA, USA). The pH of the grape juice was 2.86.

**Yeast cultures**

*Saccharomyces cerevisiae* CE10, *Candida stellata* FAW223 and *Kloeckera apiculata* CE114 were obtained from the Cornell enology culture collection at NYS Agricultural Experiment Station. The yeasts were grown on potato dextrose agar (PDA) for 3–5 days at 22°C and harvested for preparation of stock inoculum in grape juice. A calibration plot of the number of cells versus absorbance at 660 nm was prepared. The number of cells was counted under a microscope using a Neubauer ruling counting chamber (Hausser Scientific Partnership, Horsham, PA, USA) and the absorbance at 660 nm was measured using a Jenway 6300 spectrophotometer (Jenway, Dunmow, Essex, UK). A stock inoculum of 1 l in grape juice was prepared at the desired concentrations ($10^7$–$10^8$ cfu ml$^{-1}$) by adding cells from potato dextrose agar (PDA) plates to the juice and the desired concentration was adjusted by optical density measurements.

**Grape juice inoculation and processing**

Filter-sterilized grape juice was inoculated with the yeast cultures prepared in grape juice about 1 h prior to processing. The inoculation levels were $1.5 \times 10^6$, $1.3 \times 10^6$ and $3.6 \times 10^7$ cfu ml$^{-1}$ for *C. apiculata*, *S. cerevisiae* and *C. stellata*, respectively. The inoculated grape juice was processed in a dense phase CO2 pilot plant unit (Praxair, Danbury, CT, USA) (Fig 1). This flow-through system uses a diaphragm pump (A) to feed the juice from an inlet sample tank (B) through a holding tube (C) where the juice meets and statically mixes with an inlet line of pressurized CO2. A bottom-siphon liquid CO2 pressurized tank (D) is fed through at regulated pressures at or above 4.1 MPa. The flow rate was measured with a CO2 flowmeter installed on the equipment. The concentration of CO2 expressed as grams of CO2 per kilogram of juice was set at levels of 0, 85 and 170 g kg$^{-1}$ by adjusting the CO2 flow rate relative to the juice flow rate. Juice mixed with CO2 then entered a 600-ml volume temperature-controlled treatment holding tube (E) that was pressurized to preset levels. The preset temperatures were 25 and

![Figure 1. Schematic of the dense phase CO2 pilot plant unit.](image-url)
35 °C and the preset pressures were 6.9, 27.6 and 48.3 MPa. An air-driven pump (F) moved the juice through the treatment loop. After the treatment loop, the treated juice passed through two successive temperature-controlled pressure-release areas (G) that served to prevent the product from freezing upon pressure drop and allowed for evolution of CO2 as gas from the product. The preset pressures in these areas were 4 and 0.7 MPa and the temperature was set at 25 °C at both locations. As the treated juice was collected in a flask where the CO2 was released to atmosphere in gaseous state, it took about 5 min between entry of the juice into the CO2 pilot plant and its exit.

The experiment was conducted in a full factorial design with the following factors and levels: process temperature (25 and 35 °C), CO2 concentration (CO2 to juice ratios) of 0, 85 and 170 g kg\(^{-1}\) and process pressures of 6.9, 27.6 and 48.3 MPa. Experimental conditions were set for all three states of CO2 (ie gas, liquid and supercritical). The treatments with supercritical CO2 were at 35 °C/27.6 MPa and 35 °C/48.3 MPa; treatments with liquid CO2 were at 25 °C/27.6 MPa and 25 °C/48.3 MPa; and treatments with gaseous CO2 were at 25 °C/6.9 MPa and 35 °C/6.9 MPa. Each treatment was replicated twice.

**Enumeration of viable cells**
The juice samples were plated no later than 1 h after the treatment. Appropriate dilutions of samples were served to prevent the product from freezing upon pressure drop and allowed for evolution of CO2 as gas from the product. The appropriate dilutions of samples were plated no later than 1 h after the treatment.

**Sensory evaluation of grape juice**
For a preliminary sensory evaluation the filter-sterilized uninoculated grape juice was processed in the dense phase CO2 pilot plant unit. The temperature, CO2 concentration and pressure were 35 °C, 170 g kg\(^{-1}\) and 48.3 MPa, respectively. Untreated, uninoculated sterile grape juice served as a control. A triangle test was conducted using an untrained panel of 170 subjects. The subjects were given randomly coded two controls and one treated samples in random order and asked to indicate the odd one. The samples were presented to each panelist once. The panelists were asked to describe the difference in a few words and whether the difference was desirable, undesirable or neutral.

**Statistical analysis**
Analysis of variance was conducted on the log reduction data using Minitab release 13 (Minitab, State College, PA, USA). Main effects, interactions and multiple comparisons of the factors using the Tukey procedure were conducted. The probability level for the triangle test was determined according to Roessler et al.\(^{16}\)

**RESULTS**
Survival of yeast cells was significantly affected by CO2 concentration, pressure and temperature (Table 1). Concentration of CO2 was the most important factor in inactivating yeasts cells. Treatments without CO2 did not result in any significant reduction in yeast cells. On the other hand, a CO2 concentration of 85 g kg\(^{-1}\) resulted in up to a 6.5 log reduction in counts at elevated pressure and temperature (Figs 2–4). Further increases in CO2 concentration from 85 to 170 g kg\(^{-1}\) resulted in an additional 1 log reduction in counts. As pressure and temperature increased the effect of CO2 concentration became more significant. Yeast populations decreased to about an undetectable level at 27.6 and 48.3 MPa along with 85 and 170 g kg\(^{-1}\) CO2 concentrations and a temperature of 35 °C (Figs 2–4). The detection limit for yeast populations was 10 cfu ml\(^{-1}\).

The process pressure had pronounced effect on inactivation of yeast cells (p = 0.000). As pressure increased inactivation increased (Figs 2–4). For all three organisms evaluated, processing at 6.9 MPa resulted in a lower overall inactivation than higher pressures. The inactivation was lowest at 25 °C and 6.9 MPa (subcritical state of CO2). However, at higher pressure and temperature (35 °C), CO2 in the supercritical state resulted in up to a 6.5 log reduction of viable cells (Figs 2–4). Further increase in pressure from 27.6 to 48.3 MPa resulted in a greater reduction (as with K apiculata and C stellata), but overall the effect became insignificant (p > 0.10) owing to

| Table 1. Analysis of variance table showing main effects and two-way interactions for each of yeast inactivation data |
|---|---|---|---|
| Factor | Degrees of freedom | S. cerevisiae | C. stellata | K. apiculata |
| CO2 concentration | 2 | 0.000 | 0.000 | 0.000 |
| Temperature | 1 | 0.000 | 0.000 | 0.000 |
| Pressure | 2 | 0.000 | 0.000 | 0.000 |
| CO2 concentration–temperature | 2 | 0.004 | 0.000 | 0.000 |
| CO2 concentration–pressure | 4 | 0.000 | 0.136 | 0.000 |
| Temperature–pressure | 2 | 0.037 | 0.002 | 0.518 |

Dense phase CO2 processing of grape juice

Figure 2. Effect of pressure and CO2 concentration at 25 °C (A) and 35 °C (B) on survival of S. cerevisiae in grape juice (error bars represent standard deviations).

Figure 3. Effect of pressure and CO2 concentration at 25 °C (A) and 35 °C (B) on survival of C. stellata in grape juice (error bars represent standard deviations).

total inactivation at both pressures. A significant interaction between pressure and CO2 concentration was observed (p = 0.001): the pressure effect was more pronounced at higher CO2 concentrations.

Process temperature had a significant effect (p = 0.000) on inactivation of yeasts but the effect was relatively small compared with the CO2 and pressure effects. As the temperature increased the inactivation rate increased for all yeasts (Figs 2–4). Increasing the temperature from 25 to 35 °C resulted in 0.5, 1 and 2 log additional reduction in K. apiculata, S. cerevisiae and C. stellata, respectively. The effect of temperature became more apparent as the CO2 concentration increased.

There was no significant difference in the response of different yeasts to dense phase CO2 processing (p = 0.776). The initial populations in the juice were K. apiculata 1.5 x 10^9, S. cerevisiae 1.3 x 10^6 and C. stellata 3.6 x 10^5 CFU ml^-1. At sufficiently high pressure, temperature and CO2 concentration the viability of each yeast fell below the detectable level. The larger reduction in C. stellata was mainly due to the higher initial level compared with S. cerevisiae and K. apiculata.

The preliminary sensory evaluation of the grape juice treated with dense phase CO2 suggested no significant change in sensory properties compared with the control (p = 0.203). The grape juice was treated at the highest combination of processing variables (170 g CO2 kg^-1, 35 °C and 48.3 MPa). Seven of 15 subjects correctly identified the treated juice. Some subjects indicated an increased sour and slightly astringent taste whereas only two indicated that the difference was undesirable (Table 2). The treatment did not cause any changes in pH or the Brix of the juice.

DISCUSSION

CO2 in the supercritical state (>31 °C and >7.34 MPa) was more effective in inactivating yeast cells than CO2 under subcritical conditions. Treatments with supercritical CO2 resulted in the greatest inactivation of yeasts in grape juice whereas CO2 in the subcritical state caused a lower inactivation rate (Figs 2–4). A higher inactivation power of CO2 in the supercritical state has been reported in several studies. CO2 has better diffusivity and dissolving power in the supercritical state. CO2 with these enhanced properties can effectively penetrate into cells and extract cellular components, resulting in disruption of biological system in the cell.
Table 2. Sensory evaluation (triangle test) of grape juice treated with dense phase CO2

<table>
<thead>
<tr>
<th>Discrimination</th>
<th>No. of subjects</th>
<th>Direction of difference</th>
<th>Description</th>
<th>Probability level</th>
</tr>
</thead>
<tbody>
<tr>
<td>Incorrect</td>
<td>8</td>
<td>Desirable: 3</td>
<td>Less sweet</td>
<td></td>
</tr>
<tr>
<td></td>
<td></td>
<td>Neutral: 4</td>
<td>Slightly more sour</td>
<td></td>
</tr>
<tr>
<td></td>
<td></td>
<td>Undesirable: 1</td>
<td>Oxidized, caramel, cooked</td>
<td></td>
</tr>
<tr>
<td>Correct</td>
<td>7</td>
<td>Desirable: 2</td>
<td>More refreshing, less sweet,</td>
<td>0.203a</td>
</tr>
<tr>
<td></td>
<td></td>
<td>Neutral: 3</td>
<td>Carbonated soda taste</td>
<td></td>
</tr>
<tr>
<td></td>
<td></td>
<td>Undesirable: 2</td>
<td>Slightly astringent with a slight ‘bite’</td>
<td>More sour</td>
</tr>
</tbody>
</table>

*a Not significant at the 95% confidence level.

Figure 4. Effect of pressure and CO2 concentration at 25 °C (A) and 35 °C (B) on survival of K. apiculata in grape juice (error bars represent standard deviations).

the fluidity of the membrane, resulting in an increase in permeability, alteration of membrane fusion characteristics and destruction of essential domains in the membrane, all of which result in cell inactivation.

The CO2 concentration was the most important factor affecting the antimicrobial activity of the dense CO2 process. Processing under the same temperature and pressure conditions but without CO2 (ie 0 g CO2 kg\(^{-1}\)) did not affect the yeast population. The difference between the effect of an 85 and a 170 g kg\(^{-1}\) CO2 concentration was relatively small. This was due to the high inactivation rate observed at 85 g kg\(^{-1}\). The lowest CO2 concentration that the equipment allowed was 85 g kg\(^{-1}\) and this was high enough to substantially decrease the initial yeast population. Increasing the CO2 concentration to 170 g kg\(^{-1}\) resulted in reduction of yeast population to an undetectable level at 27.6 and 48.3 MPa. A CO2 concentration of 85 g kg\(^{-1}\) at temperatures and pressures above the critical point was sufficient effectively to eliminate yeasts from grape juice. Similar effects of CO2 concentration have been reported in the literature. Isenchmid et al showed that dissolved CO2 was the main factor responsible for cell death.8 CO2 concentration at or above 50 g kg\(^{-1}\) at 6 MPa and 35 °C resulted in total inactivation of S. cerevisiae.7

Process pressure and temperature also significantly affected the inactivation of yeasts in grape juice. Pressures and temperatures above the critical point were more effective than those below critical point. However, the difference between the two supercritical pressure–temperature conditions (27.6 and 48.3 MPa) was not significant at the CO2 concentrations tested. This was due to the very high inactivation of yeasts at the lower pressure. An effect of increasing the pressure to 48.3 MPa could not be detected at 35 °C. However, further inactivation by increased pressure was observed at 25 °C and 85 g kg\(^{-1}\) CO2 concentration (Figs 2–4). Reduction in cell viability with increased pressure has also been reported in the literature.8 Most previous work was conducted in batch rather than continuous systems. Up to a 6 log reduction of S. cerevisiae after exposure for 30 min to 20 MPa and 35 °C was observed5 while a 3 log reduction of the same organism was achieved after 18 h of exposure to 5.5 MPa and 25–45 °C.17 Lin et al investigated the inactivation of S. cerevisiae by CO2 in the 6.9–20.7 MPa pressure range in a batch system. 6 They obtained up to a 7 log reduction of the yeast in liquid media at 6.9 MPa for 15 min. This inactivation was greater than that observed here at 6.9 MPa, possibly owing to the greater exposure time in their study. They also reported that inactivation increased with increasing temperature as was observed in this study. A relatively lower reduction (1.5 log) of S. cerevisiae has been achieved on hydrophilic filter-paper discs upon treatment at 5.5 MPa for 60 min.11 This inactivation rate is much lower than that achieved here, possibly owing to the treatment procedure. Their treatment involved gaseous CO2 and organism on a solid support rather than in suspension. Arreola et al found that as the temperature and pressure increased,
the $D$-value for total plate counts in orange juice decreased.\textsuperscript{3}

Most of the studies reported in the literature involved relatively lower pressure and CO\textsubscript{2} concentration in batch systems\textsuperscript{1} and to our knowledge did not involve a food system except for the orange juice study.\textsuperscript{3} It has been shown that a lower CO\textsubscript{2} concentration in a continuous treatment resulted in the same degree of inactivation as in a batch treatment.\textsuperscript{7} The inactivation level in \textit{S. cerevisiae} in our study was comparable to those in earlier studies with continuous treatment.\textsuperscript{7,10} Continuous treatment results in a higher inactivation rate, possibly owing to additional cell rupture resulting from the sudden release of pressure.\textsuperscript{7} A batch system involves relatively slower pressure reduction and therefore the cell rupture is less evident.\textsuperscript{7} In addition, a continuous system may allow better mixing of CO\textsubscript{2} with the product and hence greater dissolution compared with batch treatments.

Use of SO\textsubscript{2} as an antimicrobial agent has flavor and safety drawbacks and sterile filtration can be expensive.\textsuperscript{12} Thermal pasteurization has also been suggested as an alternative to pasteurization of grape juice and wine by SO\textsubscript{2} and filtration.\textsuperscript{12} Malletroit \textit{et al} found that pasteurization in a water-bath or heat tunnel at up to 15 pasteurization units (PU; 1 PU was defined at 1 min of heating at 60°C or its equivalent) were sufficient to reduce yeast cells from 10\textsuperscript{7} cells ml\textsuperscript{-1} to an undetectable level.\textsuperscript{12} The authors suggested that thermal pasteurization can be a good alternative to chemical or filter pasteurization. Our continuous dense phase CO\textsubscript{2} processing of grape juice resulted in the same degree of yeast inactivation as obtained by thermal pasteurization in an earlier study.\textsuperscript{12} Therefore, dense phase CO\textsubscript{2} processing can be a cost-effective alternative to thermal processing of fruit juices as CO\textsubscript{2} is relatively inexpensive. However, appropriate equipment design that allows proper control of process variables such as temperature, pressure, CO\textsubscript{2} concentration and residence time needs to be developed.

Our preliminary sensory evaluation of grape juice treated at the highest levels of factors used in the experiment suggested that the treated juice were indistinguishable from the control. Only a few subjects indicated a more acidic or carbonated taste of treated juice and none of them expressed that it was undesirable. The juice used in sensory evaluation was treated at the highest combination of temperature, pressure and CO\textsubscript{2} concentration. The process did not cause a detectable change in pH of the grape juice although a slight change in pH of orange juice upon supercritical CO\textsubscript{2} treatment has been reported.\textsuperscript{3} The preliminary sensory results suggest that dense phase CO\textsubscript{2} processing does not cause deterioration of the sensory attributes of grape juice. However, more detailed sensory evaluations along with microbiological analysis are needed to optimize the process.


\textbf{CONCLUSIONS}

Continuous dense CO\textsubscript{2} processing effectively eliminated yeasts in grape juice and can be an alternative non-thermal pasteurization method for the product. A CO\textsubscript{2} concentration of 85 g kg\textsuperscript{-1} in the supercritical state was sufficient to achieve more than a 6 log reduction in yeast population. The process did not cause significant flavor changes in grape juice. Further studies may be useful to optimize the process parameters considering both the microbial and organoleptic quality of the product.

\textbf{ACKNOWLEDGEMENTS}

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\textbf{REFERENCES}


