INTRODUCTION

Liquid food products are pasteurised in order to eliminate the risk of food poisoning and increase their shelf life. Pasteurization is a process in which the liquid foods are heated to a specific temperature for a specific time to kill or deactivate the pathogenic bacteria. The liquid foods that are commonly pasteurized are whole liquid eggs (removed from shell), milk, fruit juices, almonds, cider, and beer.

Usually liquid foods are pasteurized by a low temperature long time (LTLT) process at about 145 °F (63 °C) for 30 min or a high temperature short time (HTST) process at about 162 °F (72 °C) for 15 s or an ultra-high temperature (UHT) process at about 265 to 295 °F (130 to 145 °C) for 2 to 45 s. Though the heat treatment can increase the shelf life to about 2 to 3 weeks under refrigeration (lower than 7 °C), it can cause significant reduction in physical, nutritive, sensory quality of foods and may also reduce the content or bioavailability of some bioactive compounds [1-3]. Therefore, there is a necessity for pasteurization using non-thermal techniques such as high pressure processing, irradiation, pulsed electric fields, power ultrasound, ozone, oscillating magnetic fields etc.

The use of dense phase carbon dioxide (DPCD) has been proposed as an alternative non-thermal technique for pasteurization of foods [4]. In the DPCD technique, food is contacted with (pressurized) sub- or supercritical CO₂ for a certain amount of time in batch, semi batch, or continuous equipment. Though DPCD technique has been studied on a variety of food products, it has predominantly been used for pasteurising liquid foods. Various studies have shown that DPCD can be used an effective means to inactivate microorganisms in liquid foods. Increasing research interests in this technology have aimed at developing not only safe foods but also high-quality food with "fresh-like" characteristics.

This paper aims to provide a detailed and critical review of the application of the DPCD technique for pasteurisation of liquid foods, and also to shed some light on its current challenges and opportunities for future development in the food industry.

Dense Phase Carbon Dioxide

Dense phase is the fourth phase (Solid, Liquid, Gas, and Dense) that cannot be described by the senses. When a pure compound is above critical pressure and critical temperature, the system is often referred to as a “dense phase fluid” or “super critical fluid” to distinguish it from normal gas and liquid. The term “dense phase” (DP) fluid as used here denotes those phases
of matter that remain fluid, yet are dense. The dense phase has a viscosity similar to that of a gas, but a density closer to that of a liquid. Because of its unique properties, dense phase has become attractive for food and pharmaceutical processing applications, transportation of natural gas, carbon dioxide (CO₂).

CO₂ can be more beneficially utilised in its dense form for pasteurisation of liquid foods due to its demonstrated bactericidal effects [5-7]. While other gasses (N₂O, N₂, Ar, tetrafluoroethane) have also been evaluated for bactericidal efficacy at pressures higher than atmospheric, the superior effect that CO₂ has over other gases has been attributed to its low critical point: 7.11 MPa, 31ºC, which is only slightly above room temperature, thereby eliminating thermal degradation (Table 1).

### Table 1. Properties of some supercritical fluids at the critical point [8].

<table>
<thead>
<tr>
<th>Fluid</th>
<th>Critical Temp. (ºC)</th>
<th>Critical Pressure (MPa)</th>
<th>Critical Density (g/ml)</th>
</tr>
</thead>
<tbody>
<tr>
<td>CO₂</td>
<td>31.0</td>
<td>7.11</td>
<td>0.47</td>
</tr>
<tr>
<td>N₂O</td>
<td>36.5</td>
<td>7.10</td>
<td>0.45</td>
</tr>
<tr>
<td>Water</td>
<td>374.2</td>
<td>21.50</td>
<td>0.32</td>
</tr>
</tbody>
</table>

Moreover, in the dense-supercritical phase, CO₂ has low viscosity (3-7 × 10⁻⁵ Nsm⁻²) and zero surface tension, so it can quickly penetrate porous and complex food materials. CO₂ remains the most preferable gas for killing food organisms because of its low toxicity, nonflammability, and low cost [9] making it an economically feasible option. Furthermore, as an additive, CO₂ would not negatively affect a consumer’s perception because of their familiarity with products such as carbonated beverages.

### Earlier Uses of Carbon Dioxide in Food Preservation

Because of the need for a preservation method that is safe, inexpensive, and non-destructive to heat sensitive compounds, the use of carbon dioxide has been tested as a food preservation method. The use of carbonation as a means of preserving food started as early as 1939 with the study by Brown et al. [10], where apple cider was carbonated and microbial inactivation and flavor changes were recorded. The carbonation of the juice was shown to preserve the cider for up to 3 months at approximately 21ºC with no change in flavor.

The use of carbonation was also investigated for its use in soft drinks as a preservation agent. Even at the lowest amount of gas pressure (3 volumes of CO₂ where 1 volume=1 L of CO₂ per L of beer) sterility was achieved on approximately the 20th day depending on the °Brix of the beverage [11]. Further, since 1980, many researchers have reported the bacteriostatic action and inhibitory effect of CO₂ on growth and metabolism of some microorganisms. Although carbonation with CO₂ has been shown as an effective preservative some bacteria are not affected.

Molin [12] reported that CO₂ had approximately 75% inhibitory affect on Bacillus cereus, Brochothrix thermosphacta, and Aeromonas hydrophila, and a 53% - 29% inhibitory effect on Escherichia coli and Streptococcus faecalis. Pseudomonas was found to be very sensitive while other types, such as Lactobacillus and Clostridium were less sensitive. Inhibitory rates for anaerobic bacteria were even lower. This proved that carbonation of foods alone would not inactivate all food related bacteria and subsequently brought in the need to use CO₂ under pressure.

Kamihira et al. [13] tested the sterilizing effect of CO₂ at supercritical, liquid and gaseous states on wet and dry Escherichia coli, Staphylococcus aureus and conidia of Aspergillus niger by using a supercritical fluid extraction apparatus. Since then, many studies investigated the effects of CO₂ under pressure on pathogenic and spoilage organisms, vegetative cells and spores, yeasts and molds, enzymes and their activities and food quality attributes. Eventually, it has been found that if CO₂ is pressurized, the process is no longer bacteriostatic, but bactericidal [7,14,15]. Hence, like any technique, the use of CO₂ under pressure to kill bacteria has also been tested on a wide range of food materials using different pressure and temperature conditions.

Furthermore, according to the pressure and temperature conditions used, the application of CO₂ under pressure is denoted by a few techniques. As the terminologies are interchangeably used, it might be necessary to learn the differences between these terminologies to avoid misconceptions.

- HPCD (High Pressure CO₂) Processing - use of CO₂ above atmospheric pressure
- SCCD (Super Critical CO₂) Processing - use of CO₂ in the Super Critical phase only
- DPCD (Dense Phase CO₂) Processing - use of CO₂ in the Super Critical and liquid states

### Dense Phase Carbon Dioxide Processing

Dense phase carbon dioxide (DPCD) processing is a non-thermal processing technology that utilizes carbon dioxide at pressures under 50 MPa to inactivate microorganisms i.e., pasteurise mostly liquid foods. DPCD is called as a cold pasteurization method for since it affects microorganisms through molecular effects of CO₂ without exposing foods to adverse thermal effects of traditional pasteurization (heat) and retains their fresh-like physical, nutritional, and sensory qualities [16]. DPCD processing is a collective term for liquid CO₂ and supercritical CO₂ or high pressure carbon dioxide (HPCD).
Fraser [17] was the first to show that DPCD can inactivate bacterial cells. He showed that 99% of *E. coli* numbers were rendered non-viable by a decompression of CO₂ from 500 psi to atmospheric pressure. Since then, many studies investigated the effects of DPCD on pathogenic organisms and enzymes. Works on various liquid foods have been carried on by many investigators (Table 2). In the past 2 decades, the number of research results and patents increased, and commercialization efforts intensified [18].

Table 2. Summary of the studies on microbial inactivation in liquid foods by dense phase CO₂.

<table>
<thead>
<tr>
<th>S. No.</th>
<th>Food</th>
<th>Inoculated Microorganism</th>
<th>Treatment</th>
<th>Temperature (°C)</th>
<th>System</th>
<th>Maximum Reduction</th>
<th>Reference</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>Milk</td>
<td><em>L. monocytogenes</em></td>
<td>7 MPa /1 h</td>
<td>45</td>
<td>Batch</td>
<td>3 log</td>
<td>Lin et al. [19]</td>
</tr>
<tr>
<td>2</td>
<td>Skim milk</td>
<td><em>S. aureus</em></td>
<td>9-15 MPa /2.5 h</td>
<td>25</td>
<td>Batch</td>
<td>7 log</td>
<td>Erkmen [20]</td>
</tr>
<tr>
<td>3</td>
<td>Fruit juice</td>
<td><em>E. faecalis</em></td>
<td>6 MPa/3-6 h</td>
<td>45</td>
<td>Batch</td>
<td>5 log</td>
<td>Erkmen [21]</td>
</tr>
<tr>
<td>4</td>
<td>Milk</td>
<td><em>E. faecalis</em></td>
<td>6 MPa/24 h</td>
<td>45</td>
<td>Batch</td>
<td>5 log</td>
<td>Erkmen [21]</td>
</tr>
<tr>
<td>5</td>
<td>Orange juice</td>
<td><em>L. monocytogenes</em></td>
<td>6 MPa/8 h</td>
<td>45</td>
<td>Batch</td>
<td>6 log</td>
<td>Erkmen [22]</td>
</tr>
<tr>
<td>6</td>
<td>Peach juice</td>
<td><em>L. monocytogenes</em></td>
<td>6 MPa/4 h</td>
<td>45</td>
<td>Batch</td>
<td>6 log</td>
<td>Erkmen [22]</td>
</tr>
<tr>
<td>7</td>
<td>Whole milk</td>
<td><em>E. coli</em></td>
<td>10 MPa/6 h</td>
<td>30</td>
<td>Batch</td>
<td>6.4 log</td>
<td>Erkmen [15]</td>
</tr>
<tr>
<td>8</td>
<td>Skim milk</td>
<td><em>E. coli</em></td>
<td>10 MPa/6 h</td>
<td>30</td>
<td>Batch</td>
<td>7.2 log</td>
<td>Erkmen [15]</td>
</tr>
<tr>
<td>9</td>
<td>Natural orange juice</td>
<td><em>Mould-Yeasts</em></td>
<td>30 MPa/15 min</td>
<td>28</td>
<td>Semi-continuous</td>
<td>Total inactivation</td>
<td>Spilimbergo et al. [4]</td>
</tr>
<tr>
<td>10</td>
<td>Beer</td>
<td>Yeasts</td>
<td>26.5 MPa/4.77 min 9.6% CO₂</td>
<td>21</td>
<td>Continuous</td>
<td>7.3 log</td>
<td>Folkes [23]</td>
</tr>
<tr>
<td>11</td>
<td>Grape juice</td>
<td><em>S. cerevisiae</em></td>
<td>49 MPa 170 g CO₂/kg juice</td>
<td>25</td>
<td>Continuous</td>
<td>5.5 log</td>
<td>Gunes et al. [24]</td>
</tr>
<tr>
<td>12</td>
<td>Orange juice</td>
<td><em>S. typhimurium</em></td>
<td>21 MPa/10 min</td>
<td>25</td>
<td>Continuous</td>
<td>6 log</td>
<td>Kincal et al. [25]</td>
</tr>
<tr>
<td>13</td>
<td>Orange juice</td>
<td><em>L. monocytogenes</em></td>
<td>38 MPa/10 min</td>
<td>25</td>
<td>Continuous</td>
<td>6 log</td>
<td>Kincal et al. [25]</td>
</tr>
<tr>
<td>14</td>
<td>Coconut water</td>
<td><em>Aerobic plate count</em></td>
<td>34.5 MPa/6 min 13% CO₂</td>
<td>40</td>
<td>Continuous</td>
<td>5.61 log</td>
<td>Damar [26]</td>
</tr>
<tr>
<td>15</td>
<td>Apple juice</td>
<td><em>S. cerevisiae</em></td>
<td>9 MPa/15 min</td>
<td>32</td>
<td>Multi-batch</td>
<td>4.9 log</td>
<td>Parton et al. [27]</td>
</tr>
<tr>
<td>16</td>
<td>Kava beverage</td>
<td><em>Aerobic plate count</em></td>
<td>34.5 MPa/7 min</td>
<td>30</td>
<td>Continuous</td>
<td>3 log</td>
<td>Hsieh et al. [28]</td>
</tr>
<tr>
<td>17</td>
<td>Apple juice</td>
<td><em>Aerobic plate count</em></td>
<td>10 MPa/10 min</td>
<td>36</td>
<td>Multi-batch</td>
<td>Total inactivation</td>
<td>Gasperi et al. [29]</td>
</tr>
<tr>
<td>18</td>
<td>Hami melon juice</td>
<td><em>Aerobic plate count</em></td>
<td>35 MPa/60 min</td>
<td>55</td>
<td>Batch</td>
<td>Total inactivation</td>
<td>Zhang et al. [30]</td>
</tr>
<tr>
<td>19</td>
<td>Peach and kiwi juice</td>
<td><em>S. cerevisiae/Aerobic plate count</em></td>
<td>10 MPa/15 min</td>
<td>35</td>
<td>Batch</td>
<td>Total inactivation</td>
<td>Spilimbergo and Ciola [31]</td>
</tr>
<tr>
<td>20</td>
<td>Lychee juice</td>
<td><em>Aerobic plate count</em></td>
<td>8 MPa/2 min</td>
<td>36</td>
<td>Batch</td>
<td>5 log</td>
<td>Guo et al. [32]</td>
</tr>
</tbody>
</table>

Mechanisms of Microbial Inactivation by DPCD

Though many theories were developed to explain the bacteriostatic action of CO₂, the exact inactivation mechanisms still remain to be unravelled. However, the different steps involved in the hypothetical microbial inactivation mechanism can be summarized as follows:

i) Solubilization of pressurized CO₂ in the external liquid phase

ii) Cell membrane modification

iii) Intracellular pH lowering effect

iv) Key enzyme inactivation

v) Direct (inhibitory) effect of molecular CO₂ and HCO₃⁻ on metabolism

vi) Disordering of the intracellular electrolyte balance

vii) Removal of vital constituents from cells and cell membranes.

Most of these steps will not occur consecutively, but rather take place simultaneously in a complex and interrelated manner.

Solubilization of pressurized CO₂ in the external liquid phase

Pressurized CO₂ is able to dissolve in the water content of the food matrix. As a consequence, the aqueous part of a food in contact with pressurized CO₂ generally becomes acidic due to the formation and dissociation of H₂CO₃⁻, which liberates H⁺ ions.

\[
\text{CO}_2 + \text{H}_2\text{O} \leftrightarrow \text{H}_2\text{CO}_3^- \\
\text{H}_2\text{CO}_3^- \leftrightarrow \text{H}^+ + \text{HCO}_3^-
\]

\[\text{pK}_a = 6.57\]
precursor amino acids and nucleic acids. CO₂ fulfills the role of either a biosynthetic substrate in carboxylation reactions or a
substrate, product, and cofactor, which are primary elements in the regulation of enzymatic activity.

When the applied CO₂ pressure accumulates in the cytoplasmic interior of the bacterial cells, lethal damage to the biological
system of the cells may occur. This may convert HCO₃⁻ to CO₃²⁻, which could precipitate intracellular inorganic electrolytes (such
as Ca²⁺, Mg²⁺ and similar ions) from cells and cell membranes. Since these inorganic electrolytes (apart from being important
regulators of a large number of other cell activities) help in maintaining the osmotic relationships between cells and their
surrounding media, this could have deleterious effects on the volume of cells.

Removal of vital constituents from cells and cell membranes

Several authors have suggested that accumulated CO₂ could, due to its relatively high solvating power, “extract” vital
constituents from the cells or cell membranes. The pressurized CO₂ first penetrates into the cells to build up the density to a
critical level within the cells. Then there is a sudden release of the applied pressure which disturbs or alters the structure of the
bio-membrane, leading to removal and rapid transfer of the intracellular constituents, such as phospholipids and hydrophobic
compounds out of the biological system into the extracellular environment. This extraction disturbs the balance of the biological

Intracellular pH lowering effect

Due to the increased membrane permeability, pressurized CO₂ may easily penetrate through the bacterial cell membrane
and accumulate in the cytoplasmic interior of bacterial cells. There, the relative concentrations of both dissolved CO₂ and HCO₃⁻
are in first instance controlled by internal pH buffering as a result of pH homeostasis in order to maintain a more or less constant
cytoplasmic internal pH (which is essential for optimal cell viability and cellular activity). However, if too much dissolved CO₂
enters the cytoplasm, the internal pH will start to decrease. If the internal pH is lowered too much, cell viability will be seriously impaired
and the cells also may be unable to maintain the resulting large pH difference, ΔpH (ΔpH = pH internal – pH external). Therefore,
impairment of cellular activity may coincide with both a low internal pH and the collapse of a large ΔpH[34].

Spilimbergo et al.[8] exposed a suspension of Bacillus subtilis to DPCD at 8.0 MPa and 30 °C for 5 min. The internal pH and
external pH of the suspension were determined as 3.3 and 3.2 respectively which brought up a 5-decimal reduction of B. subtilis
cells. Though the values of internal pH and external pH were very similar, these findings seemed to support the hypothesis that
upon DPCD exposure, microbial cells are unable to maintain a favourable cytoplasmic pH homeostasis and many aspects of cell
structure and function are influenced by internal pH.

Key enzyme inactivation

Enzymes, which make up most of the proteins in the cytosol, have maximal activity at the optimum pH, and their activity
decays sharply on either side of the optimum. Lowering of the cytosolic internal pH might cause inhibition and/or inactivation
of key enzymes essential for metabolic and regulatory processes, such as glycolysis, amino acid and peptide transport, active
transport of ions, and proton translocation[34]. Thus a loss over biological control of the internal pH of cells may be detrimental in
all aspects of intermediary metabolism and cellular function.

Direct (inhibitory) effect of molecular CO₂ and HCO₃⁻ on metabolism

The reaction rate of each enzymatic reaction is not only a function of the pH but also of the intracellular concentrations of its
substrates, products, and cofactors, which are primary elements in the regulation of enzymatic activity.

Carboxylation reactions are particularly important for the gluconeogenesis and the synthesis of particular biosynthetic
precursor amino acids and nucleic acids. CO₂ fulfills the role of either a biosynthetic substrate in carboxylation reactions or a
metabolic product from decarboxylation reactions. As far as decarboxylation reactions are concerned, they all appear to produce
CO₂ in the dissolved (unhydrated) CO₂ form.

The ultimate effect that the various species of CO₂ may have on microbial metabolism probably will be a function of the
relative importance of the various carboxylation and decarboxylation reactions[30].

Disordering of the intracellular electrolyte balance

Intracellular ph lowering effect
system, thus promoting inactivation \cite{13,33,36}. Lin et al. \cite{36} also suggested that the rate of inactivation could be improved by repeating the release and recharge of pressurized CO$_2$ in the pressure vessel during the treatment.

Nakamura et al. \cite{7} used SEM as an evidence for mechanical rupture of yeast cells. They observed that some cells were completely burst whereas some had wrinkles or holes on their surface. This was also confirmed by Ballestra et al. \cite{37} who demonstrated that some E. coli cells treated with DPCD at 5.0 MPa and 35°C showed some signs of deformation of cell walls. Following researches by Hong and Pyun \cite{38}, Folkes \cite{23} and Bertoloni et al. \cite{39} in the L. plantarum, E. coli and S. cerevisiae revealed DPCD treatment resulted in irreversible cellular membrane damage including leakage of intracellular materials like UV absorbing substances, enzymes and release of intracellular ions (such as Mg$^{2+}$ and K$^+$).

### Current Challenges and Future Scope

After a comprehensive review of prior research on Dense phase CO$_2$ processing method to pasteurise liquid foods, it can be observed that there are some challenges remain:

- Only vegetative bacteria have been extensively studied and are susceptible to Dense Phase CO$_2$ treatment
- The effects of different factors like temperature, pressure and equipment on deactivation of microbes can be substantial, but there is not yet a clear understanding of these effects
- The nutritional attributes of DPCD treated liquid foods in general have been poorly examined
- The shelf-life and stability of the DPCD treated liquid foods during different storage conditions has not sufficiently been studied

The future research on DPCD treatment for liquid foods may be directed towards the following line of action:

- Mathematical modelling of CO$_2$ pasteurization need to be strengthened, as it is important for elucidating the mechanisms, and for process optimization
- Combined treatments of dense phase carbon dioxide and other potential non thermal processing techniques like high hydrostatic pressure (HHP) can be studied to enhance the safety and shelf life of liquid foods

### CONCLUSION

There has been an increased interest in application of non thermal techniques to preserve liquid foods. At the same time there is also a demand for fresh-like and nutritious food. The DPCD cold pasteurisation technology is a cost-efficient, environment friendly and reliable method to preserve the quality of liquid food. Further, several batch, semi-continuous, and continuous systems have been developed for DPCD applications. However, the applicability of this technology in industrial scale has not been widely spread due to be challenges mentioned above. It is necessary to have a better understanding of the complex challenges and find out the ways that would contribute to improve the applications of DPCD for pasteurization of liquid foods.

### REFERENCE