Sterilisation and virus inactivation by Supercritical Fluids: A review

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Abstract:
While supercritical processes are developing both in the "classical" applications in food industry and in new domains related to Health Sciences, the interactions of SCFs with living micro-organisms are of growing importance. It is known for long that SCF processing does protect the feeds from oxidation and prevent bio-burden increase. Moreover, SCF were also shown to have the ability to kill most micro-organisms and to inactivate viruses. This paper intends to briefly review the present state-of-the-art in order to underline the promising future of SCF sterilization/pasteurisation and virus inactivation as an alternative "green" method to classical processes that cannot be used in certain cases (i.e. heat treatment of thermolabile products, or irradiation of bio-molecules).

Introduction

For long, high pressure treatment is used for pest control and sterilization in food industries as alternative to heat treatment that generally degrades product quality (aspect, taste, vitamin content, etc.). However, the required hydrostatic pressure for efficient sterilization is extremely high (400 to 800 MPa) with long exposition times, which leads to costs incompatible with most markets. As mentioned in early publications and patents [2,4-12], it is known for several decades that supercritical fluid exposure can be considered as a less expensive variant working at much lower pressures with CO_2, possibly added with water or ethanol or other additives like hydrogen peroxide. On the other hand, it is known that CO_2 and N_2O, even at low pressure, inhibit the growth [1] and boost the inactivation rate of micro-organisms and spores during irradiation [2] or thermal treatment: A heat treatment at 50-55°C under 6 bar of CO_2 or N_2O has the same lethal effect on several bacteria, fungi and yeasts as a heat treatment at 60-65°C in presence of air, or, in other terms, operating with this gas pressure could reduce by 50% the time of pasteurisation at a given temperature [17].

Biological effects of supercritical fluids on microorganisms

From several sources [4,5,12,15,34,35], comparison of survival curves of microorganisms in contact with a pressurised gas like nitrogen, ethane or propane, and with a sub-/super-critical fluid (CO_2, ethane, propane), clearly demonstrates that the bactericidal effect of these fluids cannot be attributed to the hydrostatic pressure in the range of tens or hundreds of bars. On the other hand, it has been recognized that gaseous CO_2 can inhibit microbial growth, leading to its use in the preservation of packed foods, although its inactivation effect seems reversible [2,15]. Moreover, this specific effect is definitely supported by the comparison of cell number decay of various micro-organisms when submitted to a hydrostatic pressure with and without CO_2 [16,19]: The decay of *E. coli* in CO_2 at 15 MPa and 35°C was similar to the one observed at 300 MPa at 20°C during the same period of time [16]. So, there is no doubt that this bactericidal effect is caused by specific interactions between the living cell and the fluid that readily dissolves inside the cell. As discussed in depth by Spilimbergo et al. [31,35,51], many authors proposed possible mechanisms, although quantification of each contribution remains unknown and some of these effects cannot be alluded when N_2O is used:

- Cell wall alteration due to a strong interaction of the fluid with the lipids,
- Inactivation of some key-enzymes resulting from pH decrease inside the cell [51], and

specific inhibition of decarboxylases by excess of CO_2, breaking the metabolic chain;
- Inactivation of certain bio-reactions caused by lipid extraction;
- Precipitation of carbonates (Ca, Mg, etc.) from bicarbonates inside the cell when the CO_2 pressure is released.

As membrane disruption and cell lysis through supercritical CO_2 rapid pressure cycles was proposed [7,13], it is not surprising that such pressure cycling [15,26,27,31,43,45] increases the sterilisation efficacy, and may considerably reduce the treatment time for obtaining a given degree of inactivation. Another support for this mechanism comes from the strong synergy observed by combining a very brief pulsed electric field pre-treatment - that renders the cell membrane more fragile - and a classical high-pressure CO_2 [38].
Obviously, pressure, temperature and treatment duration are the basic parameters controlling the survival rate as shown by many results. But, it seems also clear that the matrix plays an important role [16], as exemplified by the surprisingly results obtained by Wei et al. [12] on different food ingredients spiked with bacteria, especially the completely different treatment efficacy on the whole egg and the egg yolk only. No doubt that the presence of water increases the bactericidal effect of CO_2 [8,27], probably in relation with the resulting pH effect, as it was also shown on enzymatic reactions in supercritical CO_2 demonstrating the irreversible effect of moisture excess on enzymes [14]. Moreover, the pH of the treated stuff has a significant influence on the survival rate, an acidic pH favouring inactivation [15,28].
As spores are known to be highly resistant to heat, drying, radiation, and chemical agents, it is not surprising that inactivation by a sub-/super-critical fluid is much more difficult than with bacteria and the few results [2,4,5,8,31,35,41,50] already disclosed show a dependence on the strain, a strong effect of temperature [31,41] and the high efficacy of the addition of a small concentration of hydrogen peroxide in CO_2 [50]. In fact, spores - including the most resistant ones - can be inactivated by combination of "mild" heating (<100°C) and CO_2 treatment while a sole heat treatment would require at least 120°C [41] and high-pressure CO_2 at 35°C has no or a very limited effect [8,31,43]. As for bacteria, combination of a pulse electric field pre-treatment followed by high-pressure CO_2 treatment is very efficient on spores although each one has no significant effect when operated alone [38]. The spore inactivation mechanism is not yet understood, although it seems to be caused by disruption/perforation of the outer layers of spore structure, especially in presence of H_2O_2 additive in CO_2 [50].
Regarding viruses, it also appears that the fluid interactions with the envelop seems to be the predominant cause of inactivation as the decay of enveloped viruses is much higher than the decay of non-enveloped ones [53-55].

Sterile filtration

In two patents [3,22], the material (active pharmaceutical ingredient) is dissolved into either the supercritical fluid (CO_2, N_2O or R13) or a liquid solvent, the solution is sterilised by filtration and the solid recovered by rapid depressurisation or by addition of a supercritical anti-solvent., but no specific sterilising effect of the supercritical fluid was claimed.

Carbon dioxide sterilisation

Most recent works refer to the specific properties of CO_2, possibly added with another agent (such as H_2O_2 or ethanol) to kill micro-organism. CO_2 is preferred due to its harmless character, low cost and great availability, However, in some cases, the acidity of CO_2 can cause an undesirable effect on the treated product, especially on pH-sensitive proteins leading to the use of N_2O that seems to have a similar lethal effect on most micro-organisms.
When the feed to be sterilized is a solid, the equipment is similar to those currently used in batch extraction plants. A continuous fluid flow is much more efficient than a static contact only, as mass transfer is a significant parameter [26]. Counter-current columns or membrane

units (such as POROCRIT ®) are the most effective systems for treatment of liquids and can be operated in continuous mode [23,24,29,32,33]. The main parameters to optimise are :

- ✓ Pressure and pressurisation/depressurisation cycles
- ✓ Temperature
- ✓ Exposure time
- ✓ Moisture content
- ✓ Possibly, addition of a co-agent such as an alcohol, hydrogen peroxide, etc.
- ✓ Possibly, use of a pulse electric field.

Applications

- *Pest control*

CO_2 pressure of 10 to 50 bar is enough to kill insect eggs, larva or beetles after exposure during 10 to 20 min; this strong effect may be connected with gas action as a respiratory analeptic [7]. A recent study [40] confirmed that the most common insects and their eggs present in rice can be completely eradicated by CO_2 at low pressure conditions (25 bar).

- *Bacteria and fungi*

We would firstly refer to the excellent review by Spilimbergo and Bertucco [35]. Although not exhaustive, Table 1 gathers the results published by many authors, classified by micro-organisms, with a summary of the processing conditions. Most works were completed with the micro-organisms selected for sterility validation belonging to the three biological types: Aerobic bacteria, anaerobic bacteria and fungi or with those widely present (such as *E. coli*) or presenting a very high resistance to sterilisation (such as spores of *B. stearothermophilus* and *B. subtilis*). If not otherwise specified, experiments were run with pure supercritical CO_2.

- *Virus inactivation :*

Although Stahl et al. [5,7] found a weak inactivation (~2 $\log_{10}$) of *Coliphage* by CO_2 in the range of 2,500 bar, the same virus was completely inactivated by CO_2 at 300 bar 50°C [23].
In the 90's, two groups disclosed attractive results on plasma fractions processing. The first one [52,53] used supercritical N_2O. Most viruses, exhibiting various degrees of resistance to classical treatments, were inactivated, the efficacy is higher on enveloped viruses than on non-enveloped ones, probably due to interaction with the envelop lipids; moreover, the decrease of the bio-activity of the plasmatic factors was limited. The second group [54,55] worked with several fluids (CO_2, N_2O, propane, fluorocarbons: R22, R23, R134a, R124) and compressed nitrogen. Most results were obtained on murine-C retrovirus (MnLV), as model of HIV, and some other ones on other viruses: Supercritical N_2O, R22 and R23 are claimed to be good inactivation fluids, although the efficacy also appears higher for enveloped viruses than for non-enveloped ones. But, curiously, ethanol-added N_2O and other fluorinated fluids look less efficient although they are good lipid solvents,. Moreover, the authors also claimed no significant alteration of bovine plasma composition during processing with the different fluids and no important loss of bio-activity of clotting factors after processing with N_2O.
Another group [56,57] developed a commercial process for preparation of bone allografts that were submitted to supercritical CO_2 processing for lipid extraction: the virus load was heavily reduced and remained below the detection level for any virus type.

- *Inactivation for immunogenic preparations*

A recent patent [44] claims the use of near-critical or supercritical carbon dioxide for inactivating whole micro-organisms in order to obtain an immunogenic preparation for vaccine manufacture as exemplified by inactivation of *Salmonella typhimurium*, by supercritical CO_2. As cell walls remain intact, a positive immunologic response is expected by the inventors although complementary results are needed to demonstrate it.

Table 1: List of micro-organisms processed by supercritical fluid

Micro-organisms	Fluid and Process	References
Alicyclobacillus acidoterrestris (spores)		**32**
Aspergillus niger	Pure CO_2 : (+ 2% ethanol) : (+ 0.5% acetic acid)	**8**
Bacillus atrophaeus (spores)	CO_2 + H_2O_2 (200ppm)	**50**
Bacillus cereus	Pressure cycling	**26,27**
Bacillus cereus (spores)	Pulse electric field pre-treatment	**38,41**
Bacillus coagulans (spores)		**41**
Bacillus licheniformis (spores)		**41**
Bacillus subtilis		**5,15,31,37,42,51**
Bacillus subtilis (endospores)	Static: Static:Cycling semi-continuous : CO_2 + acetic or peracetic acid : Pressure cycling	**8,31,36,41, 43,45**
Spores of Bacillus /cereus/ subtilis/megaterium/ polymyxa/coagulans/circulans/licheniformis/macerons	Micro-bubbles	**23,24**
Bacillus stearothermophilus (endospores)	CO_2: (+ 2% ethanol): (+ 0.5% acetic acid): (+TFA)	**8,43**
Geobacillus stearothermophilus (spores)		**41**
Candida utilisis		**20**
Candida albicans		**5**
Clostridium thermocellum	N_2, ethane, propane, CO_2	**34**
Clostridia 3679 (spores)	Irradiation	**2**
Enterococcus faecium	Semi-continuous process (liquid stuff)	**28**
Escherichia coli	Irradiation: Pressure cycling or not: Pulse electric field pre-treatment : CO_2 + water: CO_2 + propanol: CO_2 + triclosan	**2,5,8,15,16,23, 25, 26,27,28,32, 38,39,40,46,48**
Kloeckera apiculata	Semi-continuous process (liquid stuff)	**28**
Kluyveromyces fragilis		**20**
Lactobacillus casei	Semi-continuous process (liquid stuff)	**28**
Lactobacillus spp.	Semi-continuous process (liquid stuff)	**28**
Lactobacillus plantarum		**16,32,33**
Lactobacillus brevis		**24**
Leuconostoc dextranicum		**19**
Leuconostoc mesenteroides		**32**
Legionella dunifii	Pressure cycling	**26,27**
Listeria monocytogenes		**12,21**
Listeria innocua	Pressure cycling	**26,27**
***Penicillium* sp.**		**40**
Pichia awry 1272		**42**
Proteus vulgaris	Pressure cycling	**26,27**
Pseudomonas aeruginosa	Pressure cycling	**5,26,27**
Pseudomonas fluorescens		**15**
Saccharomyces cerevisiae		**8,20,28,32**
Saccharomyces cerevisiae (ascospores)		**32**
Salmonella	Irradiation	**2**
Salmonella typhimurium (food)		**12**
Salmonella salford	Pressure cycling	**26,27**
Staphylococcus aureus	Pressure cycling: Pulse electric field pre-treatment: CO_2 + H_2O_2	**8,15,25,26,27, 38,47,49**
Streptococcus spp.		**28**
Yeast (naturally present)		**30**

Conclusion

Supercritical fluid sterilisation look attractive as it avoids heat processing and irradiation that cannot be used in all cases. Many results – often conflicting - were recently published, and even if it is difficult to raise firm conclusions, I would try to summarise as follows:

- Liquid food pasteurisation is operative and near to be employed at commercial scale;

- Due to bacteria spore resistance to supercritical CO_2, the use of additives seems required to reach a complete sterilisation;
- Virus inactivation would need important further work to optimise the process and obtain acceptance as a safe alternative to present techniques;
- Supercritical fluid sterilisation of bio-medical items (like implants, prostheses or medical instruments) is of special interest to prevent nosocomial infections.

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