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High pressure carbon dioxide–a non-thermal food processing technique for inactivation of micro-organisms

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For more than 20 years now, high pressure carbon dioxide (HPCD) has been proposed and studied as an alternative non-thermal pasteurization technique for various foods. This method imparts several basic advantages, mainly because of the involvement of mild conditions. A majority of the observed advantages are particularly because it permits the processing of foods at much lower temperature than the ones used in thermal pasteurization. In spite of intensified research efforts for the last couple of years, the HPCD preservation technique has not yet been implemented on a large scale by the food industry until now. Many scientific studies mainly focusing the lethality of this technique on different microorganisms have been done over the years. Most of the research aimed towards optimization of the HPCD technique to produce a desired level of stabilization for specific foods but less effort has, however, been put into the analysis of the interaction mechanism between HPCD and the structure of food, its kinetics and the effect on microorganisms is yet to be observed.

Key words : High pressure carbon dioxide (HPCD), Non thermal pasteurization, Emerging technology, Dense phase carbon dioxide (DPCD)

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INTRODUCTION

Thermal pasteurization is a well-known and ancient technique for reducing the microbial load of various foods. Traditional thermal processing, however, can destroy heat sensitive nutrients and food product qualities such as texture, flavour and colour. Especially in case of heat sensitive food products, thermal pasteurization can impart undesirable organoleptic changes in addition to some detrimental effects to the nutritional quality of the food. Due to the increased consumer demand and awareness for fresh-like and nutritious food products with high and/or maximum organoleptic quality and an extended shelf life, non-thermal processing alternatives have been proposed.

Various non-thermal methodologies that have been studied for considerable development over the traditional thermal methods for treatment to preserve the food and maintain and improve their shelf-life are pulsed electric field (PEF), high pressure processing (HPP), osmotic dehydration, A-thermal membrane processes. high intensity pulsed light technology, non-thermal processing by radio frequency electric fields, ultrasound, irradiation, microwave heating, ohmic heating.

For almost two decades now, the use of high pressure carbon dioxide (HPCD) has also been proposed as an alternative non-thermal pasteurization technique for foods. The main goal of this treatment is the elimination of the spoilage and pathogen micro-organisms by a process which does not deteriorate the quality of food. HPCD is also known as dense phase carbon dioxide (DPCD) and has gained great interest in inactivation of microorganisms in liquid foods or liquid model solutions. This review presents the importance of HPCD technique for microbial inactivation, CO_2 bactericidal action, inactivation of microbial vegetative cells, bacterial spores and also the regulatory hurdles.

CO_2 as a medium :

CO_2 is a non-toxic gas with anti-microbial properties and it is an accepted ingredient in food and beverages. It is effective for extending the shelf life of perishable foods by retarding microbial growth. The overall effect of CO_2 is to increase both the lag phase and the generation time of spoilage of microorganisms and under pressure kills it bacteria, yeasts and molds. The effect was synergistic with raised temperature, acidic pH and antagonized by lowered water activity. The medium used in this high pressure HPCD technique is either

pressurized subcritical or supercritical CO_2. The medium is brought into contact with the food for a certain amount of time in a batch, semi-batch or continuous manner.

Supercritical CO_2 exists as a single phase at a temperature and pressure above its critical point values (T_c = 31.1°C, P_c = 7.38 MPa), and in this phase it has the unique ability to diffuse through solids like a gas, and dissolve materials like a liquid (Fig. 1). Additionally, it can readily change its density upon minor changes in temperature or pressure. This state of the medium results in excellent solvent power when used in HPCD pasteurization.

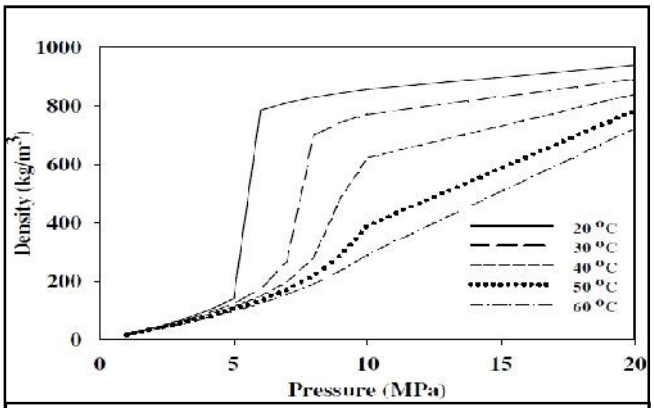

Fig. 1 : CO_2 density as a funtion of pressure and temperature

Subcritical (gaseous or liquid) CO_2 on the other hand, is CO_2 at a temperature or pressure below its thermo-dynamical critical point values. In the supercritical state, CO_2 has low viscosity (3-7 × 10^{-5} Ns/m^2) and zero surface tension (McHugh and Krukonis, 1993). Hence, it can quickly penetrate complex structures and porous materials. The most important property of carbon dioxide is the density which varies with temperature and pressure as shown in Fig 2. By changing the CO_2 density, the solvent power of the medium can be varied and thus can its capability of coming to contact with the liquid phase containing microorganisms be varied/altered.

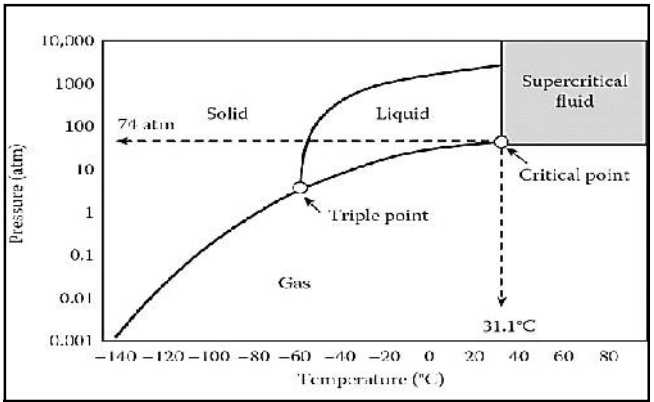

Fig. 2 : Phase diagram of CO_2

When gaseous or liquid CO_2 is heated and compressed above the critical temperature (31°C) and pressure (73 atm), it becomes a dense, highly compressible fluid that demonstrates properties of both liquid and gas. Hence, at relatively low pressure and temperatures, carbon dioxide transitions to a supercritical state. The liquid state of CO_2 conserves some of the solvent properties of the supercritical state, with low viscosity and high diffusion co-efficients, therefore, the term dense-fluid refers to both the supercritical and liquid states. The properties of supercritical CO_2 help them penetrate deeply in to the foods/substrates (Van der Velde and De Haan 1992; Ge and Yan, 2002).

Mechanism of HPCD :

The process involves a pressurization step with CO_2 for a set treatment time just to allow the penetration of the applied gas into the microbial cells, and a subsequent explosive decompression. This explosive decompression results from the rapid gas expansion within the cells microbial cells. Process temperature ranges from 20°C up to 60°C to avoid the thermal damage to processed foods while process pressure ranges between 7.0 MPa to 40.06 MPa. The temperature increase induced by the pressure built up is almost zero because of the relatively low pressure levels involved in the process. The treatment times can ranges from about 3 and 9 minutes to about 120 and 140 minutes depending on the HPCD equipment *i.e.* continuous, semi-continuous or batch type. It also depends on the kind of food that is being processed.

The different steps in this simplified and hypothetical inactivation mechanism can be summarized as listed below. These steps do not occur consecutively during the process but would take place simultaneously in a complex and inter-related manner :

- Solubilisation of pressurized CO_2 in the external liquid phase
- Cell membrane modification
- Intracellular pH decrease
- Key enzyme inactivation/cellular metabolism inhibition due to internal pH lowering
- Direct (inhibitory) effect of molecular CO_2 and HCO_3^- on metabolism
- Disordering of the intracellular electrolyte balance
- Removal of vital constituents from cells and cell membranes

Step 1: Solubilisation of pressurized CO_2 in the external liquid phase :

In foods with high water content, CO_2 (present in the reactor headspace) can dissolve in the water to form carbonic acid (H_2CO_3) which dissociates into bicarbonate, carbonate and hydrogen ionic species. Water in contact with pressurized CO_2 generally becomes acidic due to the formation and

dissociation of H_2CO_3, which liberates H^+ ions. This lowered extracellular pH may inhibit microbial growth (Valley and Rettger, 1927; Daniels *et al.*, 1985; Hutkins and Nannen, 1993). This may also diminish the microbial resistance to inactivation because of increased energy consumption to maintain pH homeostasis (Hong and Pyun, 1999).

Step 2: Cell membrane modification :

On approaching the surface of the microbial cell, aqueous CO_2 may diffuse into the membrane and may accumulate into its phospholipid inner layer (Isenschmid *et al.*, 1995). Spilimbergo (2002) found that CO_2 can be dissolved in the phospholipids of a model cell membrane at a very high extent. This accumulated amount of CO_2 in the lipid phase may then structurally and functionally disorder the cell membrane due to an order loss of the lipid chain which may increase fluidity of the membrane.

Step 3: Intracellular pH decrease :

Due to increased permeability, pressurized CO_2, may easily penetrate through the bacterial cell membrane and accumulate in the cytoplasmic interior of bacterial cells. There, the relative concentrations of both aqueous CO_2 and HCO_3^- 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 pH.

Step 4: Key enzyme inactivation/cellular metabolism inhibition due to pH (Cytoplasmic) lowering :

Enzymes, which make up most of the proteins in the cytosol, have maximal activity at the optimum pH, and their activity declines sharply on either side of the optimum. So lowering of the cytoplasmic pH might cause inhibition and/or inactivation of key enzymes essential for metabolic and regulating processes, such as glycolysis, amino acid and peptide transport, active transport of ions, and proton translocation (Hutkins and Nannen, 1993).

Hong and Pyun (2001) based on an intense investigation, showed that among the 13 enzymes identified in untreated cells, some significantly lost their activities, whereas others were only little affected by pressurized CO_2. This is because lowering of the pH induces precipitation of only those enzymes which have an acidic isoelectric pH, leaving the solubility of the enzymes with basic isoelectric points unaffected (Spilimbergo, 2002).

Step 5: Direct (Inhibitory) effect of molecular CO_2 and HCO_3^- on metabolism :

Jones and Greenfield (1982) elucidated the exact effects of HCO_3^- and dissolved (unhydrated) CO_2 on carboxylation and decarboxylation reactions. In these reactions, CO_2 fulfils 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 CO_2 in the dissolved (unhydrated) CO_2 form. Although it is clear that dissolved CO_2 can inhibit decarboxylation reactions, the inhibitory effects that CO_2 may have on the decarboxylase enzymes is, however, not clear and could be due either to product inhibition by CO_2 or to an equilibrium-based mass action effect (Jones and Greenfield, 1982).

Step 6: Disordering of the intracellular electrolyte balance:

Lethal damage to the biological system of the cells may also be produced when the applied CO_2 pressure accumulates in the cytoplasmic interior of the bacterial cells. This may convert HCO_3^- to CO_3^{2-}, which could precipitate intracellular inorganic electrolytes from cells and cell membranes (Lin *et al.*, 1993). Since these inorganic electrolytes aid in maintaining the osmotic relationships between cells and their surrounding media, this could have deleterious effects on the volume of cells.

Step 7: Removal of vital constituents from cells and cell membranes :

Pressurized CO_2 first penetrates into the cells to build up the density to a critical level within the cells after which it removes intracellular constituents to disturb or alter the structure of the bio-membrane and/or the balance of the biological system, thus promoting inactivation. This removal process appeared to be stimulated by a sudden release of the applied pressure, leading to a rapid transfer of intracellular environment (Lin *et al.*, 1992a; 1993). The inactivation rate could be improved by repeating the release and recharge of pressurized CO_2 in the pressure vessel during the treatment to improve transfer of intracellular materials out of the bacterial cells.

Systems :

Various systems for HPCD or DPCD are mentioned below:

Batch system :

In this system the sample is placed into the pressure vessel and temperature is set to the desired value. Then, CO_2 is introduced into the vessel until the sample is saturated at the desired pressure and temperature. The sample is left in the vessel for a period of time and then CO_2 outlet valve is opened to release the gas. Some systems contain an agitator to decrease the time to saturate the sample with CO_2.

Continuous micro-bubble system :

In this system, CO_2 and saline solution are pumped

through a vessel. An evaporator is used to convert CO_2 (liquid) to gas and is then dispersed into the saline solution from a stainless steel mesh filter with 10µm pore size. The micro-bubbles move upwards while dissolving into the solution. Then, the solution saturated with CO_2 is passed through a heater to reach the desired temperature and a suspension of microorganisms is pumped into it. Another coil with a heater was used to adjust the residence time.

Dense phase CO_2 pilot plant unit :

This flow-through system uses a diaphragm pump to feed the juice from an inlet sample tank through a holding tube where the juice meets and statically mixed with an inlet line of pressurized CO_2.

A bottom siphoned liquid CO_2 from the pressurized tank is fed through at regulated pressures at or above 4.1 MPa. The flow rate is measured with a CO_2 flow-meter. The concentration of CO_2 expressed as grams of CO_2 kg of juice is set by adjusting the CO_2 flow rate relative to the juice flow rate. Juice mixed with CO_2 then enters a holding tube that is pressurized to pre-determined levels. An air-driven pump moves the juice through the treatment reactor (holding tube). At the exit of the reactor, the treated sample passes through two successive temperature-controlled pressure-release areas that serve to prevent the product from freezing upon pressure drop and allowed for evolution of CO_2 as gas from the product. As the treated sample is collected in a flask where the CO_2 is released to atmosphere in gaseous state, it takes about 5 minutes between the entry and its exit.

Effects of HPCD treatment :

Gram +ve and Gram –ve bacteria have both been subjected to HPCD treatment and the effects have been studied. It has been a general observation that Gram +ve bacteria have been more difficult to deactivate as compared to the Gram –ve bacteria owing to their thick peptidoglycan layers. Although there is a difference in sensitivity between the Gram +ve and the Gram –ve bacteria, both are susceptible to HPCD this treatment.

Effect on vegetative cells of bacteria, yeast and molds :

Effect of pressure and temperature :

Generally, an increase in the pressure results in a proportionate increase in the microbial inactivation. As a consequence, at higher pressures, a shorter exposure is needed to inactivate the same level of microbial cells (Lin *et al*., 1993, 1994; Hong *et al*., 1997; Hong and Pyun, 1999). Pressure is the parameter which controls both the solubilisation rate of CO_2 and its total solubility in a suspending medium. Therefore, higher the pressure more is the CO_2 solubilisation rate and this facilitates both acidifications of the external medium as well as its contact with the cells. This stimulating effect of CO_2 pressure however, does not go on indefinitely and is limited by the saturation solubility of CO_2 in the suspending medium. Spilimbergo (2002) proved that above 10MPa pressure, the solubility of CO_2 is a weak function of pressure. Table 1 showed the effect of pressurized CO_2 for microbial inactivation.

The inactivation rate increases with increasing temperature. Higher temperatures stimulate the diffusivity of CO_2 and can also increase the fluidity of the cell membrane

Table 1: Effect of various CO_2 processing conditions on microbial inactivation (Gracia-Gonzalez *et al*., 2007)

Target microorganism	Solution	Process conditions		
		Pressure (MPa)	Temperature (^OC)	Time (min)
Escherichia coli	Synthetic medium	3.5	37-38	3
	Nutrient broth	6.2		120
	Hydrophilic filter paper disks	5	Room temp	200
	Growth medium	20.5	42	20
	Distilled water	6.18	35	120
Listeria monocytogenes	Chicken meat	13.7	35	120
	Shrimp	13.7	35	120
	Orange juice	13.7	35	120
Salmonella typhimurium	Chicken meat	13.7	35	120
Saccharomyces cerevisiae	Growth medium	6.9	25	45
	Distilled water	4	40	180
Leuconostoc dextranicum	Distilled water	4	40	240
Enterococcus faecalis	Growth medium	6.9	35	20
Lactobacillus plantarum	Hydrophilic filter paper disks	5	Room temp	200
	Acetate buffer (0.1 M)	6.9	30	60

to make penetration easier (Lin *et al.,* 1993; Hong *et al.,* 1997; Hong and Pyun, 1999). This stimulates the modification of cell membrane (Step 2) and in some cases, the phenomenon of removal of vital constituents from the cell and cell membrane (Step 7). However, above the critical temperature of the CO_2, its solubilization capacity decreases quite rapidly as the temperature increases. Too high temperatures could also prove to be cause of certain deteriorative effects in the food quality in many applications (Lin *et al.,* 1993, 1994; Hong *et al.,* 1997; Hong and Pyun, 1999). Fig. 3 showed the process of lethal action of pressurized CO_2 on bacteria.

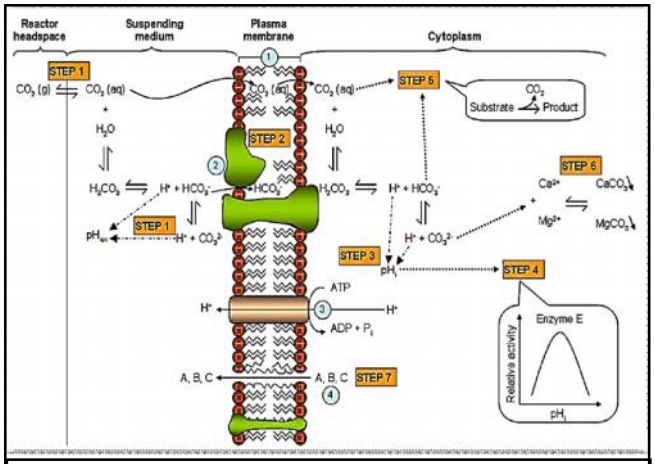

Fig. 3: Schematic diagram of how pressurized CO_2 may exert its lethal action on bacteria (Gracia-gonzalez *et al.*, 2007)

Effect of the physical state of CO_2 :

Studies have shown that supercritical CO_2 is more effective in inactivating microorganisms than CO_2 under subcritical conditions. The enhanced microbial lethality of supercritical CO_2 could be attributed to its interesting physic-chemical properties, which are in between those of liquids and gases. Supercritical CO_2 exhibits a more liquid-like density while mass transport properties are closer to that of gas. The liquid-like density allows a higher solvating power compared to the gaseous state. On the other hand, the gas-like mass transport properties enhance the diffusion rate when compared with the liquid state. The use of supercritical CO_2 as an inactivation medium would thus influence steps 2 and 7 considerably resulting in an increased disruption of biological systems.

Effect of agitation :

Agitation can enhance the solubilization of CO_2 and its contact with the bacterial cells. This would thus make cellular penetration of the medium much easier and more effective. Thus, appropriate agitation generally results in an improved microbial inactivation by HPCD. Hong *et al.* (1997) found that microbial inactivation depended on the sample size in absence of agitation.

Effect of water content :

Microbial inactivation strongly depends on the water content (or the water activity, a_w) of the medium in which the cells are suspended during HPCD treatment. Studies have shown that microbial inactivation increases with decreasing water content. Sterilization kinetics is strongly affected by the addition of water.

The reason why wet microbial cells are more prone to HPCD inactivation is probably the direct result of an increased CO_2 solubility which liberates more H^+ ions that subsequently reduce the pH of the suspending medium to lower values. In addition, swollen cell walls and membranes, because of the presence of water, become more penetrable by CO_2 and hence positively affect cell membrane modification.

Susceptibility of different microorganisms :

Microbial sensitivity to HPCD treatment varies greatly among species. However, comparisons are difficult to make because they are hampered by the different equipment, treatment media, strains, test conditions etc. In general, Gram +ve bacteria are more resistant than Gram –ve due to the composition of their thicker cell wall which thus has a negative effect on the cell wall modification step (Step 2).

Effect of initial bacterial concentration on the efficacy of HPCD :

The effectiveness of HPCD treatments is strongly affected by the initial bacterial load. Under the same conditions, the highest degree of inactivation is obtained with the lowest initial bacterial concentration. As a result, at higher initial bacterial concentrations, longer exposure times are needed to achieve the same log reduction.

Effect of the physical and chemical properties of the suspending medium :

The microbial inactivation rate is strongly affected by the constituents of the suspending media and nature of foods during HPCD treatment. The rate decreases considerably in case of complex physicochemical environments of food systems as compared to the simple solutions.

Lin *et al.* (1994) attributed the increased resistance in complex media to the lipid and fat components of the media. They proposed that the presence of fat in growth and suspending media probably led to a decreased CO_2 penetration into cells by changing the structure of cell walls and membranes. This has a negative effect on the cell membrane modification process.

Effect of initial environment pH of suspending medium :

The initial environmental pH of the cell suspension greatly affects the inactivation rate of HPCD treatments.

Various intensive studies by Haas *et al.* (1989), Hong and Pyun (1999) and Hong and Park (1999) have shown that there is an enhanced microbial inactivation with a lower initial pH of the cell suspension. The lowered pH apparently contributed to an increase in cell permeability to facilitate the penetration of CO_2 into cells.

Effect of culturing conditions and stage of cell growth :

In general, stationary phase cells are more heat and pressure resistant than log phase cells (Adams and Moss, 1995; Mackey *et al.,* 1995). Higher inactivation rates were observed when *L. plantarum* cells of the late log phase were subjected to HPCD treatments at 30°C and 6.9 MPa as compared to those of the stationary phase. Also, a higher resistance of *L. plantarum* cells to inactivation was observed when they are cultivated at temperatures lower that their optimal temperatures.

Effect of additives :

All studies conducted so far have shown that the inactivation rate increases when an additive (other than ethanol) is used. However, though the addition of a co-solvent can turn to be beneficial from a microbial point of view, the HPCD treatment carried out in such a manner can no longer be regarded as solvent free any longer thus also losing its sustainability.

Inactivation of bacterial spores :

A spore is the highly resistant dormant form of various *Bacillus / Clostridium* species. Sporulation of vegetative cells occurs under harsh environments such as poor nutrition. Spores are highly resistant to heat, UV radiation, free radicals, and chemicals because of their unique structures. They are highly dehydrated (only 10-25% water content), making it very resistant to heat and chemicals. Spores have not been studied extensively in the presence of HPCD. There have been few studies conducted on the reaction of spores during HPCD treatment. Spores are highly resistant to this treatment. Vegetative *Geobacillus stearothermophillus* cells were shown to reduce by more than 6 log cycles after 1.5 hour exposure (CO_2 at 2.75 MPa and 25°C), however, on the other hand, even with 2 hour exposure (20 MPa and 35°C, 80%) *G. stearothermophillus* spores remained viable.

Long treatment time and high temperatures are two potential problems of the HPCD sterilization technique. Even though a high degree of deactivation of spores has been realized, this usually required more than 10 hours, which is not competitive with the average time of 10 – 15 minutes for steam sterilization. Additionally the high temperatures used *i.e.* 55–90°C can easily damage heat sensitive materials in the product. Pressure cycling is a promising method to improve deactivation while lowering the temperature and time requirements. With pressure cycling of 30 cycles/hour with $\triangle P$ = 8 MPa and 36°C for 30 minutes, a 3.5 log reduction of *B. subtilis* spores was achieved. Without pressure cycling, a treatment at 36°C, 7.5 MPa for 24 hour only resulted in 0.5 log reductions (Spilimbergo 2002).

Inactivation of enzymes :

Carbon dioxide under pressure can inactivate certain enzymes at temperatures where thermal inactivation is not effective (Balaban *et al.*, 2001). Some of the enzymes which can be inactivated using this technique are pectin-esterase (causes cloud loss in some fruit juices), polyphenol oxidase (undesirable browning in fruits, vegetables, juices and sea-foods), peroxidase (important role in discolouration of foods) and lypoxygenase (chlorophyll destruction and off-flavour development in frozen vegetables). CO_2 under pressure can change isoelectric profiles and protein patterns of polyphenol oxidases while CO_2 at atmospheric pressure does not affect these properties (Chen *et al.*, 1992). High pressure is also reported to cause conformational changes in protein and enzyme molecules (Suzuki and Taniguchi, 1972). The extent of enzyme inactivation by CO_2 under pressure is affected by the type and source of the enzyme, treatment conditions such as pressure, temperature, time and treatment medium properties.

Regulatory issues :

The commercialization of foods manufactured by this novel technique appears to result in two different attitudes in regard to regulation either within the European Union (EU) or outside the EU. Under the EU, the national regulations for new products and new technologies have been replaced by the "Novel Food Regulation (NFR)". The NFR is a community regulation for novel foods and ingredients (EC No. 258/97) which is in force since 1997. Novel foods, under the EU, are those food and food ingredients that have not been used for human consumption to a significant degree within the community before May 15, 1997. The NFR legislation principally addresses food safety concerns in the context of:

– Food and food ingredients with a new molecular structure;
– Those consisting of or isolated from microorganisms, plants or animals;
– Those derived from novel production processes.

The HPCD pasteurized foods probably must be regarded under the novel foods category since they have no history of human consumption in the community so far, and have been produced by a new manufacturing process. Before a novel food can be placed on the EU market shelves, applications must be submitted in concern with scientific information and

the safety assessment report for HPCD.

The future gaps :

Within the food industry itself, there now is an increasing emphasis as well as trend toward natural food preservation technologies in response to growing consumer demand and awareness for "greener" additives and inclusions. During the last two decades, several natural non-thermal technologies have been studied worldwide for practical applications. These methodologies have shown inactivation of microbes as well as enzymes without significant adverse effects on organoleptic and nutritive properties of the products. The use of HPCD/DPCD may offer unique advantages for food preservation with minimal or desirable effects on the rheological properties of food.

In some years, HPCD treatment could become one of the most available emergent technologies. However, to meet this high expectation, consumers and stakeholders must be convinced about the improvements this new technology. This will require convincing data, and provision of clear, objective and unbiased information also including the potentially negative aspects of the technology and their limitations. The people involved in the investigation of the technique need to be ready to face criticism at each and every level and be able to explain the benefits of the technique along with proper and valid scientific proof to support their claims.

Also, for being able to replace other preservation techniques, HPCD treatment must not only improve food quality, but also promote shelf life and long-term safety by inactivating spoilage and pathogenic microorganisms. Therefore, further research is essential to demonstrate and explain the effect of HPCD preservation on the shelf-life and safety of food products. In addition, it is important that the effect of a HPCD treatment on the sensory and nutritional quality of both liquid and solid foods is more thoroughly investigated. Also the economics of the process must be assessed.

Conclusion :

High pressure carbon dioxide treatment has received much attention in the past decade in the field of stabilization of various foods. The industrial applications for this method, however, are not yet fully established. This is one difficulty that is being faced for the time being. Another major concern that the industry might face is the occurrence of vegetative bacteria, which become resistant to inactivation after several rounds of the treatment. Till date, there has been no evident information or material to support this hypothesis though, but the possibilities do exist and this might prove to be a very big hurdle in commercialization of the novel technique for microbial inactivation and increasing the stability of the food products.

All the researches and studies have shown that the microbial inactivation is governed most importantly by the penetration of CO_2 into the cells. The effectiveness of the treatment procedure improves by the enhanced transfer rate. Studies worldwide have also shown that temperature and pressure when applied simultaneously work synergistically to strongly enhance the lethal effects of the process.

The effectiveness of the treatment is influenced by several parameters like pressure, temperature, the physical state of CO_2, agitation, water activity (a_w), the properties of the suspending medium, the stage of cell growth being experienced by the microorganisms, the pH of the environment, additives etc.

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