INACTIVATION OF FOOD MICROORGANISMS BY PRESSURIZED CARBON DIOXIDE

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ABSTRACT

This work discusses the influence of several operational parameters on the lethal effect of CO_2 over *Lactococcus lactis*. Inactivation rate increased with exposure time. The raise in pressure and temperature exerted a positive effect on the inactivation of this microorganism. However, the application of N_2 at same conditions did not cause any impact. The combination of high-pressure N_2 and reduced external pH simulating the acidity achieved with dissolved CO_2 was not efficient either, as demonstrated on a typical air contaminant bacillus. These findings indicated that the lethal effect of CO_2 should be attributed to other causes rather than reduced external pH or hydrostatic pressure. Moreover, the CO_2 treatment modified the membrane as stated. Therefore, it seems that the efficiency of the CO_2 could be related to the solubilization of the membrane components by the CO_2 which is favored by the use of high pressure. Membrane fluidity could be also enhanced by the increase in temperature and the low medium pH, favoring the CO_2 attack.

INTRODUCTION

The use of pressurized CO_2 as sterilization agent has awakened high interest in the last years for the conservation of thermolabile products [1,2,3]. These investigations have demonstrated that the method is efficient to kill microorganisms such as bacteria, yeast and fungi which cause their spoilage while preserving their freshness. However, most of the investigation has been conducted using suspensions of pure cultures.

In general, the lethal effect of CO_2 is higher as pressure is raised, although most investigations have used pressures less than 200 bar. Inactivation rate is also increased with temperature; however explored values were usually less than 60ºC. Treatment time widely ranges from minutes to few hours. Dry cells are unlikely to be sterilized [1,4]. A variable amount of water is normally required. The addition of small amounts of disinfecting agents is also very useful for the completely destruction of some microorganisms [4,5].

In case of spore cells, the contact time has to be longer and coupled with higher temperatures due to the compact and difficult-to-penetrate structure of these resistant species [6,7]. An effective way to increase the inactivation rate of spores has been the application of pressurization and depressurization cycles [8].

A very important parameter is the contact mode between the CO_2 and the product to be sterilized. Most of the patents related to this technology [9,10] deals with this aspect because the conditions need to be less severe if CO_2 is fed continuously and well dispersed. However, the majority of the investigations up to now have been conducted in batch mode.

The mechanism of CO_2 actuation is not completely clear. It could be related to the decrease of the pH medium and the use of high pressure. One of the objectives of this work is to investigate the separate influence of hydrostatic pressure, external pH, and the combined

effect of both parameters on the inactivation rate of two microorganisms: an air pollutant common bacteria and *Lactococcus lactis*. This latter anaerobic lactic acid bacteria is frequently used in food industry to produce fermented products such as cheese, yogurt, butter, etc. Other parameters to be explored are the temperature and the agitation speed. The cell membrane was analyzed after the treatment to support the proposed inactivation mechanism.

MATERIALS AND METHODOLOGY

Materials. Pressurized liquid carbon dioxide at its vapor pressure (about 5 MPa), 99.998% purity, was supplied by Air Liquide. The Spanish Type Culture Collection (CECT) provided *Lactococcus lactis* ssp. l*actis,* (CECT 185, ATCC 9936). An air common bacteria was utilized to investigate the effect of external pH. Both microorganisms were cultivated in YGLPB broth at 37ºC for 24 h. The culture was diluted in fresh broth to provide a final culture concentration around 0.7 g/l containing about 10^8 colony forming units (CFU)/ml.

Apparatus. The apparatus consisted of a CO_2 feeding line, a high-pressure sterilizer and a sample collection system. The CO_2 was cooled to -15ºC in a thermostatic bath and pumped with a head-cooled membrane pump (Milroyal D, Dosapro Milton Roy). A 316 ss batch vessel of 100 ml-capacity (Autoclave engineers, Eze-seal model) was used as sterilizer. It was equipped with agitation, CO_2 and broth inlet and sampling outlet connections and a rupture disk set at 30 MPa. The temperature was controlled ± 2ºC by a heating jacket and was recorded by a thermocouple placed inside the reactor. Pressure was read in a manometer. The sample collection system was formed by a deep tube connected to a valve that permitted the extraction of culture broth aliquots at different times.

Experimental procedure. The culture broth was fed to the vessel using 50 ml sterilized syringes through a valve. A Bunsen burner was placed around to provide a sterile atmosphere. The heating jacket was connected and when the temperature was achieved, the CO_2 was fed in until reaching the desired pressure. Agitation was fixed at 400 rpm. Samples were collected at different times and kept refrigerated prior their analysis. After two hour run, stirring was stopped and CO_2 was slowly released. After each run, the vessel was filled with water and heated up to 120ºC for 20 min. to sterilize it. The water was expelled by injecting CO_2 at high pressure. By this way the equipment was kept aseptic for next run.

Determination of inactivation efficiency. To assess the inactivation efficiency of the procedure, the survival ratio was determined by a plate count agar (PCA) in samples obtained at 1 and 2 hour exposure time. The samples were serially diluted with saline solution of NaCl 9 g/l and then plated on the PCA medium to enumerate the count after incubating the plates at 37ºC for two days. The survival ratio is expressed as the mean viable count (N) for the experimental group treated with the high-pressure CO_2 at a fixed time (N_t) to the control group incubated at the same temperature for the same period (N_o). The results are presented as $\log_{10}(N_o/N_t)$. The evolution of the method efficiency with time was followed measuring the ATP in the living cells. The determination was conducted using an ATP commercial kit (ATP kit HS, Biothema) that contains the enzyme luciferase which catalyses the conversion of D-luciferin to oxiluciferin liberating CO_2 and light, consuming the ATP released from the cell. The intensity of the light was proportional to the ATP content and was determined in a luminometer (Optocomp I, MGM Instruments). Data are represented in figures as the percentage ratio between the ATP content after the CO_2 treatment (ATP) and the initial one $(ATP)_o$. It was not possible to correlate both procedures but the ATP method was very quick and permitted to observe the comparison of the effect of different experimental conditions.

RESULTS AND DISCUSSION

The following section discusses the effect of the main operating parameters affecting the high pressure CO_2 treatment on *Lactococcus lactis* that are pressure, temperature, pH and exposure time. These factors affect the environment of the microorganism. Pressure and temperature also influence the solubility and mass transport properties of the CO_2. Thus, their impact is separately examined on the efficiency of the method. The results in terms of viable cell reduction are presented in Table 1. The ATP data are plotted and discussed in the following sections. Based on the findings and observations made on the cell membrane, the mechanism of CO_2 actuation is analyzed.

Table 1. Effect of different operating conditions on the decrease of *L. lactis* viable cells.

Pressure (MPa)	Temperature (ºC)	Agitation speed (rpm)	Gas type	Log (N_o/N_{60})	Log (N_o/N_{120})
		Effect of pressure			
6.8	20	400	CO_2	0.1	0.3
10.3	20	400	CO_2	0.8	1.9
20.5	20	400	CO_2	1.2	2.1
		Effect of temperature			
10.3	20	400	CO_2	0.8	1.9
10.3	45	400	CO_2	1.2	2.0
20.5	20	400	CO_2	1.2	2.1
20.5	60	400	CO_2	1.7	2.6
		Effect of agitation speed			
10.3	45	400	CO_2	1.2	2.0
10.3	45	700	CO_2	0.8	2.0
		Effect of type of gas			
10.3	45	400	CO_2	1.2	2.0
10.3	45	400	N_2	0.0	0.0

Effect of pressure and exposure time

Table 1 shows the inactivation of *Lactococcus lactis* as function of pressure at 20ºC. The increase in pressure exerted a positive effect on the inactivation effect of CO_2. Additionally, the survival ratio decreased with exposure time. At 6.8 MPa, the reduction on living cells was not significant even after two hour exposure time. At 20.5 MPa, half time was sufficient to inactivate more than 1 log cycles of cells. Pressure controls CO_2 diffusion and solubility in the suspension. Consequently, higher pressure enhanced CO_2 contact with the cell and improved microorganism death. Likewise, increased exposure time favored the CO_2 attack and promoted *Lactococcus lactis* inactivation. These results are similar to those achieved for other microorganisms, including some of same type, e.g. *Lactobacillus* sp. [11].

The impact of pressure itself was evaluated comparing the results with the application of an inert-not-soluble gas (N_2) at equal conditions. The treatment with N_2 did not cause any effect as shown in Figure 1a. Other authors found identical result using same gas [2,12,13] or tetrafluorethane whose critical properties are similar to those of CO_2 [2]. Lin *et al*.,1993 tried with hydrostatic pressure in the inactivation of *Saccharomyces cerevisiae*, finding that the lethal effect was null even after a series of decompressions and successive recompressions [13].

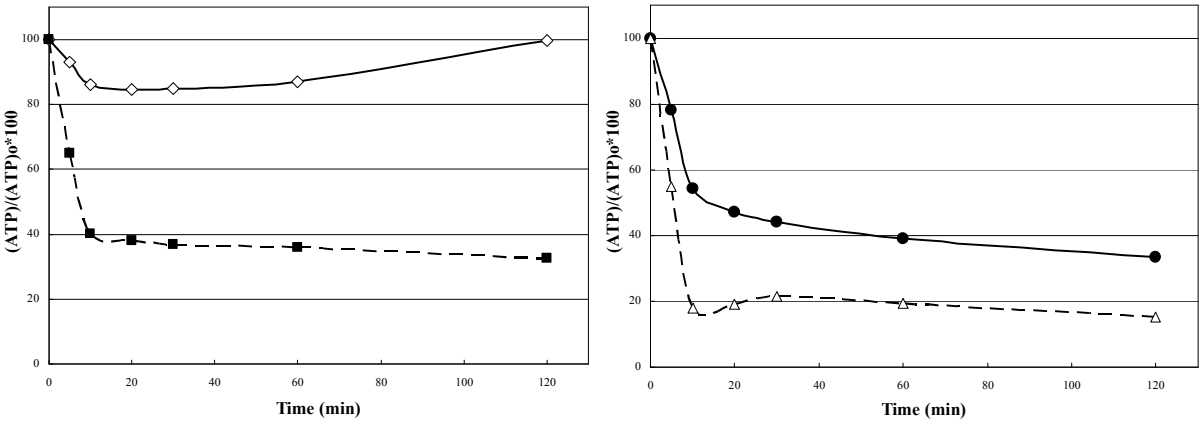

Figure 1. The influence of a) type of gas and b) temperature on the *L. lactis* living cells.

Increased agitation speed was also analyzed since pressure and treatment time can be reduced if CO_2 and membrane contact is facilitated. For example, Bertucco *et al*., 2001 compared the effectiveness of the batch versus the semicontinuous operation in the *S. cerivisiae* inactivation degree. In the semicontinuous process, a total sterilization was obtained with an operating pressure of 74 bar and exposure time of 10 minutes, while in the batch process, 60 minutes were needed with an operating pressure of 200 bars [14]. In the present work, batch mode was utilized. The only way to improve CO_2 mass transport from the gas to the liquid phase was to increase stirring speed that was augmented from 400 rpm to 700 rpm. The inactivation efficiency was not appreciably improved though (see Table 1).

Effect of temperature

For a method that pretends to be an alternative to conventional heat processes to sterilize thermolabile products, temperature should not be high. Accordingly, the investigation was conducted between 20ºC and 60ºC at 20 MPa. As expected, the lethal effect of CO_2 increased as temperature was raised (see Figure 1 b) because of the harmful effect that temperature has on biological activity. Additionally, the CO_2 diffusivity was improved favoring CO_2-membrane contact. The impact of temperature for this specific microorganism was not very significant though, since after two hour treatment at 20.5 MPa and 20ºC, the reduction on activity was 2.1 log cycles while at 60ºC, it only increased to 2.6.

Effect of external pH

It is well known that dissolved CO_2 forms carbonic acid which further reduces medium pH. *Lactoccocus lactis* is a lactic acid bacteria so its natural environment is quite acid. In fact, the pH of the suspension was usually less than 4. So the impact of external pH was evaluated in a suspension of a typical air contaminant bacillus. After CO_2 treatment, samples pH was about 5.5. To evaluate the effect of reduced pH, an organic (acetic acid) and an inorganic (phosphoric acid) were added to the initial suspension until the acidity of the medium was equivalent to the one measured in the CO_2 treated samples. Then, the solution was maintained at same temperature (40ºC) and same exposure time at ambient pressure than the treated samples with CO_2. The results are compared in Figure 2. The microorganism was not affected by the reduction in pH in the acidified solutions. It was even able to grow. Therefore, the mortal efficiency of CO_2 is not due to the decrease in the medium acidity; even if it may help to facilitate the CO_2 attack to the membrane cell [15].

To prove if CO_2 effect was due to a combination of high pressure application and reduced environment pH, another experiment was conducted applying N_2 at same pressure than CO_2 in an acidified suspension with phosphoric acid. The results are also represented in Figure 2. The use of high pressurized N_2 and reduced external pH was not efficient either, indicating that the lethal effect of CO_2 should be attributed to other causes.

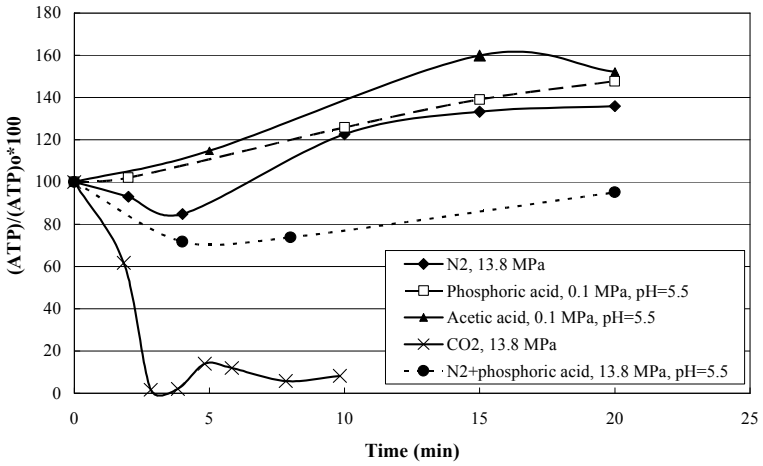

Figure 2. Effect of pressure, external pH and the combination of both factors on the inactivation rate of a typical bacillus.

Effect of CO_2 treatment on cell membrane

The aspect of the membrane cell after the CO_2 treatment was observed by scanning electron microscopy (SEM). Figure 3 shows the comparison of the *L. lactis* membrane cell before and after CO_2 treatment. The surface membrane rugosities of the treated samples indicated morphological changes that could not be related to mechanical rupture induced by the high pressure, since these modifications were not observed in the N_2 treated samples (image not shown). Bothun *et al*., 2003 also observed that *C. thermocellum* membrane lost fluidity as CO_2 density was increased [16]. In similar fashion, Ballestra *et al*., 1996 detected that CO_2 contact deformed *E. coli* cell walls allowing cytoplasm leakage [17]. Similar modification was found on the microstructure of *Lactobacillus plantarum* [15].

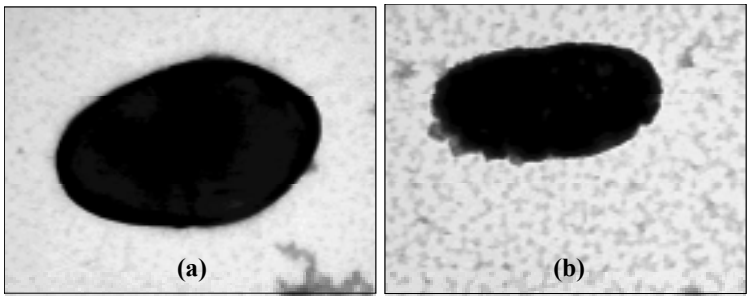

Figure 3. SEM images of a) untreated and b) CO_2 treated *L. lactis* cells at 10.3 MPa and 40ºC for 20 min.

The CO_2 at high pressure could dissolve the vital constituents of the membrane cell such as phospholipids [18] due to its high solvation capacity for this kind of compounds. And viceversa, the amount of CO_2 that can be dissolved in the lipidic membrane can be also very high [8] confirming the high potential of the CO_2 to destroy the membrane cells.

CONCLUSIONS

The use of supercritical CO_2 seems to be an efficient medium to inactivate microorganisms of different nature. This work demonstrated the effectiveness in the inactivation of *Lactococcuss lactis*. Total sterilization was not possible within the explored interval of operating conditions, but, based on previous results in other microorganisms, the inactivation rate could be enhanced passing CO_2 continuously and using a better mode of CO_2 dispersion since the method efficiency is highly dependent on the close contact between CO_2 and the membrane. The lethal effect of the supercritical CO_2 could be attributed to the cell membrane damage originated by the solubilization of the membrane components in the CO_2. This reason could explain why other gases applied at same operating conditions in a reduced pH medium can not cause microorganisms death. Some authors have hypothesized that another motive for cell death could be the drop of the intracellular pH [11]. In particular, the CO_2 could diffuse through the membrane and accumulate inside the cell, exceeding the buffer capacity of the cytoplasmatic pool and reducing the internal pH which would further cause the microorganism death. In any case, the process would be benefited at high pressures because the CO_2 diffusion coefficient in the liquid medium and its solubility are higher. High pressure may also help penetration of the CO_2 into de cell membrane and solubilization of the vital cell constituents. Similarly, elevated temperature would diminish diffusion coefficient and promote membrane fluidity which seems to be also favoured by low external pH [19].

REFERENCES

[1] HAAS, G.J., PRESCOTT, H.E., DUDLEY, E., DIK, R., HINTLIAN, C., KEANE, L., J. Food Safety, Vol. 9, **1989**, 253.

[2] DILLOW, A., DEHGHANI, F., HRKACH, J., FOSTER, N., LANGER, R., Proc. Natl. Acad. Sci. USA, Vol. 96, **1999**, 10344.

[3] ERKMEN, O. , Food Microbiol., Vol. 17, **2000**, 589.

[4] KAMIMURA, M., TANIGUCHI, M., KOBAYASHI, T., Agric. Biol. Chem., Vol. 51 (2), **1987**, 407.

[5] BACH, E., CINQUEMANI, C., SCHOLLMEYER, E., International Symposium on Supercritical Fluids, Orlando, Florida, USA, **2005**, CD Rom.

[6] ENOMOTO, A., NAKAMURA, K., HAKODA, M., AMAYA, N., J. Fermen. Bioeng., Vol. 83, **1997**, 305.

[7] BOTHUN, G.D., KNUTSON, B.L., STROBEL, H.J., NOKES, S.E., Proceedings of the Sixth International Symposium on Supercritical Fluids, Vol.2, **2003**, 1461.

[8] SPILIMBERGO, S., ELVASSORE, N., BERTUCCO, A. J., Supercritic. Fluid., Vol. 22, **2002**, 55.

[9] MARC, S., US patent 6331272, **2001.**

[10] YUTAKA, O., MITUYA, S., TAMOTE, K., KUNIHIKO, O., European patent 0786513, **1997**.

[11] HONG, S.I., PARK, W.S., PYUN, Y.R., Lebensm.-Wiss. H.-Technol., Vol. 30, **1997**, 681.

[12] ENOMOTO, A., NAKAMURA, K., NAGAI, K., HASHIMOTO, T., HAKODA, M., Biosci. Biotechnol. Biochem., Vol. 61 (7), **1997**, 1133.

[13] LIN, H., YANG, Z., CHEN, L. F., Biotechnol. Prog., Vol. 8, **1992**, 458.

[14] BERTUCCO, A., SPILIMBERGO, S., in: A. Bertucco, G. Vetter (Eds.), High pressure process technology: fundamentals and applications, Elsevier Science, Amsterdam, **2001**, 626.

[15] HONG, S.I., PYUN, Y.R., J. Food. Sci., Vol. 64, **1999**, 728.

[16] BOTHUN, G.D., KNUTSON, B.L., STROBEL, H.J., NOKES, S.E., Proceedings of the Sixth International Symposium on Supercritical Fluids, Vol.2, **2003**, 1461.

[17] BALLESTRA, P., SILVA, A.A.D., CUQ, J.L., J. Food Sci., Vol. 61 (836), **1996**, 829.

[18] LIN, H., YANG, Z., CHEN, L.F., Chem. Eng. J. Vol. 52, **1993**, B29.

[19] ERKMEN, O., Food Microbiol., Vol. 17, **2000**, 225.