INACTIVATION OF *Alicyclobacillus acidoterrestris* SPORES BY HIGH PRESSURE CO_2 IN APPLE CREAM

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ABSTRACT

Alicyclobacillus acidoterrestris (A.acidoterrestris) is a bacillus-type spore that causes significant alterations in fruit products. It is highly resistant to typical pasteurization regimes; thus, this work explored the use of high-pressure CO_2 (HPCD) for its deactivation in apple cream. The assays were conducted in a high-pressure apparatus where the cream was placed in a vessel and the CO_2 passed over under different operating conditions for distinct periods of time. The HPCD was able to deactivate the *A. acidoterrestris* population in the apple cream by four orders of magnitude at 30 ºC and 100 bar. On the other hand, the lethal effect of HPCD was independent of the thermal effect since the application of dry heat at same temperature did not cause any *A. acidoterrestris* deactivation. The important variables in terms of improving the method were the flow regime and the way to put in contact the HPCD and the cream. Finally, the HPCD treatment did not affect the most important sensorial and rheological properties of the cream; although there was a slight reduction in the vitamin C content due to thermal degradation.

INTRODUCTION

Alicyclobacillus acidoterrestris (A.acidoterrestris) is a bacillus-type spore that causes significant alterations in fruit products. For example, it produces clarified fruit juices to have a light sediment or cloudiness, but the main effect is a phenolic odor and flavor, caused by guaiacol, 2.6 dibromofenol or 2.6 dichlorophenol.

The spores are the most resistant form of bacteria; their structure is different and more complex compared to vegetative cells. Part of their resistance is due to the presence of an external protein coat. Therefore, they can survive extreme conditions for years.

The spores of *A. acidoterrestris* are found on the ground, so it is thought that the source of contamination is fresh fruit, which is often introduced into the juicing process without proper cleaning.

Juice marketeers require a maximum concentration of 100 CFU mL^{-1} of *A. acidoterrestris* in juice, although it is unusual to find concentrations above 10^3 CFU mL^{-1}. The problem is that although the concentration is low, this microorganism is difficult to deactivate by heat.

Therefore, it is of great interest to find ways to destroying it using non-thermal methods. One possibility is the application of CO_2 at high pressure (HPCD). This method provides several advantages because it is a fluid inert, nontoxic, accessible, and affordable. Under ambient conditions is a gas so it leaves no residue in the product and it is considered a GRAS solvent. This fluid can deactivate most microorganisms, bacteria, yeasts, molds and enzymes that cause food spoilage [1]. Moreover, some authors have managed to deactivate endospores

of microorganisms of the family *Bacillaceae*, although they had to combine the HPCD treatment with temperatures of around 75-90 ºC [2, 3] and/or with the use of small amounts of additives [4, 5].

However, there are factors in HPCD that could hinder the deactivation of microorganisms in food. Furukawa *et al.* 2009 demonstrated the influence of salts and sugars on the inactivation of *Geobacillus stearothermophilus* spores, which both exerted a protective effect in proportion to their concentrations in the solute [6].

The aim of this work was to study the use of HPCD in the inactivation of *A. acidoterrestris* in apple cream and to evaluate the influence of a wider range of operating conditions than were previously used. The effects of the environment and pH were also investigated. Since the feasibility of this method of food preservation depends on the effects of CO_2 on the treated product, a further goal of this paper was to analyze how the treatment affected the most important quality properties of the cream.

MATERIALS Y METHODS

We used apple cream (Andros brand, from France), inoculated with 10^6 CFU g^{-1} of a suspension of *A. acidoterrestris* (ATCC 49025) spores from the Spanish Type Culture Collection. CO_2 purity> 99.95% was supplied by Carburos Metalicos, Spain.

The assays were conducted in a high pressure apparatus with CO_2 continuous flow. The cream was placed in the vessel equipped with agitation and temperature control. The CO_2 previously pressurized and heated, entered from the bottom and passed over the cream under different operating conditions for distinct periods of time. When the end of the operating time was reached, the depressurization began very slowly. The vessel containing the cream was taken to a sterile laminar-flow chamber for subsequent microbiological and physicochemical analyses.

The degree of deactivation of *A. acidoterrestris* was measured as the logarithm of recount of CFU $g^1 1$ before (N_0) and after treatment (N). The standard deviation in the readings was + / - 0.7 log.

A Tamson TC 9 concentric-cylinder narrow-space ($\delta R_1^{-1} < 0.1$) viscometer was used to measure the viscosity of the cream; where we could vary the applied shear stress (τ) measuring the shear rate (γ) caused. The standard deviations for the rheological parameters were + / - 0.02 in the consistency index (k) and + / - 0.04 in the behavior index (n).

The ascorbic acid concentration was semi-quantitatively measured by observing the color change in reactive strips (Merck, Spain), which indicate a range of ascorbic acid concentrations, from 0-2000 mg L^{-1}. Color was measured by observing the samples using the naked eye and assigning a value scale: --- if no color change was observed, x if the color of the cream was slightly changed, xx if browning occurred and xxx if the cream turned black. The pH was measured using a Basic 20 + pH meter (Crison, Spain). The standard deviation was + / - 0.05.

RESULTS AND DISCUSSION

Effect of operation variables

As shown in Figure 1, the HPCD treatment was able to reduce the population of *A. acidoterrestris* in apple cream until four orders of magnitude at 30ºC. This result is somewhat surprising because the inactivation of bacillus-type sporulated organisms usually requires

temperatures in the range of 70-90 °C. When we compared these results with those obtained from the heat treatment, it was found that the CO_2 had lethal effect of its own. The reason why the HPCD treatment has lethal effect even at low temperatures could be that, in media with a high water activity, it promotes the activation and germination of spores [2, 3, 6] . This phenomenon would be strongly associated with a reduced pH.

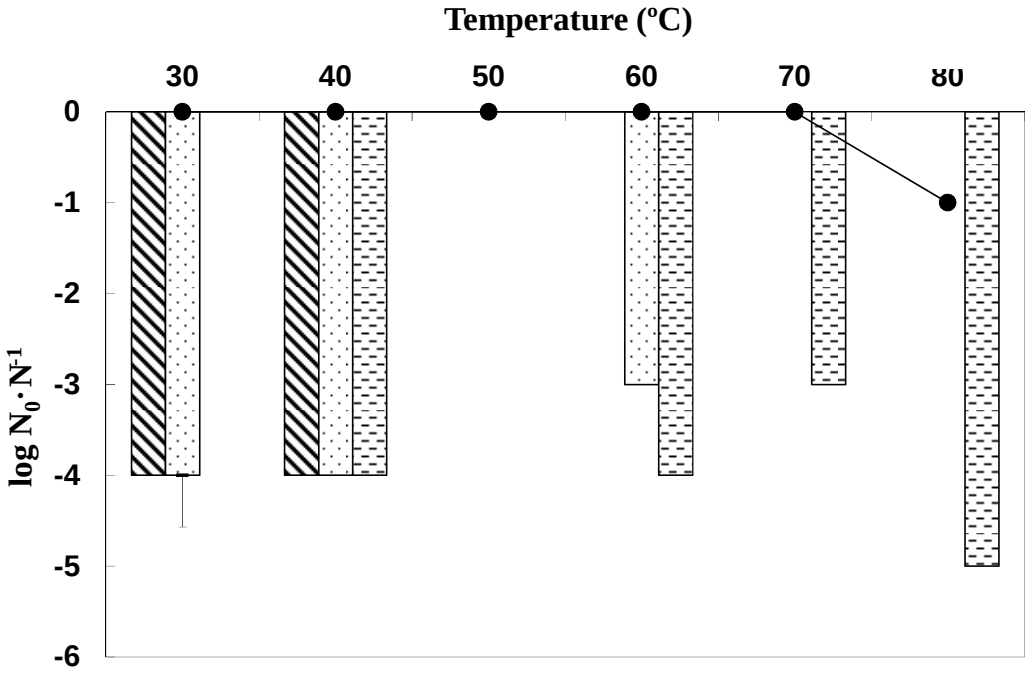

Figure 1. Inactivation of *A. acidoterrestris* by HPCD under various pressures and temperatures and with heat treatment. Other operating conditions: treatment time, 30 minutes, flow rate of CO_2, 4 g min^{-1}, agitation speed: 500 rpm. ▨ 100 bar, ⁘ 150 bar, ▤ 350 bar, ● 1 bar (atmospheric pressure).

No significant differences were found in the deactivation of *A. acidoterrestris* at CO_2 pressures between 100 and 350 bar since increasing the pressure within this interval did not reduce significantly the external pH (the cream was already very acid) or caused damage to the structure of the spore. Other authors obtained similar results [7, 8].

There was also no benefit increasing the duration of the treatment up to 120 minutes (results not shown). Therefore, the rest of the assays were run for 30 min.

Effect of the medium (the cream)

Table 1 shows a summary of the deactivation of *A. acidoterrestris* in cream and other media, such as apple and orange juice and sterile water, in which the spores were dispersed. The pH of each of the media and the method of exploration, i.e., the treatment conditions, the operation mode and manner of CO_2 introduction are included in this table.

The results obtained for the *A. acidoterrestris* deactivation in apple cream are lower than those obtained in orange, apple juice and sterile water. This difference was attributed primarily to the viscosity. In short, apple cream was more viscous than juice or water and this may result in a less effective level of contact with CO_2. This phenomenon could explain that

total deactivation of *A. acidoterrestris* occurred in orange juice with a continuous supply of CO_2 trough a membrane contactor that effectively dispersed it into very fine bubbles [9]. Therefore we investigated the variables affecting the contact between cream and CO_2. Table 2 shows the results.

Table 1. Summary of the conditions, method of operation and degree of deactivation achieved in the HPCD treatment of *A. acidoterrestris* in different media.

Sample	Medium	pH	Inactivation conditions						Inactivation (log $N_0.N^{-1}$)	Reference
			Temperature (°C)	Pressure (bar)	Time (min)	Operation mode	Agitation speed (rpm)	CO_2 introduction form		
1	Oranje juice	--	45	75	2	Continuous	--	Membrane contactor	-6 (Total)	[9]
2	Apple juice	3,5	65	100	40	Semicontinuous	--	Directly from the bottom	-6 (Total)	[10]
3	Apple cream	3,6	60	100	30	Semicontinuous	500	Directly from the bottom	-4	Our work
4	Apple cream	3,7	30	100	30	Semicontinuous	500	Directly from the bottom	-4	Our work
5	Sterile water	5,8	30	100	30	Semicontinuous	500	Directly from the bottom	-5	Our work
6	Citric acid solution	3,60	30	100	30	Semicontinuous	500	Directly from the bottom	-6 (Total)	Our work

First, it was found that increasing the stirring speed significantly improved the degree of deactivation of *A. acidoterrestris* in the cream. This was primarily due to increased turbulence favoring contact between the CO_2 and cream. An improvement was also achieved by doubling the flow rate of CO_2. However, increasing the amount of CO_2 in contact to the cream was only efficient when it was due to increased turbulence (compare samples 3 and 4) and not as a consequence of increasing the treatment duration (compare samples 5, 6 and 7)

Table 2. Influence of variables related to the effectiveness of the contact between the CO_2 and apple cream in the deactivation of spores of *A. acidoterrestris*. Other operating conditions: pressure, 150 bar; temperature, 30 ºC.

Sample number	Agitation speed	CO_2 flow rate	CO_2 mass	Treatment duration	log (N_0 N^{-1})
	(rpm)	(g min^{-1})	(g)	(min)	
Effect of increased agitation speed					
1	**150**	2	60	30	-2.2
2	**500**	2	58	30	-3.1
Effect of increased flow rate					
3	500	**2**	58	30	-3.1
4	500	**4**	119	30	-4.0
Effect of increased CO_2 mass					
5	500	4	**119**	30	-4.0
6	500	4	**177**	45	-4.1
7	500	4	**243**	60	-4.1

Effect of HPCD treatment on cream quality

Table 3 shows the measurements of the apple cream after various HPCD and thermal treatments (1 bar at atmospheric pressure) for the sensory and rheological parameters, the vitamin C content and the pH level. The cream darkened with increasing the temperature, pressure and duration with HPCD treatment. The color change from 50 °C and above was significant, and this was also true for the samples treated with heat alone. As the presence of enzymes was not detected in this cream, such changes could be attributed to chemical processes, mainly Maillard reactions and caramelization associated to high temperatures.

Vitamin C is directly related to the antioxidant capacity of fruit products. The data shown in Table 3 indicate that all of the samples treated with CO_2 contained lower amounts of vitamin C. However, the vitamin C content also decreased in the samples treated with heat, so it could be deduced that the reduction in vitamin C content was mainly due to heat denaturation.

The apple cream had a pseudoplastic behavior that was not altered by HPCD treatment under mild conditions. If temperature increased, lower values were obtained for n and greater for , due to water evaporation during the passage of CO_2.

The dissolution of CO_2 in water forms carbonic acid, which lowers the pH. However, Table 3 shows that the pH value did not decrease significantly in any of the apple cream samples treated with HPCD, essentially because the pH level was already low. Hence, any changes in the quality parameters cannot be attributed to this factor.

Table 3. Quality variables of apple cream after HPCD and heat treatment (indicated as treated at atmospheric pressure).

Sample	P (bar)	T (ºC)	t (min)	Color	K	n	pH	Vitamin C (mg L^{-1})
Cream as received + 10% *A. acidoterrestris*					0.56	0.42	3.69	200
Samples treated at atmospheric pressure								
1	1	30	60	=	0.53	0.34	3.67	100
2	1	40	60	x	0.68	0.29	3.68	100
5	1	60	60	xx	0.53	0.33	3.50	100
Samples treated by HPCD								
8	100	30	30	=	0.54	0.39	3.66	100
9	100	60	30	xx	1.16	0.23	3.36	100
10	100	80	30	xxx	0.58	0.30	3.61	100
11	150	60	30	xx	0.70	0.41	3.74	100
14	300	70	60	xxx	0.97	0.21	3.66	100
15	350	45	120	xxx	0.71	0.36	3.65	100

CONCLUSIONS

The HPCD treatment is effective in deactivating spores of *A. acidoterrestris* in apple cream. This application does not require the use of either a high temperature or pressure since these parameters did not significantly affect the treatment. Therefore, CO_2 can be applied at near ambient temperatures and very moderate pressures (≤ 100 bar), i.e., at much milder conditions than required for pasteurization or high hydrostatic pressures, respectively. In this way, the concentration of *A. acidoterrestris* can be reduced by up to 4 log cycles, a reduction that could be enough since the pollutant load of this endospore does not usually exceed 1000

CFU g^{-1} in creams that are used as raw materials in industrial practices. Under these conditions, the HPCD treatment does not affect the most important sensory and rheological properties.

The really important variables in terms of improving the effectiveness of the method are the flow regime and the contact between the HPCD and the cream. In this sense, a continuous operation, promoting good mixing and dispersion of CO_2, would be the objective for scaling up the process.

There is sufficient technological maturity regarding the availability of engineering companies that can design and construct high-pressure installations. For all of these reasons, HPCD treatment as a method of "sterilization" for fruit creams or juices is promising and ready to be scaled up.

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