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Original

Pre-treatment of thermoduric spores in CO_2 modified atmosphere and their survivability during food extrusion

Pré-tratamento de esporos termodúricos em atmosfera modificada de CO_2 e sua sobrevivência ao processo de extrusão

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Abstract

The aim of this experiment was to evaluate how susceptible spores become to mechanical damage during food extrusion after being submitted to CO_2. *B. stearothermophilus* spores sowed to corn and soy mix were submitted to 99% CO_2 for 10 days and extruded in a single-screw extruder. The treatments were: T1 - spore-containing samples, extruded at screw rotational speed of 65 rpm and barrel wall temperature of 80 °C; T2 - as T1, except for screw rotational speed of 150 rpm; and T3 - as T2, except that samples were submitted to the modified atmosphere. The results for cell viability, minimum and maximum residence times, and static pressure were T1 - 19.90 ± 3.24%, 123.3 ± 14.50 seconds; 203.3 ± 14.05 seconds; 2.217 ± 62 kPa; T2 - 21.42 ± 8.24%, 70.00 ± 5.77 seconds; 170.00 ± 4.67 seconds; 2.310 ± 107 kPa; and T3 - 11.06 ± 2.46%, 86.00 ± 7.23 seconds; 186.00 ± 7.50 seconds; 2.403 ± 93 kPa, respectively. It was concluded that the extrusion process did reduce the cell count. However, screw rotational speed variation or CO_2 pre-treatment did not affect cell viability.

Keywords: *shear; feed; Bacillus stearothermophilus.*

Resumo

Foi avaliada a viabilidade de esporos durante a extrusão quando previamente submetidos ao CO_2. Esporos de *B. stearothermophilus* foram semeados em mistura de milho e soja e submetidos à atmosfera modificada de 99% de CO_2 por 10 dias, sendo processados em extrusora de rosca simples. Os tratamentos foram: T1 - amostra contendo esporos, extrusada com velocidade de rotação da rosca em 65 rpm, temperatura na parede do cilindro de 80 °C; T2 - idêntico a T1, exceto pela velocidade de rotação da rosca em 150 rpm; T3 - idêntico a T2, exceto que as amostras foram submetidas ao CO_2. Os resultados de viabilidade celular, tempos mínimo e máximo de residência, e pressão estática foram: T1 - 19,90 ± 3,24%, 123,3 ± 14,50 seconds; 203,3 ± 14,05 seconds; 2.217 ± 62 kPa; T2 - 21,42 ± 8,24%, 70,00 ± 5,77 seconds; 170,00 ± 4,67 seconds; 2.310 ± 107 kPa; e T3 - 11,06 ± 2,46%; 86,00 ± 7,23 seconds; 186,00 ± 7,50 s; 2.403 ± 93 kPa, respectivamente. Concluiu-se que a extrusão reduziu a viabilidade celular, e não pôde ser creditada ao calor. A alteração da rotação da rosca ou pré-tratamento com CO_2 não afetou a viabilidade dos esporos.

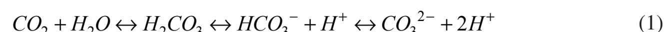
Palavras-chave: *cisalhamento; ração; Bacillus stearothermophilus.*

1 Introduction

Several authors have demonstrated that extrusion can reduce the viability of unicellular organisms, and they attributed such effect to heat (BOUVERESSE et al., 1982; QUÉGUINER et al., 1989; LIKINAMI et al., 1990; KELLEY; WALKER, 1999). However, studies with heat-resistant microorganisms indicate that shear stress may be involved in microbial load reduction during the extrusion process indicating that mechanical forces might be involved in cell rupture (KAUFFMAN; HATCH, 1977; BULUT; WAITES; MITCHELL, 1999; KELLEY; WALKER, 2000; OKELO et al., 2006). Nevertheless, we were not able to visualize ruptured cell; we observed reduced count of *B. stearothermophilus* spores when submitted to shear stress in a capillary rheometer (FRAIHA; FERRAZ; BIAGI, 2008) and food extruder (FRAIHA, 2008) confirming that mass flow during these processes may damage single-cell structures.

The inhibitory effect of carbon dioxide (CO_2) on the growth of some microorganisms is well known (DIXON; KELL, 1989), and it is widely used for microbial control in many commercial applications.

CO_2 dissociates in water as follows (Equation 1):



$$CO_2 + H_2O \leftrightarrow H_2CO_3 \leftrightarrow HCO_3^- + H^+ \leftrightarrow CO_3^{2-} + 2H^+ \quad (1)$$

and it permeates biological membranes freely, and once inside the cell, it bonds to water releasing H^+, which may change intra-cell pH. It is suggested that the CO_2 inhibitory effect may be linked to changes in permeability and structure of cell membrane and to changes of enzymatic synthesis at the cytoplasm (DIXON; KELL, 1989). Some authors also demonstrated that pressurized CO_2 causes cell disruption when pressure as low as 5 MPa is suddenly released (SHIMODA et al., 2001; DEBS-LOUKA et al., 1999; LIN; YANG; CHEN, 1992). During food extrusion processes, similar level of pressure on the food mass is observed, followed by a sudden pressure drop at the die exit.

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We hypothesize that bacterial spores become more susceptible to viability reduction due to structural damage when submitted to a food extrusion process after pre-treatment of cells with $CO_{2.}$

This experiment aimed at evaluating the pre-treatment of spores with a modified atmosphere of CO_2 and their survivability during food extrusion.

2 Materials and methods

2.1 Sample preparation

The experimental material was prepared at the Agricultural Engineering School, UNICAMP (Campinas, Brazil). The spore suspension of *Bacillus stearothermophilus* ATCC 7953 was acquired from Fundação André Tosello (Campinas, Brazil).

Two hundred kilograms of corn and soy grains were manually mixed at a ratio of 70:30 (mass) and ground in a hammer mill with a 1.5 mm mesh sieve. The corn and soy mix average geometric diameter was 542.39 ± 24.84 μm, determined according to ANSI/ASAE S319.3 method (AMERICAN..., 2004).

A 10 kg subsample was vacuum packed to later serve as a vehicle for the microorganisms; the remainder was 200 $g.kg^{-1}$ hydrated with water and vacuum packed in 20 kg plastic bags resting for 12 hours at ambient temperature (28 °C) for uniform distribution of moisture content. The final moisture content was 301.60 ± 0.5 $g.kg^{-1}$ (mean ± standard error), determined by gravimetric method, after drying in a forced-air oven for 24 hours at 105 °C to constant weight.

In order to avoid any interference from natural microbial load, the material was sterilized with gamma irradiation at 25 kGy, at Instituto de Pesquisas em Energia Nuclear (IPEN) at University of São Paulo (São Paulo, Brazil). The material was then kept frozen at -30 °C for 72 hours before the experiments, when it was thawed at ambient temperature.

The spore suspension was dispersed in a 0.85% saline solution and mixed, using a kitchen mixer, with the grain subsample purposely reserved to this aim, so as to obtain a final mass moisture-content of 300 $g.kg^{-1}$ (wet basis) and a final spore concentration of 10^6 colony forming units (CFU) per 5 g. This sample was stained with a phthalocyanine-based organic dye (Xadrez, Sherwin-Williams, Brazil), so as to ease its identification. After sowing, 300 g aliquots of this material were packed in aluminum bags, sealed and kept under refrigeration at 5 °C until the experiments. Some samples were packed under modified atmosphere of 99% CO_2 (Selovac, 200B, Brasil). The CO_2 content was confirmed with gas chromatography (model 3400 GC, Varian, Palo Alto, USA). The manipulation of the material was carried out in laminar flow hood to prevent contamination.

To assess the residence time distribution in the extruder, a colored phthalocyanine-based tracer was prepared. Several samples composed of a mixture of 100 mg of phthalocyanine-tracer and 100 mg of the not-sowed sterile corn-soy grain mix were individually packed in small plastic bags, which were afterwards kept under refrigeration at 5 °C until used.

2.2 Extrusion experiment

The extrusion experiments were carried out at Embrapa Agroindústria de Alimentos (Rio de Janeiro, Brazil). A Brabender 19/20 DN single-screw extruder (Duisburg, Germany) was used, featured with temperature control in three zones along the barrel, a pressure sensor immediately preceding the internal die wall, and a feeding screw and main screw with speed control. The barrel had an inner diameter of 20 mm, was internally grooved over its entire length, and the length to diameter ratio was of 20:1. A smaller barrel, with 70.6 mm in length and 10 mm inner diameter was screwed up to the main barrel serving as a mounting support to a third electrical heating resistance, the pressure sensor, and the die. The pressure sensor was screwed up to the wall of the smaller barrel, so as to be in contact with the extruded mass and register the static pressure (Figure 1).

For descriptive purposes herein, the screw length was arbitrarily divided into three equal parts denominated feeding, compacting, and rheological zones. The die inner diameter and thickness were 3.8 and 23.1 mm, respectively. Specifications for the screw are given in Table 1 thus allowing the estimation of nominal apparent shear rate based on the following (Equation 2):

$$\dot{\gamma} = \pi N D Y^{-1} \tag{2}$$

where Y represents the screw channel depth in the rheological zone, N stands for screw rotational speed (rpm), D is the barrel diameter (20 mm), and the nominal apparent shear rate $\dot{\gamma}$ is expressed in second.

Routine procedures were common to all treatments. The extrusion screw was turned on and adjusted to the test rotational speed; next, the helicoid feeder was also turned on and adjusted to 25 rpm; all three heating zones were set to 80 °C. The not-sowed sterile sample was used to bring the extruder up to steady state regime, which was recognized when temperature, pressure, and extruded flow stabilization. Then, a spore-containing sample calculated so as to fill 1.5 times the nominal volume of the extruder was extruded. In order to prevent any dilution effects of the sowed sample on the not-sowed one, half of the sowed sample was allowed to flow before 50 g collecting for microbiological analysis. After each treatment, the screw and barrel were cleaned up, using a steel brush, sponge, and soap. After the removal of every residue from barrel and screw, ethanol 80% was sprayed over them and wipe out with a dry sponge, and then it was dried out with pressurized air. Since bacterial spores were used, their multiplication and the contamination of the extruder were assumed no-existent since a special procedure is required for their germination (ORSON; SORRELS, 1992). All disposable materials were incinerated to avoid any accidental contamination.

The treatments followed the same operational parameters, except for the screw rotational speed that was 65 rpm for T1 and 150 rpm for T2 and T3. One of the mechanisms proposed to explain how CO_2 kills bacterial cells is its direct effect on their metabolism by disrupting the physicochemical properties of the membranes and direct inhibition of metabolic pathways (HOTCHKISS; WERNER; LEE, 2006). Thus, samples in T3 were

submitted to modified atmosphere of 99% CO_2 for a period of time arbitrarily chosen of 10 days prior to the experiment in order to allow CO_2 diffusion into the cells and its effects on them.

The mass temperature was measured with the extruder under operation, with a thermocouple of 1.25 mm diameter and 90 mm length; it was introduced through the die to reach a point close to the die exit (Figure 1).

2.3 Estimation of residence time distribution

The residence time distribution was estimated using the phthalocyanine-tracer. Under steady state operation, the feeder hopper was emptied, and the phthalocyanine-indicator was put at the end of the feeder screw, so as to make it immediately fall into the feeding zone. A chronometer was started and the feeder hopper was again completed with the not-sowed sample. The samples were collected at the die exit in 10 seconds intervals. These extruded samples were then dried in a forced-air oven, at 50 °C for 12 hours and ground in a disk-type mill (Perten 3600, Huddinge, Switzerland).

The color of extruded samples was analyzed in a colorimeter (MiniScan XE Plus, HunterLab, USA) using the CIELab scale, and the values *a* (red-green scale) and *b* (yellow-blue scale) were registered. The intensity (C) of the phthalocyaninecolor (blue) was determined as follows (Equation 3) (SEKER, 2004):

Table 1. Screw dimensions.

Thread thickness, mm	3.8
Shaft diameter	
Feeding zone, mm	12.2
Compacting zone, mm	13.9
Rheological zone, mm	16.1
Thread angle, °	55
Thread diameter, mm	18.8

$$C_i = \left|\frac{a}{b}\right| \quad (3)$$

Assuming that the concentration of phthalocyanine and the blue color of the samples were directly proportional, the residence time distribution of tracer is given by Equation 4:

$$E(t) \cong \frac{C_i}{\sum_{i=0}^{\infty} C_i \Delta t} \quad (4)$$

The accumulated amount of tracer is given by Equation 5:

$$F(t) \cong \frac{\sum_{i=0}^{t} C_i \Delta t}{\sum_{i=0}^{\infty} Ci \Delta t} \quad (5)$$

2.4 Recovery and spores count

After the treatments were performed, the recovery of spores from the extrudates was carried out. One gram of each sample was diluted in 49 mL of a 0.85% sterile saline solution vigorously stirred with a kitchen mixer; a 5 mL aliquot was pipeted into a sterile TDT tube and submitted to double boiler, at 100 °C for 30 minutes; next it was rapidly cooled in an ice bath to induce the spores to germinate. The dextrose tryptose agar medium was prepared with distilled water, its pH was corrected to 7.1 using NaOH 0.1 N, and it was sterilized in autoclave at 121 °C, per 20 minutes. The spore counting followed the plate direct-reading method, incubated at 50 °C per 48 hours, according to the methodology described by Orson and Sorrels (1992). Spore counting was performed in four replicates. The results were expressed as percentage of viability and defined as the number of post-treatment viable microorganisms (N) to the number of viable microorganisms in the original sample (No) ratio multiplied by 100.

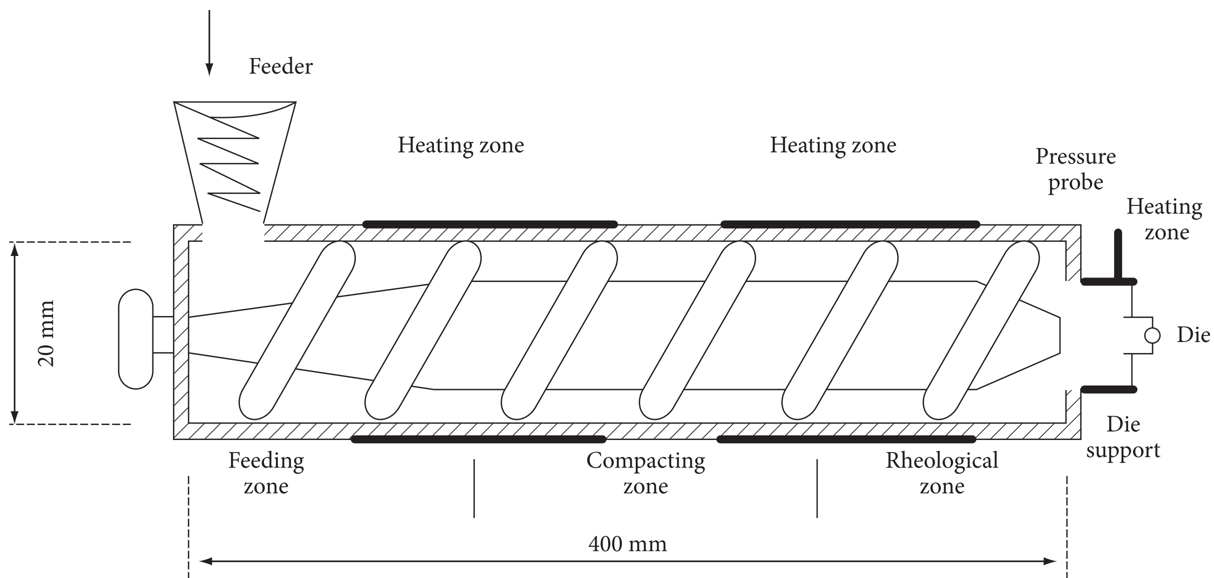

Figure 1. Schematic representation of the single-screw extruder used in this experiment, point at die - mass temperature determination.

2.5 Statistical analysis

The experimental data were analyzed using the General Linear Model (GLM) using Minitab Statistical software (version 14.1). The data were tested for normal distribution using the Kolmogorov-Smirnov test (LILLIEFORE, 1967). After analysis of variance, the treatments were compared using Tukey test. Null hypotheses were rejected when the significance level was less than 5% ($p < 0.05$). The data were presented as means of the observations followed by their standard errors.

3 Results and discussion

Table 3 presents means for cell viability, static pressure, minimum, and maximum residence times and mass temperatures according to the treatments.

Cell viability was not affect by screw rotational speed variation ($p > 0.05$). We have reported similar results elsewhere (FRAIHA, 2008), and we hypothesize that the volume of material submitted to flow velocity gradient does not change with screw rotational speed change. In a single-screw extruder configuration like the one used herein, the flow profile of food mass is to be laminar, and we have demonstrated that corn and soy mix rheological characteristic is to satisfy such flow profile (FRAIHA; FERRAZ; BIAGI, 2008). During mass flow throughout the screw, a mass velocity gradient is generated and varies from zero at the barrel wall to a maximum at the screw axis. In a laminar flow, regardless of its velocity, shear strain will fall upon a portion of the flowing mass, which might be estimated based on the rheological characteristic of the material. Thus, the amount of spores subjected to shear effects during mass flow remains unchanged as screw speed varies, which explains the lack of difference between treatments.

The CO_2 atmosphere did not affect cell viability either ($p > 0.05$). Despite the lower permeability of the cell membrane of the spores compared to vegetative cells, it is known that spores are not completely impermeable (BLACK; GERHARDT, 1962). In view of the static pressure obtained herein, it was expected that CO_2 diffusion into the cell would alter its chemical composition and wall structure enough to result in cell rupture after immediate pressure release at the die exit. Other authors have observed cell rupture after a sudden release of CO_2 atmosphere at static pressures of similar order of magnitude of those reached in this work (SHIMODA et al., 2001; DEBS-LOUKA et al., 1999; LIN; YANG; CHEN, 1992; LIN et al., 1991). However, these researchers kept cells under pressure for several minutes, whereas the present process was much shorter.

Debs-Louka et al. (1999) demonstrated that CO_2 effect on *E. coli* and *Saccharomices cerevisae* survival is dependent on the moisture level of the medium, in which they were submitted to the treatment suggesting that cell wall permeability to CO_2 depends on local moisture. Black and Gerhardt (1962) have pointed out that the wall permeability of spores and their thermal resistance might be linked to the low water content in these cells. Bearman et al. (1982) stated that among all *Bacillus* species, *B. stearothermophilus,* ATCC 7953 presented the highest $D_{100\,°C}$ value of 579 minutes, and suggested that its low water content is related to its thermal resistance.

In this experiment, the samples were submitted to CO_2 for 10 days under natural pressure (101,3 kPa), along with low water content of *B. stearothermophilus* ATCC 7953 (BLACK; GERHARDT, 1962), may have prevented gas diffusion into the cells. A different approach for further trials would be to insert CO_2 into the extruder simultaneously with samples, what could result in forced gas diffusion into the cells. Also, as discussed above, one shall make sure that some turbulence is to occur as mass flows through the extruder in order to guarantee that every portion of the mass will be exposed to the velocity gradient zone and shear stress. Such turbulence may be a result of design changes of the barrel-screw set, like flow breakers, reversed threads, or high-shear screw.

The samples were not submitted to heat pre-conditioning and were extruded at an initial temperature of nearly 20 °C. The results for mass temperature at the die exit (Table 2) reveal that there was no temperature difference between the treatments, which was below 80 °C. *B. stearothermophilus* spores evidenced to be heat-resistant when sowed into the corn-soy grain mixture, with values of $D_{121,1\,°C}$ and z of 14.2 minutes and 23.7 °C, respectively (FRAIHA; FERRAZ; BIAGI, 2008). The heat effect on spore viability reduction was negligible in all treatments at 80 °C, for the residence time the mass was subjected to extrusion since the estimated $D_{80\,°C}$ is 747 minutes. The viability reduction

Table 2. Treatments.

Treatment	Temperature (°C)	Screw rotational speed (rpm)	$\dot{\gamma}$ (second)
T1	80	65	1,047.0
T2	80	150	2,416.6
T3*	80	150	2,416.6

* T3 - samples were submitted to 99% CO_2 for 10 days; $\dot{\gamma}$ - apparent nominal shear rate.

Table 3. Means of dependent variables.

Treat	Cell viability (%)	Static pressure (kPa)	Min. residence time (s)	Max. residence time (s)	Mass temperature (°C)
T1	19.90 ± 3.24^a	$2,217 \pm 62^a$	123.3 ± 14.50^a	203.3 ± 14.05^a	78.2 ± 1.1^a
T2	21.42 ± 8.24^a	$2,310 \pm 107^a$	70.00 ± 5.77^b	170.00 ± 4.67^a	77.8 ± 0.8^a
T3	11.06 ± 2.46^a	$2,403 \pm 93^a$	86.00 ± 7.23^b	186.00 ± 7.50^a	78.6 ± 1.2^a

Mean ± std error; min - minimum, max - maximum. Different letters in the column indicate different means ($p < 0.05$).

observed in these treatments can be credited to shear stress generated by screw rotation and mass flow through the die.

4 Conclusions

Pre-treatment of *Bacillus stearothermophilus* spores in a 99% CO_2 modified atmosphere does not affect cell viability during food extrusion. Heat is not the sole phenomena to explain cell death during food extrusion, a mechanical damage of the cells might be involved.

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