Inactivation of *Bacillus amyloliquefaciens* Spores by a Combination of Sucrose Laurate and Pressure-Assisted Thermal Processing

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ABSTRACT

The aim of this research was to study the effect of sucrose laurate ester (SL) on enhancing pressure-assisted thermal processing (PATP) inactivation of *Bacillus amyloliquefaciens* Fad 82 spores. *B. amyloliquefaciens* spores (∼10⁶ CFU/ml) were suspended in deionized water, solutions of 0.1, 0.5, and 1.0% SL, and mashed carrots without or with 1% SL. Samples were treated at 700 MPa and 105°C for 0 (come-up time), 1, 2, and 5 min and analyzed by pour-plating and most-probable-number techniques. Heat shock (80°C, 10 min) was applied to untreated and treated samples to study the germination rates. Results were also compared against samples treated by high pressure processing (700 MPa, 35°C) and thermal processing (105°C, 0.1 MPa).

Among the combinations tested, SL at concentrations of 1.0% showed the best synergistic effect against spores of *B. amyloliquefaciens* when combined with PATP treatments. In the case of high pressure and thermal processing treatments, SL did not enhance spore inactivation at the conditions tested. These results suggest that SL is a promising antimicrobial compound that can help reduce the severity of PATP treatments.

Pressure-assisted thermal processing (PATP) is an emerging alternative sterilization technology to process shelf-stable low-acid food products. PATP combines elevated pressures (600 to 900 MPa) and temperatures (90 to 121°C) for short holding times (up to 5 min) (21). Bacterial spores are known to be resistant to high pressure at ambient temperatures (26) and various approaches have been proposed for spore inactivation using pressure-heat combinations. A two-stage strategy (8, 13) has been proposed wherein the spore samples are treated at 60 to 100 MPa and moderate temperatures (<30°C) with an extended holding time. Subsequently, samples are subjected to moderate pressures (<300 MPa) and temperatures to inactivate spores that have germinated at the first stage. With this approach, the possibility of a small fraction of highly resistant spores remaining dormant after the treatment cannot be ruled out (24). Many other authors utilized a combination of high temperature and pressure to inactivate a variety of *Bacillus* and *Clostridium* spores (2, 3, 14, 15, 21). Pressure treatments (500 to 600 MPa) applied at high temperatures (<60°C) release dipicolinic acid (DPA) from spores, which triggers spore germination (6, 19). Nonetheless, some of these spores may slowly complete the germination process due to a potential damage of some components of the spore germination system, such as their cortex lytic system (2, 3, 6, 19, 31). Under PATP conditions, DPA is also released from the spores (2, 14, 15). Subramanian et al. (28) applied Fourier transform infrared spectroscopy to spore suspensions treated by PATP (700 MPa, 121°C) and thermal processing (0.2 MPa, 121°C) and found that spores could be differentiated by their biochemical composition, especially by their content of DPA and secondary protein structure. DPA bands from PATP-treated spores decreased during the initial stages (mainly come-up time), whereas thermal processed spores showed only slight differences in their DPA bands but significant changes in their secondary protein structure. Rajan et al. (21) reported that the inactivation of *Bacillus amyloliquefaciens* inoculated in egg patties increased as the pressure of the treatment increased at a given temperature; however, synergy between heat and pressure diminished at high temperatures, and heat became the dominant contributor to lethality.

Very limited studies to date have investigated the use of antimicrobial compounds in combination with PATP to reduce the severity of the PATP treatment. Sucrose esters are commonly used as emulsifiers in the food industry. Several researchers have studied the sporostatic effect of these compounds and their ability to inhibit vegetative cell and mold growth (4, 16, 29). Moreover, sucrose laurate ester (SL) and sucrose palmitic acid esters have been found to have a synergistic effect with high pressure at mild temperatures to inactivate *Bacillus* spores (11, 25). Nevertheless, enhanced effects of these compounds on spore inactivation under PATP treatment conditions are largely unknown.
The objective of the current work was to investigate the potential synergetic effects of SL with thermal processing (TP), high pressure processing (HPP), and PATP on *B. amyloliquefaciens* spore inactivation. A low-acid food product (mashed carrots) was tested to assess the potential food matrix protective effect against spore inactivation under PATP conditions.

**MATERIALS AND METHODS**

**Bacterial strain.** *B. amyloliquefaciens* TMW 2.479 Fad 82 was kindly provided by Dr. M. Gänzle (Department of Agricultural, Food and Nutritional Science, University of Alberta, Edmonton, Alberta, Canada). Stock culture was stored at −20°C in Trypticase soy broth (TSB; Difco, BD, Sparks, MD) containing 40% (vol/vol) glycerol. Cells were incubated with TSB supplemented with 0.6% yeast extract (TSBYE; Difco, BD) at 32°C for 24 h prior to spore crop preparation.

**Preparation of spore suspension.** After two transfers, 100 μl of vegetative cells was spread plated onto Trypticase soy agar (TSA; Difco, BD) supplemented with 10 ppm of MnSO₄ (Fisher Scientific, Pittsburgh, PA). The inoculated plates were incubated at 32°C until more than 95% of the population, examined by phase contrast microscopy, appeared as bright spores (up to 14 days).

Spores were collected by flooding the surfaces of the plates with 9 ml of cold deionized water (DW) and scraping culture growth with sterile plastic spreaders. The spore suspension was washed four times with cold DW in conjunction with differential centrifugation ranging from 2,000 to 8,000 × g for 20 min at 4°C. The spore pellets were finally resuspended in cold DW, sonicated for 10 min at room temperature (SM275HT, peak power 270W; Crest Ultrasonic, ETL Testing Laboratory, Cortland, NY), heated at 80°C for 10 min of holding time (volume of sample, 5 ml; come-up time, 1.33 min) to kill vegetative cells, and stored at 4°C. The final concentration of the spore crop was ~10⁹ CFU/ml and was used within 3 months after harvesting.

**SL solution preparation.** Four stock solutions containing 0.11, 0.55, 1.1, and 10% SL (Ryoto Sugar Ester L-1695, Mitsubishi-Kagaku Foods Corporation, Tokyo, Japan) were prepared in DW at room temperature. Solutions were sterilized by autoclaving at 121°C for 15 min until use.

**Sample preparation.** Spore suspensions were sonicated (SM275HT, ETL Testing Laboratory) for 10 min at room temperature to minimize spore clumping. Subsequently, the spore suspensions (0.25 ml) were transferred into sterile plastic pouches (5 by 3.5 cm; Fisher Scientific) and mixed with one of the following (2.25 ml or g); sterile DW, a solution of 0.11, 0.55, or 1.1% SL, sterile mashed carrots (MC; Nature’s Goodness, Del Monte Foods, San Francisco, CA), and MC supplemented with 10% SL. The sample pouches were sealed using an impulse heat sealer (American International Electric, Whittier, CA). As much air as possible was removed from the bag. For MC experiments, the pouch containing inoculated MC sample was packaged in a second plastic pouch. DW and MC pouches were held for 60 min at 4°C prior to exposure to various pressure-heat treatments as described below. For thermal processing experiments, sonicated spore suspensions were transferred to custom-fabricated aluminum thermal death time disks (0.12 ml) and mixed with DW or a solution of 1.1% SL (1.08 ml). The disks containing spore suspensions were held for 60 min at 4°C prior to the thermal treatments.

**HPP, HPP and PATP experiments were performed using a high-pressure unit (PT-1, Avure Technologies Inc., Kent, WA) that processes samples up to 700 MPa and 130°C (21). An intensifier (M-340 A, Flow International, Kent, WA) was used to generate the desired pressure. The pressure chamber is a 54-ml stainless steel cylinder placed inside a temperature-controlled bath that was maintained at an appropriate temperature (Table 1) to guarantee isothermal process conditions throughout the pressure-holding times. Food-grade propylene glycol (57-55-6, Avatar Corporation, University Park, IL) was used as the heating and pressure-transmitting medium. Whereas the pressure come-up time depended on the target pressure (Table 1), the depressurization occurred in less than 3 s. The bath, sample, and chamber temperatures were recorded every 1 s with a K-type thermocouple sensor (model KMQSS-040U-7, Omega Engineering, Stamford, CT) and a pressure transducer (model 3399 093 006, Tecsis, Frankfurt, Germany). A data acquisition computer was used to record data. Samples in pouches were treated at 700 MPa and 35°C or 105°C for 0 (come-up time), 1, 2, and 5 min (Table 1).

Prior to HPP and PATP treatments, the sample pouch was placed inside a holder consisting of a 10-ml polypropylene syringe (model 309604, Difco, BD) covered with at least three layers of insulating material (Sports Tape; CVS Pharmacy Inc., Woonsocket, RI). Water was used to fill the rest of the sample holder to ensure that the immediate vicinity of the sample pouch had similar heat of compression characteristics as the sample inside. The sample holder was preconditioned at the desired pre بشأن المحتوى المكتوب في الصفحة التي تم تقديمها، هل هناك أي عناصر تبدو غير واضحة أو نسبية أو غير معترف بها؟ إذا كان الأمر كذلك، فما هو؟
temperature for 2 min using an ice-water bath (for high pressure experiments) or hot-water bath (Isotemp 928, Fisher Scientific) for PATP experiments. After preconditioning, the sample holder was immediately loaded into the pressure chamber and the vessel was closed. Sample temperature history was continuously monitored (Fig. 1). The pressurization started when the sample temperature reached the predetermined value $T_2$ (Fig. 1). This temperature was estimated based on a trial-and-error experimental approach using the following equation proposed by Rajan et al. (21) and Nguyen et al. (17):

$$T_2 = T'_3 - (CH \times \Delta P + \Delta T_H)$$

where $T'_3$ is the target temperature (degrees Celsius), CH is the heat of compression value of the sample (defined as temperature increase per 100 MPa during sample pressurization) and $\Delta P$ is the pressure applied (megapascals). $\Delta T_H$ is the temperature gain by the test sample during loading within the pressure chamber as well as during pressurization.

**TP experiments.** TP experiments were performed using custom-made thermal death time disks (18-mm diameter and 4.5-mm height) developed at Washington State University (12). Sample temperature was controlled by placing a K-type thermocouple (Omega Engineering) attached to a data logger (IOtech, Cleveland, OH) into an aluminum tube filled with DW or a solution of 1% SL. The following approach was used so that heat-processed samples had a thermal history similar to that of PATP samples. First, the sample disks were preheated in a water bath at 53°C for 2 min and then transferred to another water bath at 62°C. Once the samples reached 61.5°C (after 30 to 40 s), they were transferred to a circulating oil bath (28 liters; Fisher Scientific) at 118°C until the temperature reached $\sim 103$ to 104°C. Then, sample disks were finally placed in another oil bath at 105.5°C to reach the treatment temperature with a come-up time similar to that of PATP treatments (Fig. 2 and Table 2). The first disk was removed immediately after reaching 105°C and determined as 0 min (come-up time). The remaining disks were removed at different holding times.

**FIGURE 1.** Pressure-temperature treatment history for samples processed at 0.1 MPa and 105°C (a) or at 700 MPa and 105°C (b) for 5 min. $T_1$, preprocess temperature; $T_2$, immediately before treatment; $T_3$, immediately after reaching target pressure or temperature; $T_4$ to $T_5$, during holding time; $T_5$, depressurization or cooling.
The following treatments were tested to determine if Bacillus amyloliquefaciens spores suspended in deionized water (DW) or sucrose laurate (SL; 0.1, 0.5, and 1.0%) and treated with pressure-assisted thermal processing (700 MPa, 105°C for up to 5 min). SL + PATP, SL added prior to PATP treatment; PATP + SL, SL added after PATP treatment. Dashed horizontal line represents the method’s minimum detection level of viable spores. Means (n = 6) with different letters are significantly different (P < 0.05). □, Dormant spores; ■, germinated spores.

Sampling protocol. The following tests were treated to establish whether synergistic effect between SL and PATP, TP, or HPP treatments occurred during and/or after treatments: (i) SL at atmospheric pressure (0.1 MPa; SL control); (ii) PATP, TP, and HPP without SL (controls); (iii) SL added to samples prior to PATP, TP, and HPP treatments; (iv) SL added to the samples after PATP (PATP + SL), TP (TP + SL), and HPP (HPP + SL) treatments; and (v) SL added in the recovery agar (SLA) or broth (SLB).

The effects of PATP, TP, and HPP combined with SL were considered synergistic if the Log reduction of the treatment combined with SL \((\log(N_0/N_{t})_{A+SL})\) was significantly (\(P < 0.05\)) higher than the sum of the Log reductions of the treatment \((\log(N_0/N_{t})_{A})\) and the SL \((\log(N_0/N_{t})_{SL})\):

\[
\log(N_0/N_{t})_{A+SL} > \log(N_0/N_{t})_{A} + \log(N_0/N_{t})_{SL}
\]

where \(N_{t}\) is the number of viable spores after the treatment, \(N_0\) is the number of viable spores before treatment and \(A\) is the treatment applied. All samples were stored at 4°C until being analyzed. Experiments were run three times with duplicate analyses each time.

Microbiological analyses. Pouches and thermal death time disk surfaces were sanitized with a 10% solution of commercial bleach prior to being aseptically opened. In the case of spores suspended in DW and solutions of 1% SL, two aliquots of each sample (1 ml each) were transferred to 9 ml of 0.1% peptone water (PW; Difco, BD). In the case of spores suspended in MC and MC supplemented with 1.0% SL, the content of each sample (2.5 g) was homogenized with 22.5 ml of PW for 2 min using an electromagnetic blender (Seward Lab Stomacher, Norfolk, UK) at room temperature. Two 10-ml aliquots were transferred to test tubes. Heat sensitivity was used as a criterion of germination (18, 30). Test tubes containing the first dilution were immersed for 10 min (DW samples) or 30 min (MC samples) in a water bath at 80°C (come-up time, 2 min 20 s) to inactivate germinated spores. Heat-treated and non–heat-treated samples were diluted 10-fold with PW and appropriate dilutions were poured plated with TSA supplemented with 0.6% yeast extract (TSYE) and TSYE supplemented with 0.1, 0.5, or 1% SL. The plates were incubated at 32°C for 48 and 120 h, respectively. Colonies were counted with a dark-field Quebec colony counter (Leica Microsystems, Richmond Hill, Ontario, Canada). The detection limit of spore enumeration was 10 CFU/ml or g.

The most-probable-number technique (MPN; 3 tubes, TSYE with or without SL (7)) was used for treated samples when spores were not detected by the direct plating method (i.e., <10 CFU/ml or g). Tubes containing treated samples were kept at 32°C for 15 days to allow the recovery of the remaining population. The detection level was 0.5 Log MPN/ml or g.

**TABLE 2. Temperature histories during thermal processing (0.1 MPa and 105°C for up to 5 min) of mashed carrots**

<table>
<thead>
<tr>
<th>Treatment temp (°C)</th>
<th>Holding time (min)</th>
<th>Come-up time (min)</th>
<th>Preprocess (T_r, °C)</th>
<th>Immediately before treatment (T_2, °C)</th>
<th>Immediately reaching target temp (T_h, °C)</th>
<th>During holding time (T_h-T_s, °C)</th>
<th>Cooling time (min)</th>
<th>Cooling (T_s, °C)</th>
</tr>
</thead>
<tbody>
<tr>
<td>105</td>
<td>0</td>
<td>0.89 ± 0.24</td>
<td>51.35 ± 0.25</td>
<td>61.25 ± 1.16</td>
<td>105.01 ± 0.05</td>
<td>105.01 ± 0.05</td>
<td>0.91 ± 0.21</td>
<td>18.75 ± 0.25</td>
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<tr>
<td></td>
<td>1</td>
<td>0.89 ± 0.24</td>
<td>51.35 ± 0.25</td>
<td>61.25 ± 1.16</td>
<td>105.01 ± 0.05</td>
<td>105.01 ± 0.05</td>
<td>0.91 ± 0.21</td>
<td>18.75 ± 0.25</td>
</tr>
<tr>
<td></td>
<td>2</td>
<td>0.89 ± 0.24</td>
<td>51.35 ± 0.25</td>
<td>61.25 ± 1.16</td>
<td>105.01 ± 0.05</td>
<td>105.01 ± 0.05</td>
<td>0.91 ± 0.21</td>
<td>18.75 ± 0.25</td>
</tr>
<tr>
<td></td>
<td>5</td>
<td>0.89 ± 0.24</td>
<td>51.35 ± 0.25</td>
<td>61.25 ± 1.16</td>
<td>105.01 ± 0.05</td>
<td>105.01 ± 0.05</td>
<td>0.91 ± 0.21</td>
<td>18.75 ± 0.25</td>
</tr>
</tbody>
</table>

\(^a\) Values are means ± standard deviations of three independent replicates.
**Statistical analyses.** Analysis of variance was performed using the general linear models procedure of SAS software (SAS System for Windows, 8.02, SAS Institute Inc., Cary, NC). Student-Newman-Keuls and Tukey tests were used to obtain paired comparisons among sample means. The level of significance was set at $P < 0.05$.

**RESULTS AND DISCUSSION**

**Synergistic effect of SL and PATP.** *B. amyloliquefaciens* spore inactivation by the combination of PATP and SL at different concentrations (0.1, 0.5, and 1.0%) and application sequences (SL + PATP and PATP + SL) is shown in Figure 2. In the absence of PATP treatment, none of the SL concentrations used were effective against *B. amyloliquefaciens* spores. However, the addition of SL prior to PATP treatment was found to be synergistic at all SL concentrations. Among the SL concentrations tested, 1.0% showed the highest synergistic effect, enhancing *B. amyloliquefaciens* spore inactivation between 0.5 and 1.2 Log units, depending on the holding time applied. Spores suspended in 0.5 and 1.0% SL solutions were reduced to the detection level ($\leq 10$ CFU/ml) after a 2-min treatment at 700 MPa and 105°C. DW and 0.1% SL samples required a longer holding time (5 min) to achieve the same reduction (data not shown). The synergistic effect of SL and high pressure treatments has been previously studied in spore inactivation, but at lower pressure and temperature conditions. Shearer et al. (25) investigated the fate of *Bacillus cereus* and *Bacillus coagulans* spores suspended in milk and beef with <1% SL and treated at 392 MPa and 45°C for 10 min. Days later, they found that the combination of SL and high pressure enhanced spore inactivation compared to each treatment alone.

**FIGURE 3.** Populations of *Bacillus amyloliquefaciens* spores subjected to thermal processing (TP; 0.1 MPa, 105°C) or high pressure processing (HPP; 700 MPa and 35°C) with sucrose laurate (1% SL) added before or after the treatment. (a) Thermal processing; (b) high pressure processing. DW, deionized water; SL + treatment, SL added prior to treatment; treatment + SL, SL added after treatment. Dashed horizontal line represents the method’s minimum detection level of viable spores. Means (n = 6) with different letters are significantly different ($P < 0.05$). □, Dormant spores; ■, germinated spores.
Pressure treatment reduced the populations of *B. cereus* and *B. coagulans* spores by 3.0 and 5.5 Log units, respectively, compared with only 1.0 Log unit reduction in the absence of the emulsifier. Stewart et al. (27) reported a dramatic inactivation of the initial *Bacillus subtilis* spore population \(10^6\) CFU/ml suspended in McIlvaine citrate phosphate buffer and treated at 404 MPa and 45°C for 15 min when 0.1% SL was used at pH 6. Moreover, it is important to underscore that the strain used in this study, *B. amyloliquefaciens* TMW 2.479 Fad 82, has previously been found to be highly resistant to elevated temperature and pressure treatment (2, 15). Thus, these results show that SL could be a promising additive to help reduce the severity of PATP treatments.

When SL was added after PATP treatment (Fig. 2), spore inactivation was similar or slightly lower than that of DW samples. These results suggest that the enhanced effect of SL and PATP on spore inactivation mainly occurred when spores were directly in contact with SL under PATP conditions. Spores may be coated by deposition of SL under high pressure, changing their surface hydrophobicity and water permeability, and thus bringing about sporostasis even at low concentrations of SL (11).

Another important result to highlight was that no considerable germination was detected in any of the untreated or PATP-treated samples (Fig. 2). These results are in agreement with those of Ahn and Balasubramaniam (1), who found no germination of *B. amyloliquefaciens* TMW 2.479 Fad 82 spores when present in DW and treated with PATP. Despite the fact that heat shock has been commonly used to determine the percentage of spores that have germinated by high pressure treatments (18, 30), this assay may not be suitable for detecting low levels of germination. Alternatively, germination may be detected by using a suitable nucleic acid stain (e.g., SYTO 16) before analyzing the samples by flow cytometry. Black et al. (5) found that SYTO 16 did not penetrate into dormant spore cores, but germinated spores were stained well.

**Effect of SL on TP and HPP.** The combined effects of SL either with TP or HPP are shown in Figure 3. In the case of TP (Fig. 3a), none of the holding times tested (come-up time, 1, 2, and 5 min) showed any inactivation, but rather a slight increase in spore viability was detected. The addition of SL after applying TP also produced negligible effect. Likewise, HPP (Fig. 3b) had no effect on the *B. amyloliquefaciens* spore populations. No significant germination \((P < 0.05)\) was observed in any of the samples tested.

**Synergistic effect of SL and PATP against spores suspended in MC.** Figure 4 shows the synergistic effect SL and PATP against *B. amyloliquefaciens* spores in MC (pH 5.4), alone or supplemented with 1.0% SL. The combinations of SL and PATP showed a synergistic effect ranging from 0.4 to 1.1 Log units, depending on the holding time applied. However, when comparing the food matrix data (Fig. 4) to DW data for equivalent treatment conditions (Fig. 2), a food matrix protective effect against spore inactivation can be observed. Population of spores suspended in MC with 1.0% SL decreased to the detection level \(<10\) CFU/ml) after 5 min of PATP treatment. However, only a 2-min pressure holding time was required to inactivate the spores to below the detection limit for spores suspended in DW with 1.0% SL (Fig. 2). Shearer et al. (25) reported higher inactivation for *Bacillus sp., Alicyclobacillus sp.,* and *Clostridium sporogenes* spores suspended in food matrices such as milk, beef gravy, orange juice, and...
tomato juice, when different concentrations of sucrose esters were added to the samples before being treated at high pressures and mild or elevated temperatures. In HPP, the protective effect of solutions such as glucose and NaCl and food components such as fat, carbohydrates, and proteins against spore and vegetative cell inactivation has been extensively studied (9, 10, 19, 20, 22). On the other hand, very limited studies reported a food matrix protective effect during PATP treatment. Reddy et al. (23) reported that the protective effect of crabmeat depended on the type of Clostridium botulinum strain and the treatment applied. For C. botulinum strain 62-A spores, none of the treatments tested (414 to 827 MPa, 60 to 75°C, 5 to 20 min) were more effective in crabmeat than phosphate buffer. Rajan et al. (21) did not observe the food matrix protective effect when B. stearothermophilus spores suspended in egg patties were treated at 700 MPa and 105°C. The effect of food matrix under PATP conditions is evident in this study, but more systematic studies are needed to understand the influence of food composition during PATP treatment.

FIGURE 5. Populations of Bacillus amyloliquefaciens spores in deionized water (DW) or mashed carrots (MC) when treated with pressure-assisted thermal processing (PATP; 700 MPa, 105°C for up to 5 min) in combination with sucrose laurate (SL) solutions (up to 1.0%) and recovered on SL-containing agar medium (SLA). (a) DW medium; (b) MC. SL+PATP, SL added prior to PATP treatment; PATP+SL, SL added after PATP treatment. Dashed horizontal line represents the method’s minimum detection level of viable spores. Means (n = 6) with different letters are significantly different (P < 0.05). □, Dormant spores; ■, germinated spores. An asterisk (*) indicates statistically significant spore germination (P < 0.05).
Significant spore germination was detected in all MC samples treated for 1 min and in the samples treated for 2 min without SL (Fig. 4). These results are in agreement with the hypothesis that pressure treatments, applied at elevated temperatures, mainly trigger spore germination by releasing spore DPA content (5, 15, 19). In this case, the heat shock assay was effective to determine the presence of germinated spores. It is possible that some of the germinated spores could be protected by the food matrix against the high temperature and pressure at short holding times.

**Effect of adding SL to the recovery medium.** The presence of SL in the agar medium reduced the recovery of *B. amyloliquefaciens* spores in untreated and in TP-, HPP-, and especially in PATP-treated samples (Figs. 5 and 6). For instance, exposure of spores to 1.0% SL in the recovery medium after subjecting them to a PATP treatment of 1 min at 700 MPa and 105°C produced an extra reduction of 3.1 Log units in DW (see Figs. 2 and 5) and 3.9 Log units in MC (see Figs. 4 and 5) samples, respectively. In fact, this important sporostatic effect has been reported by several
authors. Hayakawa et al. (11) found that high pressure treatments increased the sporostatic effects of some sugar esters even at very low concentrations (0.1 to 10 ppm) on spores of *B. steatorhermophilus*. Shearer et al. (25) observed that the effect of SL appeared to be inhibitory rather than lethal on Bacillus and Aicylocbacillus spores. Moreover, Stewart et al. (27) found that 0.5% SL added to the agar medium inhibited the germination and outgrowth of *C. sporogenes* spores.

**MPN results.** As previously discussed, the spore population suspended in 0.5 and 1.0% SL was reduced to the detection level (<10 CFU/ml) after a 2-min treatment at 700 MPa and 105 °C (Fig. 2). However, after incubating the tubes without adding SL at 32 °C for 15 days, recovery and growth were detected in all samples (Table 3). In the case of samples treated by PATP for 5 min, the recovery was not detected for 1.0% SL samples. For MC samples supplemented with 1.0% SL and treated for 5 min by PATP, the recovery was 2.9 Log MPN/ml after incubating the tubes at 32 °C for 15 days. Some researchers reported that under high pressure and high temperature conditions, spores may slowly complete the germination process due to recovery from damage to their germination system (1, 3, 5, 31). It is important to emphasize that when SL was added to the medium in MPN tubes, recovery and growth were not detected in most of the samples (Table 3).

It may be concluded that SL showed a synergistic effect with PATP to inactivate *B. amyloiliquefaciens* spores and was also effective in inhibiting the recovery of the remaining spore population.

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