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14. ABSTRACT
This project involves an Industry/University Cooperative Research Center consortium between The Ohio State University, the University of California, Davis, and North Carolina State University to assist in advancing food processing and packaging technology and will greatly benefit the US food industry and military personnel. This industry driven consortia center, CAPPS-Center for Advanced Processing and Packaging Studies, focuses on industrially relevant research directed toward coupling microbial life sciences with process and package

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Fourier-transform infrared microspectroscopy and multivariate analysis of *Bacillus amyloliquefaciens* spore inactivation during pressure-assisted thermal processing

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Running Head: Spore inactivation by PATP through FT-IR microspectroscopy

Keywords: Pressure-assisted thermal processing, thermal processing, high pressure processing, *Bacillus amyloliquefaciens*, spore inactivation, FT-IR spectroscopy, infrared spectroscopy

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Abstract

Changes in spore composition of *Bacillus amyloliquefaciens* grown in different sporulation media (TSAYE and NAYE) as influenced by pressure-assisted thermal processing (PATP), high pressure processing (HPP), and thermal processing (TP) were investigated using Fourier-transform infrared spectroscopy (FT-IR). Additional experiments were carried out to compare and contrast the biochemical changes during single- and double-pulse PATP treatments with equivalent holding times. FT-IR spectra discriminated spore samples grown in two sporulation media as well as treated by different processing techniques (PATP, HPP, and TP). PATP and TP treatments caused major changes in calcium dipicolinate (CaDPA) structures as determined by FT-IR bands at 1381, 1415, and 1442 cm^{-1}. These bands corresponds to the contribution of COO^- vibration of CaDPA chelate, the interaction of Ca^{2+} with COO^- group, and pyridine ring vibration of DPA (dipicolinic acid), respectively. Ratio of peak heights at 1381 cm^{-1} and 1442 cm^{-1} indicated the higher amount of CaDPA release by the double-pulse treatment. In addition, impact on amide bands (1540-1650 cm^{-1}) of protein were detected in TP and PATP treated samples. While FT-IR spectra were able to predict microbiological lethality for PATP (single-pulse) and TP treatments, it did not predict lethality changes during double-pulse PATP treatment. This may be possibly due to differences in mechanism of inactivation during single- and double-pulse treatment.

Introduction

Consumers demand minimally processed shelf-stable low-acid foods, with better color, texture, appearance, nutritional values, with minimal or no preservatives. During traditional thermal processing, the product is heated by conduction or convection process and the severity of thermal treatment adversely degrade product quality and destroy heat sensitive ingredients. The food industry is investigating alternative sterilization technologies that can meet consumer demand for minimally processed safe low-acid food products. Among the alternative sterilization technologies investigated, pressure-assisted thermal processing (PATP) gained industrial interest in the recent years. It has a potential for manufacturing shelf-stable low-acid foods such as soups, mashed carrots, coffee, and tea (Balasubramaniam et al., 2008). In February 2009, its application for sterilization of low-acid shelf-stable products was approved by U.S. Food and Drug Administration (FDA) (Anonymous, 2009). The process preserves low-acid foods by using a combination of pressure (500-700 MPa) and temperature (90-121°C) over a short holding time (<10 min). One of the unique advantages of this technology is its ability to provide rapid and uniform temperature increase in the treated food as a result from heat of compression. Expansion cooling also occurs upon depressurization. This limits severity of thermal effect and provides superior product quality.

Application of pressure treatment at ambient temperature effectively inactivates variety of vegetative pathogenic and spoilage microorganisms. On the other hand, pressure in combination with heat is needed for bacterial spore inactivation. Number of earlier studies documented the effectiveness of PATP on inactivation of various bacterial spores including *Bacillus subtilis*, *B. amyloliquefaciens*, *Clostridium botulinum*, *C.*

69 *sporogenes*, and *Geobacillus stearothermophilus* (Margosch et al., 2006; Reddy et al.,
70 2006; Ahn et al., 2007; Zhu et al., 2008; Akhtar et al., 2009; Bull et al., 2009). Pressure
71 pulsing or cycling reported to further enhance spore lethality (Hayakawa et al., 1994;
72 Meyer et al., 2000). Ratphitagsanti et al. (2009) reported that *B. amyloliquefaciens* TMW
73 2.479 Fad 82 lethality was enhanced by 2-4 log CFU/ml when double-pulse treatment
74 was employed over single-pulse treatment at 600 MPa-105℃ for equivalent holding
75 times. Very limited studies have been conducted to evaluate the mechanistic factors
76 contributing to enhanced lethality during double-pulse treatment.
77 Advances in FT-IR spectroscopic instrumentation and multivariate analysis
78 provide capability for rapid detection, identification, and characterization of spoilage and
79 pathogenic microorganisms. FT-IR spectroscopy allows the simultaneous data collection
80 from all frequencies, thus improving its sensitivity. Other advantages of FT-IR
81 spectroscopy include simplicity, rapidity, and high throughput (Naumann 2000). Specific
82 spectral patterns based on chemical and biological composition of samples are obtained
83 and these unique biochemical fingerprints are the key for discrimination and
84 identification among different biological specimen. The FT-IR microspectroscopy (FT-IR
85 coupled with microscope) is effective to detect, identify, and classify various
86 microorganisms at strain levels (Perkins et al., 2005; Schiza et al., 2005; Ngo Thi and
87 Naumann, 2007; Brooke et al., 2008). Subramanian et al. (2006) employed attenuated
88 total reflectance (ATR)-IR spectroscopy as an analytical tool to predict the viable spore
89 counts after thermal and PATP treatments with correlation coefficient (r) of > 0.99 and
90 standard errors of cross-validation ($10^{0.2}$ – $10^{0.5}$ CFU/ml). The authors also found
91 correlation between PATP spore resistance and calcium dipicolinate (CaDPA) content of

the spores when five different bacterial strains were investigated (Subramanian et al., 2007).

The objective of this study was to employ ATR-FTIR microspectroscopy to differentiate biochemical changes of bacterial spores during the inactivation by pressure-assisted thermal processing (especially single- and double-pulse treatments), high pressure processing, and thermal processing.

Materials and Methods

Spore production

Spores of *B. amyloliquefaciens* TMW 2.479 Fad 82 was provided by M. Gänzle from Department of Agricultural, Food and Nutritional Science, University of Alberta, Canada. Activation and isolation of the bacterial culture was done by the 3-phase-streak plate method on trypticase soy agar supplemented with 0.6% yeast extract (TSAYE; Becton, Dickinson and Company, Sparks, MD). The plate was then incubated in an aerobic condition at 32°C for 24 h. An isolated single colony was selected and transferred to a test tube containing trypticase soy broth supplemented with 0.6% yeast extract (TSBYE; Becton, Dickinson and Company). The spore crops were grown in two different sporulation media (Table 1). The first batch of the spore crop was prepared by spread-plating the 100 µl portions of *B. amyloliquefaciens* culture on TSAYE supplemented with 10 ppm $MnSO_4 \cdot H_2O$ (Fisher Scientific, Pittsburgh, PA) (Ahn et al., 2007; Ratphitagsanti et al., 2009). The second spore crop was grown on nutrient agar supplemented with 0.6% yeast extract (NAYE; Becton, Dickinson and Company) and 10 ppm $MnSO_4 \cdot H_2O$ (adapted from Mazas et al., 1995 and Cazemier et al., 2001). The inoculated plates on

115 TSAYE were aerobically incubated at 32°C for 10-14 days, whereas those of NAYE
116 were incubated for 3-5 days to obtain 95% sporulated population. The sporulation was
117 verified by using a phase-contrast microscopy. The surface of inoculated plates was
118 flooded with 10 ml of cold sterile deionized water and the spores were scraped with
119 disposable plastic spreaders. The spore suspension was washed five times by differential
120 centrifugation that ranged from 2,000 to 8,000 x *g* for 20 min each at 4°C. Spore pellets
121 were resuspended in sterile deionized water to obtain ~10^9 spores/ml inocula. The
122 suspension was sonicated for 10 min (SM275HT, Crest, ETL Testing Laboratory,
123 Cortland, NY) following by heat treatment at 80°C for 10 min to destroy any remaining
124 vegetative cells. Both spore suspensions were stored in a 4°C refrigerator.

125 **Sample preparation for PATP, HPP, or TP treatment**

126 For PATP and HPP experiments, spore suspensions (0.2 ml) were inoculated in
127 deionized water (1.8 ml) and packaged in sterile polyethylene pouches (5cm x 2.5cm, 01-
128 002-57, Fisher Scientific) at inoculum levels of ~$1.6x10^8$ CFU/ml for TSAYE crop and
129 ~$2.5x10^8$ CFU/ml for NAYE crop. The pouches were then heat-sealed by an impulse heat
130 sealer (American International Electric, Whittier, CA). After manually mixed the sample
131 content, all pouches were submersed into an ice-water bath (4°C). Samples were treated
132 within 2 h after preparation.

133 Sample preparation for TP experiments were done by aseptically transferred 0.2
134 ml spore suspension of *B. amyloliquefaciens* and 1.8 ml sterile deionized water into an
135 aluminum-TDT-tube to obtain the final spore concentration of ~$2.3x10^8$ and ~$3.4x10^8$
136 CFU/ml for TSAYE and NAYE crops, respectively. All aluminum-TDT-tubes containing

137 the spore suspension were kept in an ice-water bath (4°C) up to 2 h before thermal
138 treatments.

139 **Combined pressure-thermal treatment**

140 Combined pressure-thermal experiments were conducted using a microbial kinetic
141 tester (PT-1 test unit, Avure Technology Inc., Kent, WA). The spore samples were
142 treated to various combined pressure-thermal treatment conditions by adapting published
143 methods reported earlier (Ahn et al., 2007; Ratphitagsanti et al., 2009). The come-up time
144 of the PT-1 unit was 0.67±0.08 min. The sample pouch was placed inside a 10-ml
145 polypropylene syringe (model 309604, Becton, Dickinson and Company) wrapped with
146 insulating material (Sports Tape, CVS® Pharmacy Inc., Woonsocket, RI). The rest of the
147 syringe was filled with ~8 ml of water to ensure uniform temperature within the syringe
148 during treatment.

149 For PATP treatments, the syringe containing spore sample was heated to a pre-
150 processing temperature of 58ºC using water bath (Isotemp 928, Fisher Scientific) for 2
151 min. Preheated syringe was immediately loaded into the PT-1 unit. The pressure chamber
152 was suspended in 105°C propylene glycol (Houghto-Safe-620-TY, Houghton
153 International Inc., Troy, MI) bath. The propylene glycol was also used as the pressure-
154 transmitting fluid within the PT-1 unit. PATP experiments were conducted at 600 MPa-
155 105°C up to 8 min pressure holding times. Additional experiments were conducted to
156 compare the microbial efficacy of single- and double-pulse treatments at an equivalent
157 pressure holding time of 3 min. During double-pulse treatment, samples were taken for
158 microbial and FT-IR analyses after various stages (C_1, D_1, B_2, C_2, and D_2; Figure 1B).
159 Spore sample pouches subjected to pressure-thermal treatment were immediately

160 immersed into an ice-water bath to cool the samples. Microbial analysis was conducted
161 on the same day of experiments, while FT-IR samples were kept in a refrigerator up to 24
162 h before the analysis.
163 High pressure processing experiments (600 MPa-35°C up to 70 min holding time)
164 were also performed by using a similar approach to that of PATP samples. The pre-
165 processing temperature for HPP spore samples was 4.9±0.8°C. An untreated spore
166 suspension of each spore crop was used as controls.
167 **Thermal treatment**
168 Thermal inactivation of spores was conducted at 105°C-0.1 MPa using a 35L
169 heating bath circulator (NESLAB EX-35 Digital One, Thermo Fisher Scientific Inc.,
170 Waltham, MA). Bath oil (Temperature range: -7 to 176°C, O220, Fisher Scientific) was
171 used as heating fluid. The aluminum-TDT-tubes containing spore samples were pre-
172 heated for 2 min at 58°C water bath before further subjected to 105°C oil bath. Sample
173 temperature was monitored by inserting a K-type thermocouple attached to a data logger
174 into a control aluminum cell containing sterile deionized water without spores. The
175 thermal come-up time to reach 105°C was 2.33 min. The tubes were removed from the
176 oil bath after the come-up time and different holding times (up to 240 min) and then
177 immediately immersed into an ice-water bath. Standard plate count method was used to
178 enumerate spores surviving the thermal treatment as well as untreated spore suspensions
179 on the same day of processing. Samples for FT-IR analysis were kept in a refrigerator and
180 the FT-IR analysis was carried out within 24 h after thermal treatments.
181 **Enumeration of spore survivors**

Spore samples grown on different sporulation media (TSAYE and NAYE supplemented with 10 ppm $MnSO_4$) and processed by various treatments were enumerated as follows. Sample contents (1 ml) were serially diluted in 0.1% peptone water and then spread-plated in duplicate on TSAYE. After incubation at 32°C up to 72 h, the viable count of spore survivors were enumerated.

Fourier-transform infrared microspectroscopy

FT-IR analysis of treated spore samples was modified from Subramanian et al. (2006) and Männig et al. (2008). Aliquots (500 µl) were centrifuged at 13,000 rpm and 4°C for 4.5 min. After removing the supernatant, the spore pellet was washed with 100 µl sterile deionized water and re-centrifuged at the same condition. The pellet was then re-suspended with 5 µl sterile deionized water, applied onto hydrophobic Neo-grid filter membrane (NGFM; Neogen Corporation, Lansing, MI). The NGFM filters with deposited spores were then vacuum-dried to form a thin film. Infrared spectroscopic studies were carried out by using an Excalibur 3500GX FT-IR microscopy spectrometer (Varian, Palo Alto, CA) in Attenuated Total Reflectance (ATR) mode. The spectra were collected from the wavenumbers of 4000 to 700 cm^{-1} (mid-infrared) region with a total of 128 scans at a resolution of 8 cm^{-1}. The FT-IR spectrometer was equipped with a PERMAGLOW mid-IR source, an extended-range KBr beam splitter, and a deuterated triglycine sulfate detector. Aliquot from each treated sample were applied onto three individual spots on the membrane and two measurements were taken at different locations on each spot. At least two to five independent replications of PATP, HPP, and TP treatments were carried out resulting in 12-30 spectra per sample per treatment time (six spectra per replication).

205 **Multivariate analyses**

206 Pirouette® (version 3.11, Infometrix Inc., Woodville, WA) comprehensive
207 chemometrics modeling software was employed to transform the spectra to their 2nd
208 derivatives using a Savitzky-Golay polynomial filter (five-point window), mean-centered,
209 and vector-length normalized. Classification analysis of samples processed at various
210 conditions was further analyzed using principal component analysis algorithm (soft
211 independent modeling by class analogy; SIMCA). Principal component analysis extracts
212 information from the data set onto few dimensions, which are accounted for maximum
213 possible variance (Mark, 2001). The spectral wavenumbers and their associated
214 functional groups responsible for the classification of the spores could be identified using
215 the discriminating power plot based on the measure of variable importance by
216 minimizing the difference between samples within clusters and maximizing those from
217 different clusters (Dunn and Wold, 1995). Correlation between specific spectral
218 information (900 – 1800 cm^{-1} region) and spore survivor curve (obtaining from the
219 standard plate count) were determined using partial least squares regression (PLSR),
220 which utilized large number of dependent variables to predict the viable spores surviving
221 the treatments. A nonlinear iterative partial least-squares (NIPALS) algorithm was
222 employed. PLSR with cross-validation (iterative recalculation of the model omitting a
223 different sample point each time) was used to test for the model sensitivity.

224 **Statistical analysis**

225 Statistical Analysis System software (SAS 9.1, SAS Institute Inc., Cary, NC) was
226 used for data analysis. Independent variables were treatment (PATP single- and double-
227 pulse, TP, and HPP), holding time, and sporulation media (TSAYE and NAYE), whereas

log reduction (Δ log CFU/ml) in response to the treatments served as the dependent variable. The data was analyzed by the SAS program with the general linear model (GLM) procedure. The mean comparison were evaluated with the Tukey's test at a 5% significant level ($P = 0.05$).

RESULTS AND DISCUSSION

Pressure and temperature history during PATP pulsing treatments and spore lethality

Figure 1 provides sample pressure and temperature history of single- and double-pulse treatment at 600 MPa-105°C for 3 min holding time. Process temperature of the single-pulse treatment was well maintained at 105°C as the external glycol bath was heated to the same temperature. Even though both single- and double-pulse treatments had an equivalent time of 3 min under pressure, double-pulse treatment had higher total treatment time (~ 5.4 min) which included pause time between two pulses (1 min) as well as an additional pressurization time for 2nd pulse (0.7 min). This longer treatment time during double-pulse may also lead to higher process temperature values during the 2nd pulse. For example, during 1st pulse holding time (1.5 min), the process temperature (105.5 ± 0.4°C) was maintained. Upon depressurization of the 1st pulse, the temperature dropped to 77°C. Subsequently during pause time between pulses (D_1-B_2), spore sample temperature increased to ~78°C due to heat transfer from the surrounding glycol bath which was kept at 105.5°C. This resulted in higher process temperature of 112 ± 0.9°C during the 2nd pulse holding time (C_2-D_2). It is further worth to note that the temperature history obtained during double-pulse treatment likely further influenced by pressure

equipment design parameters (such chamber volume, pressurization rate, chamber insulation characteristics etc). The current study utilized a pressure chamber volume of ~20 ml and had relatively faster pressurization rate (~14 MPa/s). Accordingly, care must be taken in extrapolating the results of this study to larger pilot scale equipments.

At the equivalent 3 min holding time, enhanced spore lethality were observed in the double-pulse treatment (Table 2). Spores grown from TSAYE and NAYE media provided additional 2.6 log reduction from the double-pulse treatment than that from the single-pulse PATP treatment (600 MPa-105°C for 3 min). It is worth to note that the majority of the spore inactivation during double-pulse treatment (Table 2) took place during the 2nd pulse holding time where the spores were subjected to 600 MPa-112°C treatment for 1.5 min.

Combined pressure-thermal resistance influenced by sporulation media

Among the two sporulation media, differences in spore resistant property were observed. Spore crops grown on NAYE produced higher PATP and TP resistant spores. $D_{105°C\text{-}0.1\ MPa}$ values of the spore crop grown on TSAYE and NAYE were 28.1±1.2 min and 36.8±1.5 min, respectively. Similarly, D values of PATP treated spores grown on TSAYE and NAYE media were 1.0±0.1 min and 1.4±0.2 min at 600 MPa-105°C.

It has been well-documented that sporulation media impact spore heat resistance (Cazemier et al., 2001; Mah et al., 2008). Figure 2A shows that FT-IR could differentiate the two spore crops based on their resistant properties as influenced by different sporulation media. The corresponding peaks differentiated the properties among these two spore crops were found at 1388 and 1577 cm^{-1} from the discriminating power plot

(data not shown). These bands are associated to the stretching bands of COO^- group of Ca-DPA chelate and the C-N vibrations of the pyridine ring, respectively. The discriminating power is a measure of variable importance (i.e., IR frequency), which contributes to the development of the SIMCA pattern recognition and classification (Dunn and Wold, 1995). Results indicated that different levels of Ca-DPA chelate being inherited in the spore cores during sporulation. Moreover, FT-IR could also be used to ensure consistency and reliability of pressure-thermal resistance of each new spore crop. Differences in resistant property among TSAYE grown spores from various spore crop preparation were observed (Figure 2B). This may facilitate the evaluation of process resistance of the untreated spore crops before being treated.

Classification of bacterial spores treated by thermal and pressure-assisted thermal processing

Hydrophobic grid membrane filters (HGMFs) overlaid on a selective medium was previously used to isolate a single colony of *Salmonella* serovars (Männig et al., 2008). In this current study, a protocol was developed for the classification of bacterial spores treated by various processing methods by combining a hydrophobic grid membrane filters with infrared spectroscopy (Subramanian et al., 2006). Use of the hydrophobic grid membrane enabled direct spectroscopic observation of the biochemical changes in treated spores. The membrane confined spores within the barriers of the grid, while vacuum drying helps to limit the interference from water absorption bands. This sample preparation method was simple and improved signal intensity.

296 Figure 3A and 3B provides a sample SIMCA model illustrating consecutive
297 changes in PATP and TP treated bacterial spores. FT-IR spectroscopy was clearly able to
298 discriminate changes in untreated spore samples against that of PATP or TP treated
299 samples. In TP treated samples, the biochemical changes gradually occurred over 240
300 min holding time (Figure 3B). Based on interclass distance (≤ 3), the treated samples with
301 similar changes in biochemical properties were grouped together resulting various
302 distinct clusters (Figure 3A and 3B). Interclass distances are Euclidian distances between
303 centers of clusters, which could be used as an indicator in SIMCA classification model
304 (Kvalheim and Karstang, 1992). In general, large interclass distances (above 3)
305 demonstrate well separation among the classes. PATP resulted in rapid lethality over a
306 short time. Similarity in biochemical composition of the treated spores was observed after
307 2 min-PATP treatment as indicated by the same cluster containing the treated samples
308 from 2-8 min holding times (Figure 3A). As expected, pressure alone (600 MPa-35^{o}C, up
309 to 70 min) did not clearly distinguish the untreated and treated spore samples into groups
310 since *B. amyloliquefaciens* spores were not inactivated at this condition (data not shown).
311
312 **Biochemical changes associated with PATP treated spores**
313 The spectral wavenumbers and the associated functional groups that were
314 responsible for the classification of the spores in SIMCA class projections were identified
315 using the discriminating power plot (Figure 4). The higher the value of the discriminating
316 power, the greater is the influence of that wavenumber in classifying the samples that are
317 in the model (Lavine, 2000). In comparison to untreated control, PATP caused
318 predominant changes in the region of 1384, 1415, 1446 cm^{-1} in both TSAYE and NAYE

319 grown spores. The identified bands represent changes in dipicolinic acid (pyridine-2,6-
320 dicarboxylic acid; DPA) structure, especially the contribution of COO^- vibration of
321 CaDPA chelate (1384 cm^{-1}), the COO^- stretching vibration in the presence of Ca^{2+} (1415
322 cm^{-1}), and the DPA pyridine ring vibration (1442-1446 cm^{-1}) (Byler and Farrell, 1989;
323 Cheung et al., 1999; Goodacre et al., 2000; Perkins et al., 2005). DPA is always chelated
324 with divalent cations, especially calcium ions in a 1:1 ratio as CaDPA. This unique
325 component is only present in bacterial spores and it represents about 5-10% of the dry
326 weight of *Bacillus* spores (Setlow et al., 2006).
327 Highly resistant PATP-treated NAYE grown spores showed additional
328 discriminating power peaks at 1350 cm^{-1} (absorption due to lipids) and 1577 cm^{-1} (C-N
329 vibrations of the DPA pyridine ring or COO^- group of acidic amino acid residues of spore
330 proteins) (Cheung et al., 1999; Wolfangel et al., 1999; Sahu et al., 2006) (Figure 4).
331 NAYE grown spores also caused changes in the protein region, specifically at 1635 cm^{-1}
332 (amide I of β-pleated sheet of secondary proteins), 1543 cm^{-1} (amide II involved
333 stretching vibration of C-N groups), and 1273 cm^{-1} (amide III band of proteins or CaDPA
334 band) (Helm and Naumann, 1995; Cheung et al., 1999; Schiza et al., 2005).
335 Table 3 presents discriminating power values at selected CaDPA bands (~1377-
336 1384, ~1411-1415, and ~1438-1446 cm^{-1}) and the corresponding log reduction of
337 bacterial spores grown in different sporulation media during PATP, TP, and HPP. In
338 general, discriminating power values increased with increase in holding times, within the
339 same treatment condition and sporulation media. Although this observation appeared to
340 hold good in most cases, the variation in the band intensity during TP holding times was

341 also observed. This might be due to the heterogeneity of spore populations as well as
342 variability in pressure-thermal histories during PATP and TP replications.
343
344 **Contrast between single- and double-pulse PATP treatments**
345 Figure 5 compares the discriminating power in classification of bacterial spores
346 grown in TSAYE and NAYE media and subjected to single- and double-pulse PATP
347 treatments. In general, regardless of the growth media, both single- and double-pulse
348 treatments showed similar changes on the bands associated to CaDPA chelate, in
349 particular at 1377-1381, 1411, and 1435-1442 cm^{-1} (Figure 5). However, single-pulse
350 treatment produced significantly higher discriminating power values than double-pulse
351 treatment. According to Cheung et al. (1999), the ratio of peak heights at wavenumbers
352 1379 and 1443 cm^{-1} could be used as an indicator of the CaDPA levels in bacterial spores.
353 The higher the ratio, the more the CaDPA exists in the spores. Presence of high levels of
354 CaDPA chelate, the relatively low content of core water, and the saturation of spore DNA
355 with a group of small acid-soluble proteins (SASPs) play major roles in spore resistance
356 properties (Setlow and Setlow, 1995). Specifically, the DNA-α/β-type SASP complex
357 was the primary contributor to spore thermal stabilities, while divalent cations (Ca^{2+},
358 Mn^{2+}, Mg^{2+}) and DPA provide synergistic effect on stability protecting the spore DNA
359 against heat (Setlow et al., 2006). Both sporulation media demonstrated higher ratio of
360 peak height associated to COO^- vibration of CaDPA (~1377-1381 cm^{-1}) and pyridine ring
361 vibration (~1435-1442 cm^{-1}) in the single-pulse treatment. The peak ratios of single- and
362 double-pulse treatments were 3.0 and 2.5 for TSAYE grown spores, as well as 1.5 and
363 1.2 for NAYE grown spores, respectively. The ratio suggested less CaDPA release from

364 the single-pulse treatment than that of the double-pulse treatment, indicating less lethality
365 at the equivalent holding time.
366
367 **Biochemical changes associated with TP treated spores**
368 At 105°C-0.1 MPa, it took more than 70 min to obtain any measurable spore
369 inactivation (1.1-2.5 log reduction) in both spore crops. After 180 min holding time at
370 105°C, TSAYE grown spores were inactivated to undetectable level and NAYE grown
371 spores had about 4.7 log reduction. FT-IR analysis of the TP spores indicated that
372 discrimination was largely influenced by the similar bands observed from the PATP
373 treatment, specifically at 1384, 1411, and 1442 cm^{-1} (Figure 6). Biochemical changes
374 among particular samples could be gradually monitored (data not shown). Three distinct
375 groups of PATP treated samples were classified based on their interclass distance.
376 Samples were grouped together when the interclass distance was lower than 3.
377 Differences observed between the untreated control and the 1st group of treated samples
378 (0, 2, 5 min) was mainly associated with the interaction of the Ca^{2+} with COO^- groups
379 and the stretching of COO^- group of CaDPA chelate (1419 and 1381 cm^{-1}). When
380 comparing changes between the 1st group (0, 2, 5 min) and the 2nd group (30, 70, 120
381 min), it was evident that bands associated with clustering of samples were related to the
382 DPA pyridine ring vibration (~1446 cm^{-1}), which became very prominent for
383 discrimination of the 2nd group (30, 70, 120 min) from the 3rd group (180, 240 min) (data
384 not shown).
385 Figure 7 demonstrates the influence of specific PATP and TP treatments that yield
386 comparable 3 log reduction to *B. amyloliquefaciens* TMW 2.479 spores (NAYE grown).

It required at least 120 min at 105°C-0.1 MPa to achieve ~3 log reduction, while 5 min holding time at 600 MPa-105°C PATP treatment provided similar result. Both PATP and TP specifically acted upon CaDPA chelate (1276, 1373, 1411, and 1612 cm^{-1}) and its pyridine ring (1438 and 1573 cm^{-1}) (Figure 7). FT-IR results supported that there were similar changes happening in the structural level of DPA and CaDPA among the two treatments at the same lethality. However, the bands obtained from PATP were clearly much higher in discriminating power values (~45,000 arbitrary units) than that from TP treatment (~14,000 arbitrary units). This indicated that the biochemical changes of bacterial spores taking place during PATP was at the greater intensity than TP.

Quantification of spore survivors from infrared spectra and validation of PLSR models

Surviving spore populations of *B. amyloliquefaciens* TMW 2.479 after PATP and TP treatments could be estimated by cross-validated PLSR models using spectral region (900-1800 cm^{-1}) (Figure 8-9). The leave-one-out cross validation generally removes one sample from the training set, performed PLSR on the remaining samples. Then, it predicts the log spore survivors from the left-out sample and sums up the error until the total samples in the training set is analyzed. Good correlation on spore survivors obtained from the standard plate count and the mid-infrared spectral regions was found for both PATP and TP treatments (Figure 8-9). High coefficients of correlation ($r > 0.96$) and low standard errors of cross-validation (SECV ~ $10^{0.16}$ – $10^{0.26}$ CFU/ml) were obtained from all PLSR models.

To verify whether or not single- and double-pulse treatments followed similar mechanism of inactivation, the developed PLSR model based on single-pulse PATP treatment was used to predict the spore survivors during various stages of the double-pulse treatment. Figure 5.10 shows the predicted spore survivors by FT-IR spectra and the experimental values obtained from the standard plate count during each stage of double-pulse treatment of *B. amyloliquefaciens* TMW 2.479 spores grown on different sporulation media. FT-IR spectra microbial lethality model based on single-pulse could not predict double-pulse lethality changes during 2^{nd} pulse pressure holding time (D_2), possibly due to differences in respective spore inactivation mechanisms. It is further possible that some of the biochemical changes were not detected during double-pulse treatment by FT-IR microspectroscopy. The single-pulse based PATP lethality model was successful in predicting (< 0.6 log CFU/mL) up to C_2 (2^{nd} pulse come-up time; Figure 5.1.B and Figure 5.10) only. More studies are needed using advanced high resolution spectroscopic techniques such as Raman to further understand the nature of biochemical changes under these conditions.

Conclusions

A study was conducted to investigate PATP, HPP, and TP treatment effects on biochemical changes of *B. amyloliquiefaciens* spores. Both PATP and TP caused rise of CaDPa bands at approximately 1384, 1415, and 1442 cm^{-1}. The intensity of these bands in general increased with increasing treatment times. For an equivalent log reduction, PATP showed higher intensities than that of TP. Changes in lipids and polypeptides were also evident in PATP-treated highly resistant NAYE grown spores. Release of CaDPA

from the spore core served as a key component indicating the inactivation of *B. amyloliquefaciens* TMW 2.479 spores. FT-IR spectra of the bacterial spores were not only influenced by the processing conditions, but also by the sporulation media.

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Table 1. Approximate formula per liter of media used for sporulation

Media component	Amount (g/L)
Difco™ Tryptic soy agar (TSAYE)	
Pancreatic digest of casein	15
Papaic digest of soybean	5
Sodium chloride	5
Agar	15
Yeast extract	6
Difco™ Nutrient agar (NAYE)	
Beef extract	3
Peptone	5
Agar	15
Yeast extract	6

Both media were supplemented with 10 ppm $MnSO_4$.
Source: Becton, Dickinson and Company (Sparks, MD)

Table 2. Log reduction of *Bacillus amyloliquefaciens* TMW 2.479 spores after single- and double-pulse treatments at 3 min equivalent pressure holding time

Total treatment time*	Log reduction (log CFU/ml)	
	TSAYE grown†	NAYE grown†
Single-pulse		
0.7 min (B-C; after come-up time)	0.2±0.3 ^{A}	0.3±0.1 ^{A}
3.7 min (B-D; after holding time)	-3.2±0.1 ^{E}	-2.5±0.3 ^{D}
Double-pulse		
0.7 min (B_1 -$C_{1;}$ after 1st pulse come-up time)	0.2±0.3 ^{A}	0.3±0.1 ^{A}
2.2 min (B_1-$D_{1;}$ after 1st pulse)	-1.5±0.2 ^{BC}	-1.1±0.2 ^{B}
3.2 min (B_1-$B_{2;}$ after pause time)	-1.6±0.2 ^{C}	-1.2±0.1 ^{BC}
3.9 min (B_1-C_2; after 2nd pulse come-up time)	-2.2±0.3 ^{D}	-1.6±0.3 ^{C}
5.4 min (B_1-$D_{2;}$ after 2nd pulse)	-5.8±0.3 ^{G}	-5.1±0.3 ^{F}

*See Figure 1A and 1B for nomenclature. Total processing time includes pressure come-up time, pressure holding time, and depressurization time.

†Initial population of untreated controls: TSAYE -1.6 x 10^8 CFU/ml; NAYE - 2.5 x 10^8 CFU/ml.

Means with the same letter are not significantly different.

Table 3. Discriminating power values at bands associated to CaDPA structures and log reduction corresponding to treatments.

Treatment condition and holding time	TSAYE grown spores					NAYE grown spores				
	Wavenumber (cm^{-1})			Ratio of peak height $\frac{1384}{1442}$ cm^{-1}	Δ log CFU/ml	Wavenumber (cm^{-1})			Ratio of peak height $\frac{1384}{1442}$ cm^{-1}	Δ log CFU/ml
	~1377-1384	~1411-1415	~1438-1446			~1377-1384	~1411-1415	~1438-1446		
PATP (600 MPa-105°C)										
0 min	67	179	48	1.4	-0.1±0.2 ^{AB}	212	621	8	26	0.1±0.2 ^{AB}
0.5 min	348	1399	645	0.5	-0.7±0.2 ^{BCD}	938	6192	59	16	-0.1±0.2 ^{AB}
1 min	1325	2433	2851	0.5	-1.5±0.2 ^{E}	2216	10829	168	13	-0.9±0.0 ^{CDE}
2 min	1304	3226	2301	0.6	-2.5±0.3 ^{F}	4195	11146	620	7	-1.7±0.0 ^{E}
5 min	2369	4152	3016	0.8	-5.1±0.3 ^{I}	4919	31230	570	9	-3.4±0.2 ^{G}
8 min	1417	2779	2943	0.5	-5.8±0.3 ^{J}	4363	42730	657	7	-5.2±0.1 ^{IJ}
TP (105°C-0.1 MPa)										
0 min	51	260	103	0.5	0.2±0.2 ^{A}	8	20	22	0.3	0.3±0.2 ^{A}
2 min	140	229	184	0.8	0.2±0.0 ^{A}	63	271	241	0.3	0.3±0.2 ^{A}
5 min	1956	1524	969	2.0	0.2±0.2 ^{A}	190	680	309	0.6	0.3±0.1 ^{A}
30 min	608	1335	1411	0.4	-1.1±0.0 ^{DE}	583	2164	1554	0.4	-0.1±0.1 ^{ABC}
70 min	750	2501	2359	0.3	-2.5±0.1 ^{F}	743	2446	2516	0.3	-1.1±0.4 ^{DE}
120 min	1001	2884	1949	0.5	-3.9±0.1 ^{GH}	2641	9643	6334	0.4	-2.6±0.5 ^{F}
180 min	798	6725	4179	0.2	< D.L.	6430	28580	30803	0.2	-4.7±0.4 ^{HI}
240 min	1040	4888	3136	0.3	< D.L.	4571	46611	21886	0.2	-5.1±0.1 ^{IJ}
HPP (600 MPa-35°C)										
0 min	n/a	n/a	n/a	-	-0.1±0.1 ^{AB}	n/a	n/a	n/a	-	0.02±0.0 ^{AB}
5 min	n/a	n/a	n/a	-	0.2±0.0 ^{A}	n/a	n/a	n/a	-	0.1±0.1 ^{AB}
30 min	n/a	n/a	n/a	-	0.2±0.1 ^{A}	n/a	n/a	n/a	-	0.2±0.1 ^{A}
70 min	n/a	n/a	n/a	-	0.03±0.1 ^{AB}	n/a	n/a	n/a	-	0.3±0.1 ^{A}

< D.L. represents spore survivors under method detection limit (< 10^2 CFU/ml).
n/a: data not available. SIMCA could not differentiate the treated samples into distinctive groups.
Means with the same letter are not significantly different.

FIGURE LEGENDS

Figure 1. Sample pressure (----------) and temperature (———) histories during single- (A) and double-pulse (B) treatment. Processing times include pre-process time in a conditioning bath and pressure chamber (A-B_1), pressure come-up time (B_1-C_1 and B_2-C_2), pressure holding time (C_1-D_1 and C_2-D_2), and depressurization time (< 2 s). Glycol bath temperature was maintained at 105.5°C. Time pausing between two pulses (D_1-B_2) was kept at 1 min.

Figure 2. Soft independent modeling by class analogy on resistant property of untreated *Bacillus amyloliquefaciens* TMW 2.479 spores as influenced by different sporulation media (A) and different spore crop preparations using TSAYE as a sporulation medium (B)

Figure 3. Soft independent modeling by class analogy of different class projections of *Bacillus amyloliquefaciens* TMW 2.479 spores (NAYE grown) after pressure-assisted thermal processing at 600MPa-105°C (A) and thermal processing at 105°C-0.1 MPa (B).

Figure 4. Discrimination power plot in classification of pressure-assisted thermal processing treated *Bacillus amyloliquefaciens* TMW 2.479 spores grown on two different media.

Figure 5. Comparison of single- (SP) and double-pulse (DP) treatments on *Bacillus amyloliquefaciens* TMW 2.479 spores processed at equivalent holding time for 3 min; TSAYE grown spores (A) and NAYE grown spores (B).

Figure 6. Discrimination power plot in classification of *Bacillus amyloliquefaciens* TMW 2.479 spores grown on two different media after thermal processing at 105°C-0.1 MPa.

Figure 7. Comparison of discriminating power plot of *Bacillus amyloliquefaciens* TMW 2.479 spores grown on NAYE at 3 log reduction after TP (105°C, 0.1 MPa, 120 min) and PATP (600 MPa, 105°C, 5 min) treatments.

Figure 8. Cross-validated (leave-one-out) partial least squares regression plots for spore inactivation by pressure-assisted thermal processing; TSAYE grown spores (A) and NAYE grown spores (B).

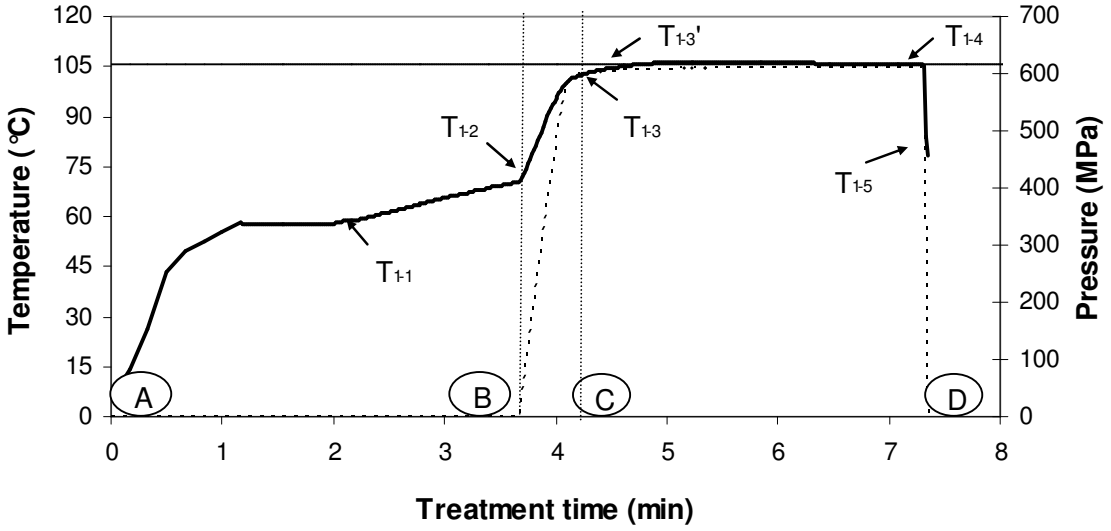
Figure 9. Cross-validated (leave-one-out) partial least squares regression plots for spore inactivation by thermal processing; TSAYE grown spores (A) and NAYE grown spores (B).

Figure 10. Predicting microbial efficacy of double-pulse PATP treatment for *B. amyloliquefaciens* TMW 2.479 spore survivors based on single-pulse FT-IR spectra model. Predicted spore survivors by FT-IR spectra (_____) and measured spore

survivors by standard plate count (◆); TSAYE grown spores (A) and NAYE grown spores (B). Refer to Figure 1B for nomenclature during the double-pulse treatment.

FIGURE 1

(A)


Temperature (°C)
Pressure (MPa)
Treatment time (min)
T1-1
T1-2
T1-3
T1-3'
T1-4
T1-5
A
B
C
D

(B)

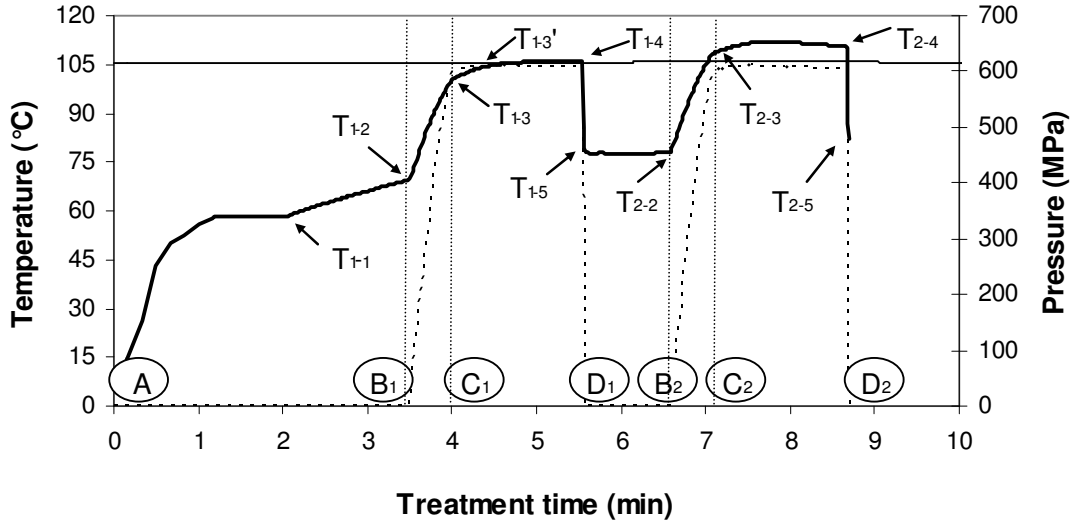
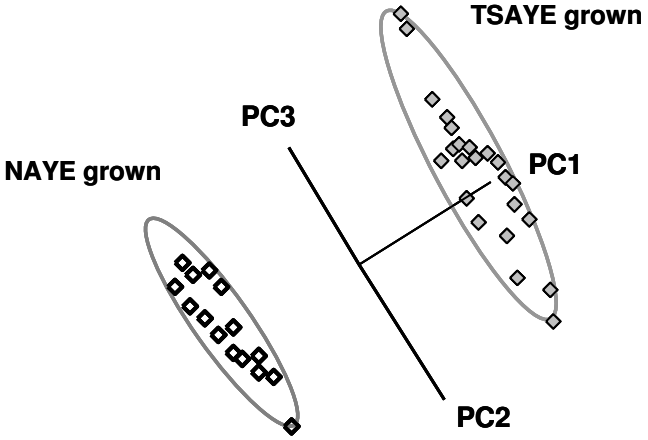

Temperature (°C)
Pressure (MPa)
Treatment time (min)
T1-1
T1-2
T1-3
T1-3'
T1-4
T1-5
T2-2
T2-3
T2-4
T2-5
A
B1
C1
D1
B2
C2
D2

FIGURE 2

(A)


TSAYE grown
PC3
NAYE grown
PC1
PC2

(B)

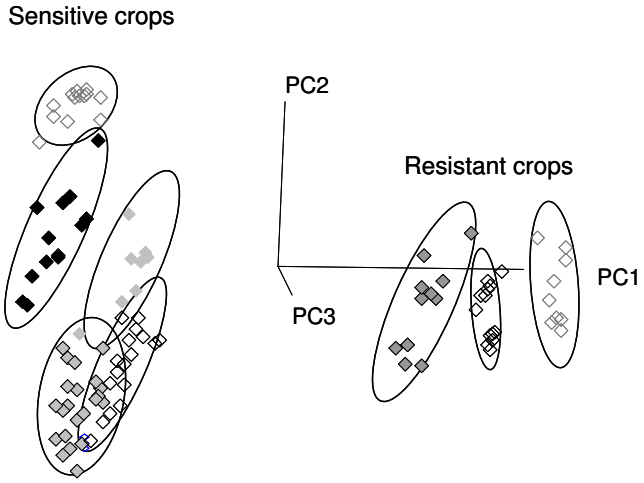
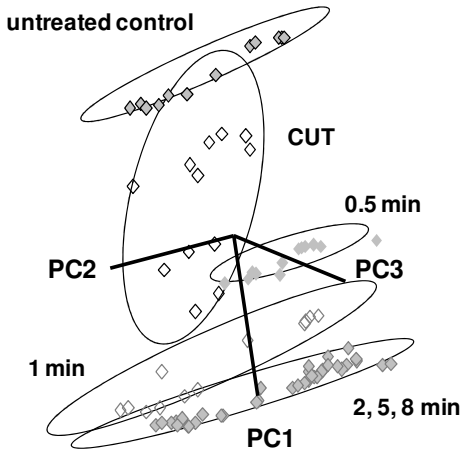

Sensitive crops
PC2
Resistant crops
PC1
PC3

FIGURE 3

(A)


untreated control
CUT
0.5 min
PC2
PC3
1 min
2, 5, 8 min
PC1

(B)

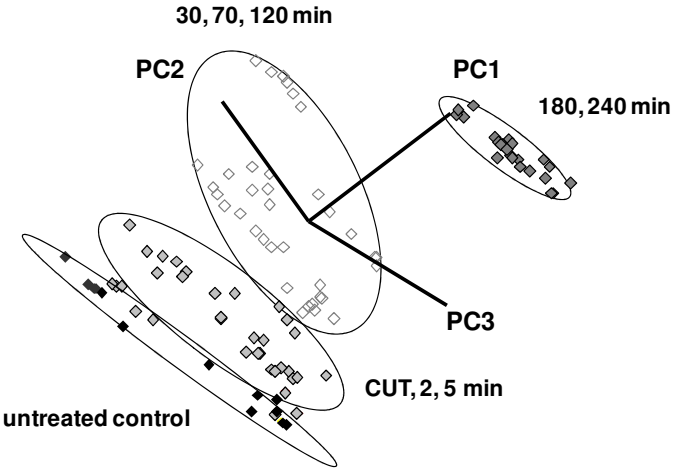

30, 70, 120 min
PC2
PC1
180, 240 min
PC3
CUT, 2, 5 min
untreated control

FIGURE 4

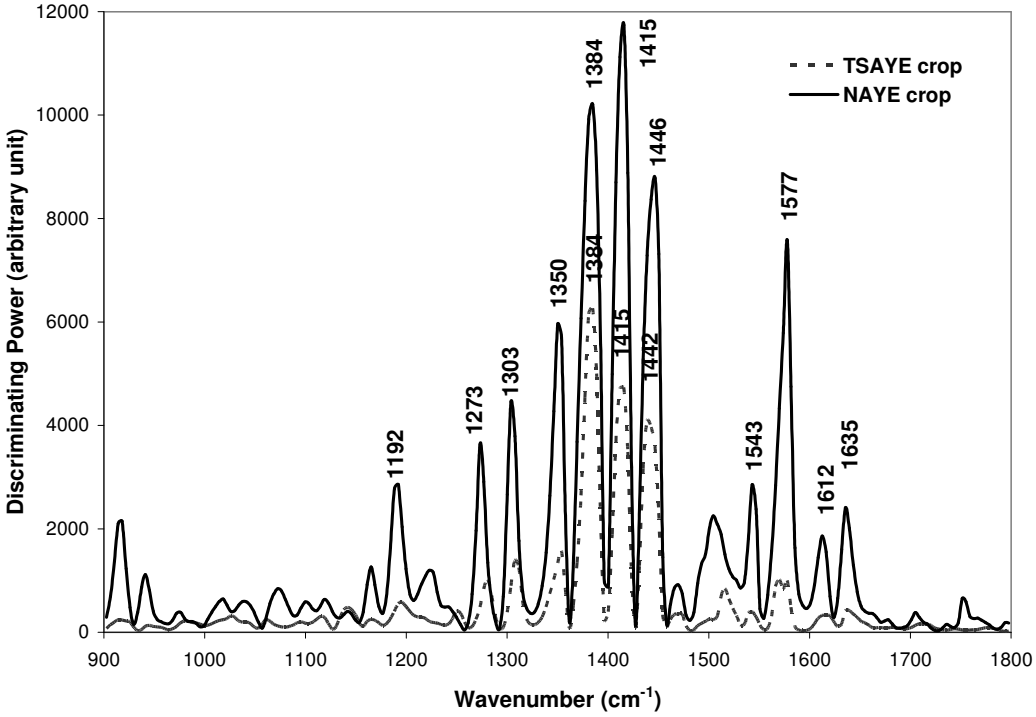
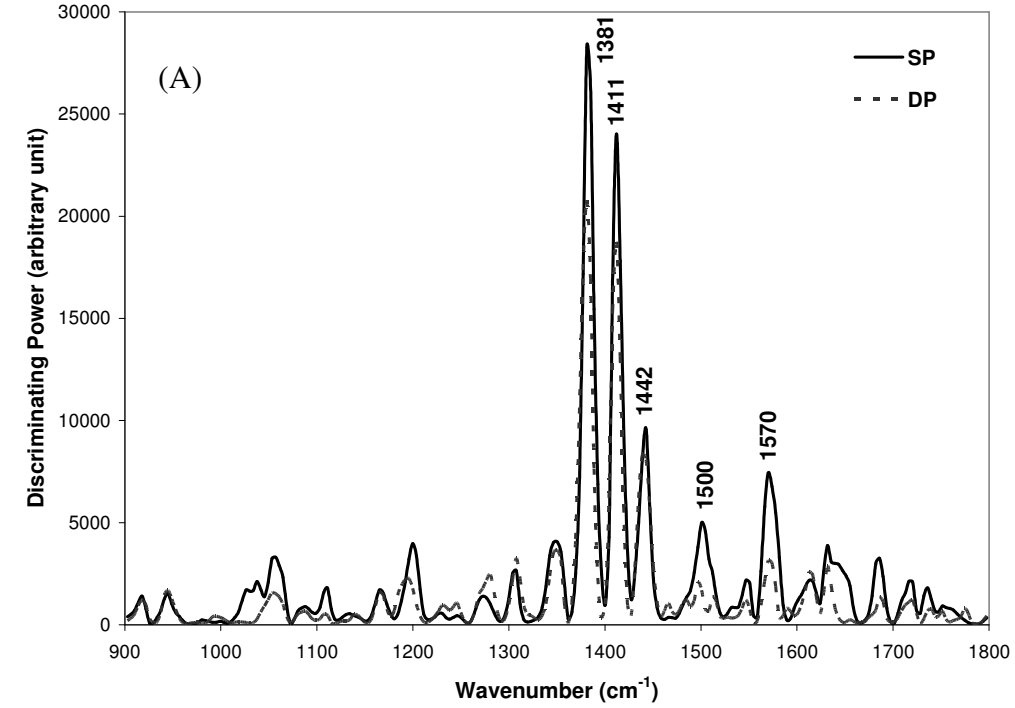

Discriminating Power (arbitrary unit)
Wavenumber (cm-1)
TSAYE crop
NAYE crop
1192
1273
1303
1350
1384
1384
1415
1415
1442
1446
1543
1577
1612
1635

FIGURE 5


(A)
SP
DP
Discriminating Power (arbitrary unit)
Wavenumber (cm-1)
1381
1411
1442
1500
1570

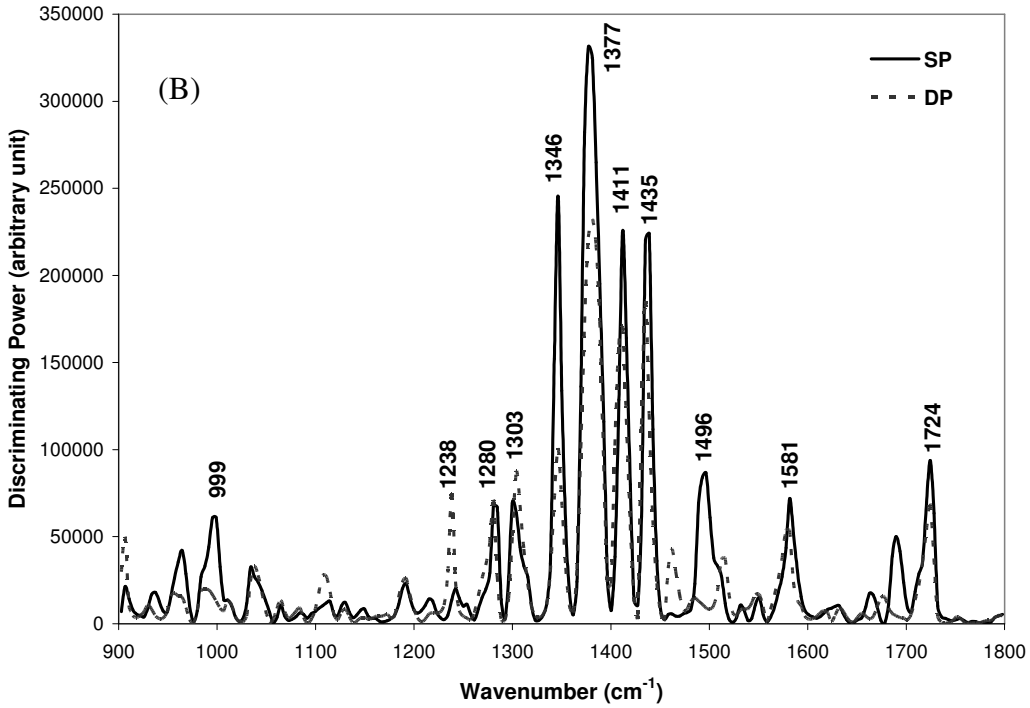

(B)
SP
DP
Discriminating Power (arbitrary unit)
Wavenumber (cm-1)
999
1238
1280
1303
1346
1377
1411
1435
1496
1581
1724

FIGURE 6

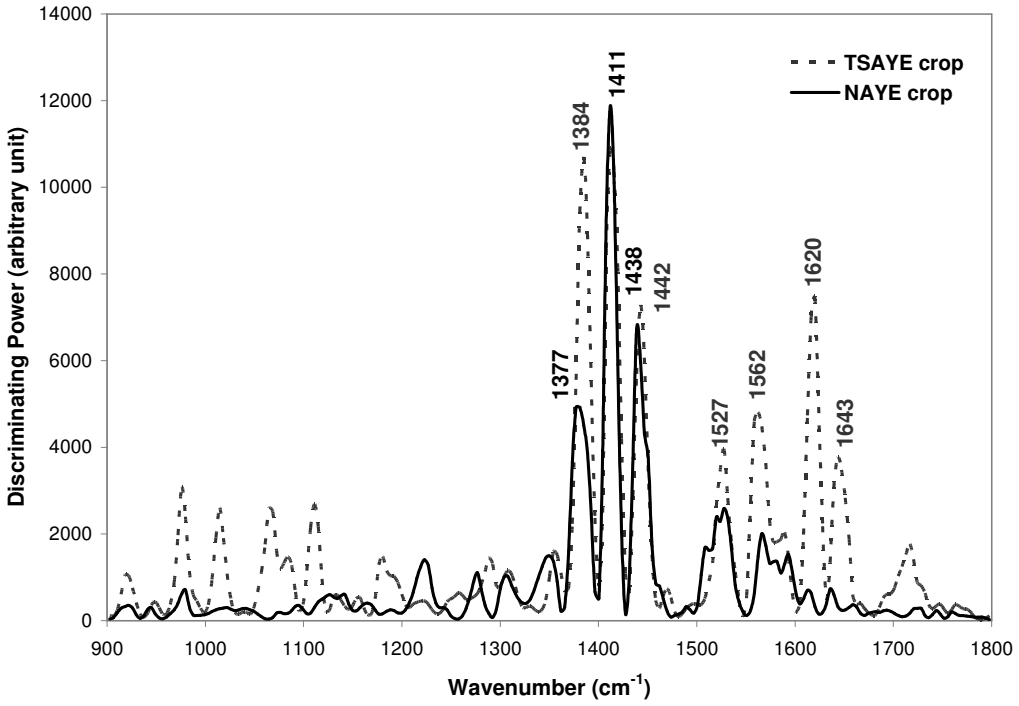

14000
12000
10000
8000
6000
4000
2000
0
900
1000
1100
1200
1300
1400
1500
1600
1700
1800
Discriminating Power (arbitrary unit)
Wavenumber (cm-1)
TSAYE crop
NAYE crop
1377
1384
1411
1438
1442
1527
1562
1620
1643

FIGURE 7

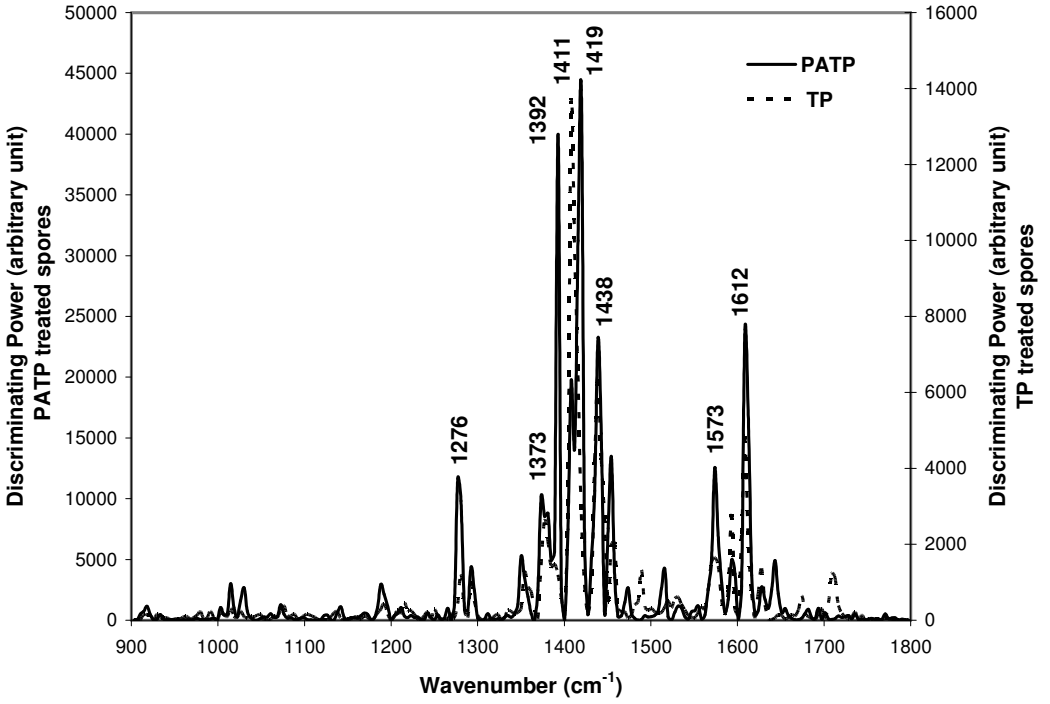
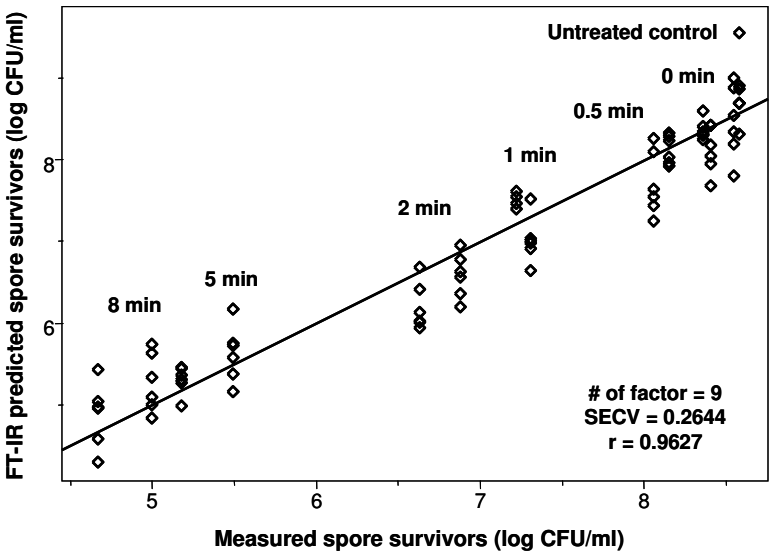

PATP
TP
1276
1373
1392
1411
1419
1438
1573
1612
Discriminating Power (arbitrary unit)
PATP treated spores
Discriminating Power (arbitrary unit)
TP treated spores
Wavenumber (cm-1)
0
5000
10000
15000
20000
25000
30000
35000
40000
45000
50000
0
2000
4000
6000
8000
10000
12000
14000
16000
900
1000
1100
1200
1300
1400
1500
1600
1700
1800

FIGURE 8

(A)


Untreated control
0 min
0.5 min
1 min
2 min
5 min
8 min
# of factor = 9
SECV = 0.2644
r = 0.9627
FT-IR predicted spore survivors (log CFU/ml)
Measured spore survivors (log CFU/ml)
5
6
7
8

(B)

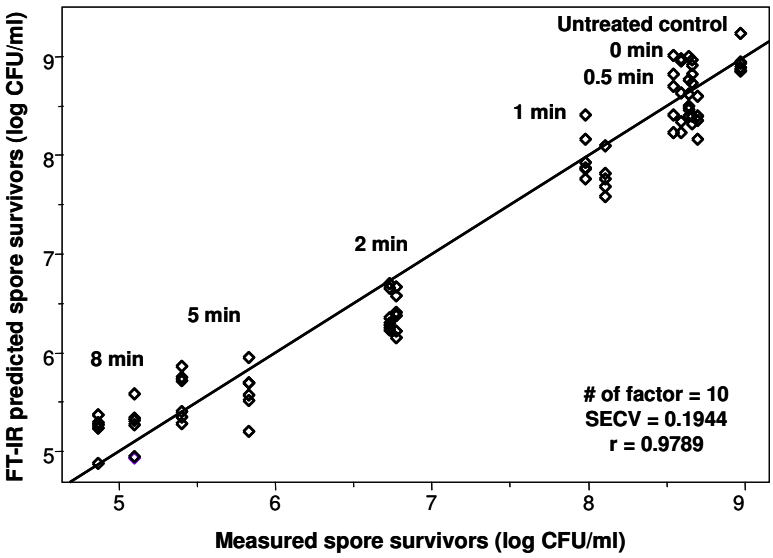
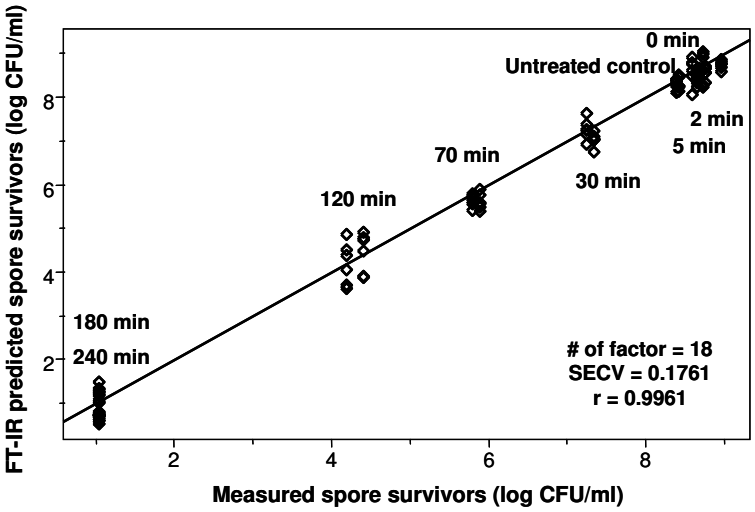

Untreated control
0 min
0.5 min
1 min
2 min
5 min
8 min
# of factor = 10
SECV = 0.1944
r = 0.9789
FT-IR predicted spore survivors (log CFU/ml)
Measured spore survivors (log CFU/ml)
5
6
7
8
9

FIGURE 9

(A)


FT-IR predicted spore survivors (log CFU/ml)
Measured spore survivors (log CFU/ml)
0 min
Untreated control
2 min
5 min
30 min
70 min
120 min
180 min
240 min
# of factor = 18
SECV = 0.1761
r = 0.9961

(B)

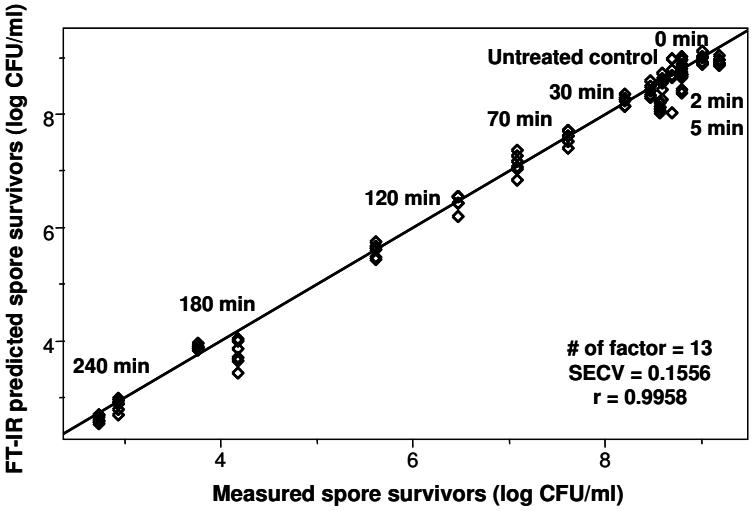
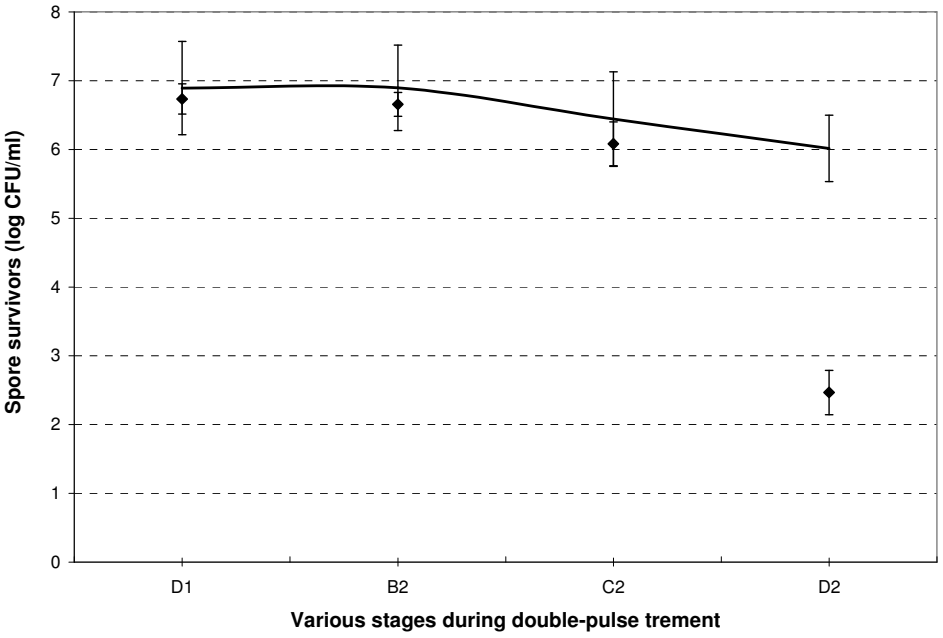
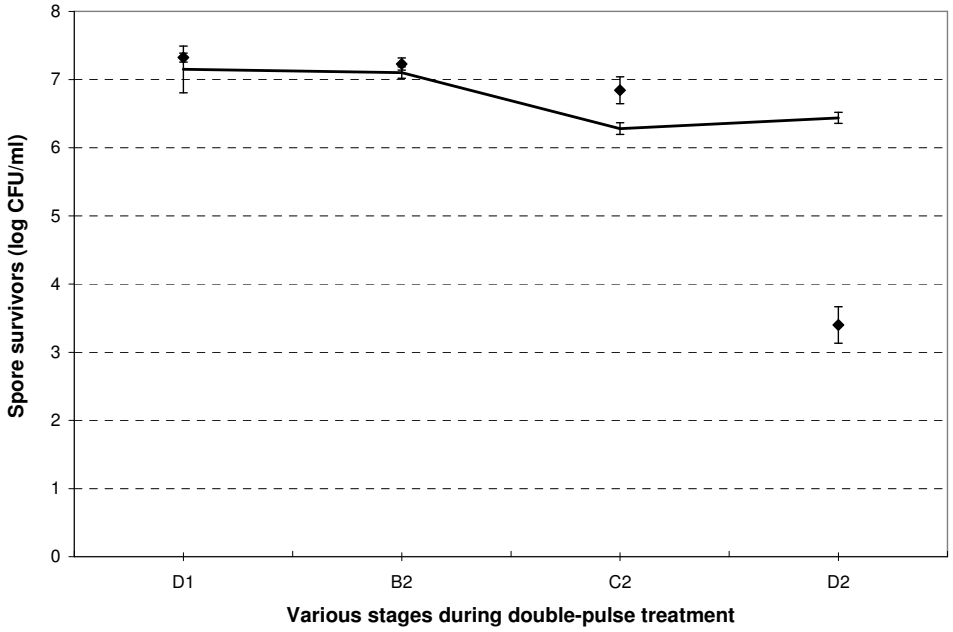

FT-IR predicted spore survivors (log CFU/ml)
Measured spore survivors (log CFU/ml)
0 min
Untreated control
30 min
2 min
5 min
70 min
120 min
180 min
240 min
# of factor = 13
SECV = 0.1556
r = 0.9958

FIGURE 10

(A)


Spore survivors (log CFU/ml)
0
1
2
3
4
5
6
7
8
D1
B2
C2
D2
Various stages during double-pulse trement

(B)


Spore survivors (log CFU/ml)
0
1
2
3
4
5
6
7
8
D1
B2
C2
D2
Various stages during double-pulse treatment

CAPPS Project Progress Report

Project Title: Study on food constituent protective effects and spore injury during pressure-assisted thermal processing of a low-acid food

Project Manager/Collaborator: Dr. V.M. (Bala) Balasubramaniam and Dr. Ahmed E. Yousef, The Ohio State University

Post-doctoral associate: Dr. Hossein Daryaei and, The Ohio State University

Description: Pressure-assisted thermal processing (PATP) provides a viable alternative to conventional thermal sterilization of foods to produce commercially sterile shelf-stable low-acid products with superior quality. During PATP, foods are subjected to a combination of high pressure (500-700 MPa) and temperatures (90-121ºC) for specific holding times. It is hypothesized that process lethality can be enhanced by addition of certain antimicrobial compounds to foods prior to PATP treatment.

This study will investigate combined pressure-thermal resistance of spores in the presence of selected antimicrobial compounds.

Previous Work: Our earlier CAPPS-funded work on sucrose esters (SE) showed some lethality enhancement when combined with PATP. Presence of 1% sucrose laurate enhanced the lethality of PATP against *Bacillus amyloliquefaciens* spores suspended in sterile deionized water (DIW) and mashed carrot by 0.55 to 1.2 log units, depending on the holding time applied. Population of viable spores in SE solutions decreased to <100 CFU/ml after 2 min treatment at 700 MPa and 105ºC. On the other hand, addition of nisin (a heat and pressure stable bacteriocin) at concentrations of up to 2000 IU ml^{-1} did not enhance the lethality of PATP under the experimental conditions tested.

Related Work Elsewhere: A number of researchers have reported the inactivation and growth inhibition effects of several antimicrobial compounds against various vegetative microorganisms and spores under moderate temperature and pressure conditions. Most of the compounds studied, showed an inhibitory effect (prevention of outgrowth and recovery during storage) and their effectiveness in enhancing PATP lethality against bacterial spores is yet to be investigated.

Progress to date:

- **Combined effect of PATP and antimicrobial compounds on the inactivation of *B. amyloliquefaciens* Fad 82 spores in HEPES buffer (a screening study)**

The purpose of this research was to screen the lethal effect of PATP against *B. amyloliquefaciens* spores in the presence of the selected antimicrobial compounds suspended in 50 mM HEPES buffer. About 20 antimicrobial compounds were screened todate. The HEPES buffer was chosen due to its strong buffering capacity and minimal pH shift during PATP treatment. Unless specified, the compounds were examined at pH 7.0.

The stock solutions of antimicrobial compounds were freshly prepared at room temperature on the day of experiment and filter-sterilized using a membrane filter (0.22 µrn) before adding to spore suspension.

- **Monoglycerides:** Monolaurin ($C_{12:0}$), monomyristin ($C_{14:0}$), monolinolein ($C_{18:2}$) and monolinolenin ($C_{18:3}$) were tested at concentration of 5 g/l. The stock solutions of monoglycerides were prepared in 95% propylene glycol.
- **Sodium salt preservatives:** Sodium benzoate and sodium nitrite were tested at concentrations of 0.5 and 2 g/l, respectively. The stock solutions were prepared in HEPES buffer.
- **Chelating agents:** Ethylenediaminetetraacetic acid **(**EDTA) was tested in the form of sodium salt (Na-EDTA) at concentration of 2 g/l. The stock solution of EDTA was prepared in HEPES buffer and its pH was adjusted to 7.0 by 10 M sodium hydroxide. In a separate trial, the lethal effect of 2 g/l EDTA at pH 5.0 combined with PATP treatment against spores was also tested.
- **Surfactants:** Sodium dodecyl sulfate (SDS) and saponin were tested at concentration of 2 g/l. the stock solutions were prepared in buffer.
- **Natural polymers:** Chitosan (a semi-synthetic polymer formed by alkaline deacetylation of chitin**)** was tested at concentration of 2 g/l and pH 6.0 and 5.0. It was insoluble at higher pH values. To prepare the stock solution, chitosan was initially dissolved in acetic acid at pH 5.0.
- **Antibiotics:** Tylosin solution was added to spore suspension at the level of 5 ml/l.
- **Bacteriocins:** Commercial pediocin (from *Pediococcus acidilactici*) and nisin (from *Lactococcus lactis*) were tested at concentrations of 5 µg/ml and 1 mg/ml (1000 IU/ml), respectively. The stock solutions were prepared in HEPES buffer at pH 7.0. Nisin was initially dissolved in 50% ethanol before adding to HEPES buffer.
- **Protein based:** Lactoferrin (an iron-binding milk protein) and lysozyme (an antibacterial enzyme) were tested at concentrations of 0.5 and 2 g/l, respectively.
- **Sucrose esters:** Sucrose stearate and sucrose palmitate were tested at concentration of 5 g/l.
- **Phenolic compounds of essential oils:** Eugenol, cinnamaldehyde, limonene and carvacrol at concentration of 0.5 g/l, and thymol at concentration of 0.1 g/l were tested. To prepare the stock solutions, Eugenol, cinnamaldehyde and limonene were dissolved in 50% ethanol, and carvacrol and thymol in 95% ethanol.

Spore preparation and PATP treatment: *Bacillus amyloliquefaciens* TMW 2.479 Fad 82 spores were used in this study, due to their high pressure-thermal resistance. The spores were prepared according to the method of Rajan *et al.* (2006) using nutrient agar as the sporulation medium supplemented with 0.6% yeast extract and 10 ppm $MnSO_4 \cdot H_2O$ and stored in DIW (containing about 10^9 CFU/ml) at 4°C until used. For PATP treatment, appropriate aliquots of spore suspension were aseptically added to 50 mM sterile HEPES buffer containing antimicrobial compounds at selected concentrations (or without antimicrobial compounds as controls) in sterile pouches to obtain ~ 4.7 x 10^8 CFU/ml. The heat-sealed pouches were stored at 4^oC for 1 h and treated in duplicate at 600 MPa and 105^oC for 2 min in a high pressure microbial kinetic tester (PT-1, Avure Technologies Inc., Kent, WA) as described by Rajan *et al.* (2006). The 2-min treatment time at 600 MPa

and 105^{o}C was selected so that efficacy of antimicrobial compounds in enhancing PATP lethality against spores can be estimated. All experiments were independently repeated in a separate trial. PATP-treated and untreated samples were enumerated within 3 h after treatments.

Key results and observations:

- Efficacy of 20 antimicrobial compounds in combination with PATP treatment have been tested.
- No noticeable changes in the pH of HEPES buffer was detected after PATP treatment.
- No considerable germination was detected during PATP treatment in any of samples with or without added antimicrobial compounds. This was confirmed by applying a heat shock at 80°C for 15 min to a proportion of the first dilution during viable count procedure and comparing the result with non-heat-shocked spore count.
- PATP treatment of *B. amyloliquefaciens* spores at 600 MPa and 105^{o}C in 50 mM HEPES buffer (pH 7.0) in the absence of antimicrobial compounds did not result in any significant inactivation (only 0.4 log reduction was achieved).
- Among the different chemical and natural antimicrobial compounds tested in this screening study, the following compounds showed promising potential (**Figure 1**)
    - lysozyme
    - SDS at PH 7.0,
    - EDTA at pH 5.0 and
    - chitosan at pH 5.0 and 6.0 were capable to significantly enhance the PATP lethality.

  The initial population of *B. amyloliquefaciens* spores in buffer was reduced by approximately 1.2 and 1.0 log units in the presence of 2 g/l lysozyme and SDS, respectively at pH 7.0; and 2.6 log units in the presence of 2 g/l chitosan at pH 6.0. Addition of 2 g/l chitosan to spore suspension at pH 5.0 resulted in a 3.5 log reduction under PATP conditions. Under the experimental conditions, 2 g/l sodium salt of EDTA (Na-EDTA) at pH 7.0 did not sufficiently enhance the PATP lethality; however, the presence of EDTA at pH 5.0 reduced the spore count by approximately 2.4 log units.
- It is interesting to note that certain antimicrobial compounds, including monoglycerides (especially monolaurin) previously reported effective during thermal processing were not sufficiently enhanced PATP lethality against *B. amyloliquefaciens* spores, possibly due to differences in their mechanisms of action against bacterial spores during PATP.

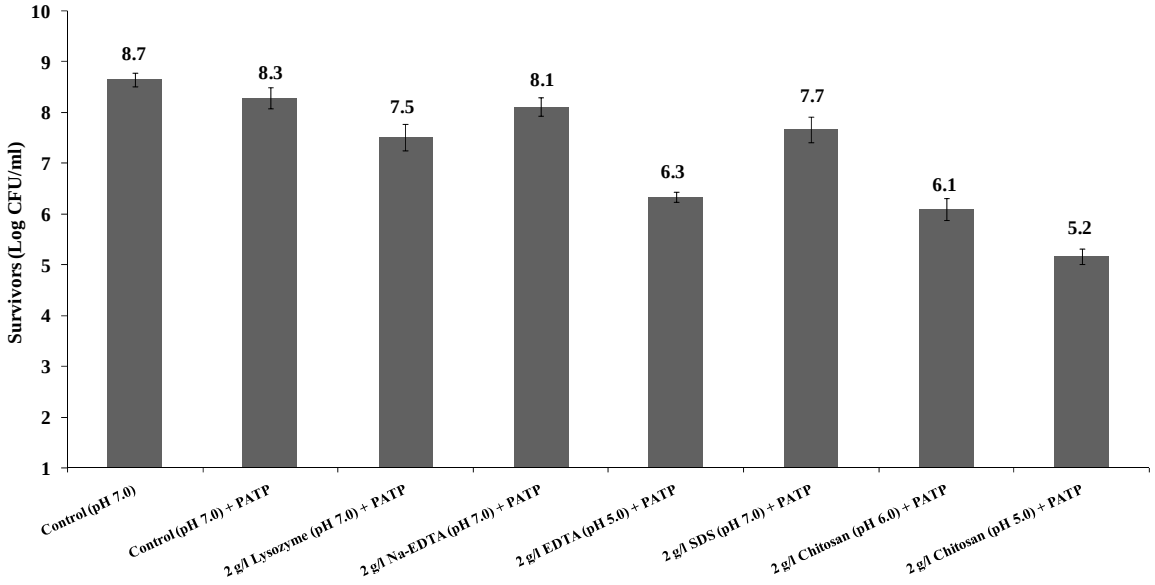


Fig.1 Enhancing PATP (600 MPa and 105^{o}C, 2 min) lethality against *B. amyloliquefaciens* spores in 50 mM HEPES buffer in the presence of selected antimicrobial compounds. The initial inoculum level was ~ 4.7 x 10^{8} CFU/ml. Bar graphs represent the mean of spore counts obtained from analyses of duplicate pouches of two PATP trials.

Conclusions:

B. amyloliquefaciens is a highly pressure-thermal resistance spore. The findings to date demonstrate that the efficacy of PATP against these spores could be enhanced by addition of certain antimicrobial compounds to spore suspension prior to treatment. This could possibly provide a method to process foods at less severe heat and pressure conditions and thus preserve quality attributes.

Ongoing research:

This research will continue to:

- Investigate the efficacy of selected pre-treatments to sensitize the spores prior to PATP treatment. This will include evaluating the effect of pre-treatment temperature on pressure-heat resistance of spores in the presence of antimicrobial compounds.
- Identify similar promising compounds (enzymes, surfactants and natural polymers) and other potential natural antibacterial compounds such as fermented pollen, grapefruit extract and liquid smoke and CO_2 that may enhance the lethality of PATP.
- Examine combinations of certain antimicrobial compounds for enhancing PATP lethality. Individual or combinations of antimicrobial compounds showing greater synergy with PATP will be selected to reduce PATP severity.

Bibliography:

Rajan, S., Ahn, J., Balasubramaniam, V.M., and Yousef, A.E. 2006. Combined pressure-thermal inactivation kinetics of *Bacillus amyloliquefaciens* spores in mashed egg patties. *Journal of Food Protection 69(4),* 853-860.

Relevant Publications from past CAPPS funded efforts:

Ratphitagsanti, W., Ahn, J., Balasubramaniam, V.M., and Yousef, A.E. 2009. Influence of pressurization rate and pressure pulsing on the inactivation of *Bacillus amyloliquefaciens* spores during pressure-assisted thermal processing. *Journal of food protection 72(4),* 775-782.

Ratphitagsanti, W., De Lamo-Castellvi, S., Balasubramaniam, V.M., and Yousef, A.E. 2009. Efficacy of pressure-assisted thermal processing, in combination with organic acids, against *Bacillus amyloliquefaciens* spores suspended in deionized water and carrot puree. *Journal of food protection* (Submitted).