

Inactivation of Baroduric Bacteria Isolated by High Hydrostatic Pressure from *Pickled Cowpea*

Xuemei Li¹, Dong Zhao¹, Anjun Chen¹, Tiantian Lin² & Biao Pu¹

¹ College of Food Science, Sichuan Agricultural University, Yaan, China

² College of Forestry, Sichuan Agricultural University, Yaan, China

Correspondence: Biao Pu, College of Food Science, Sichuan Agricultural University, Yaan 625014, China. Tel: 86-139-0816-0854. E-mail: pubiao2002@yahoo.com.cn

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Abstract

In this study, *Pickled Cowpea*, a typical lactic acid fermented vegetable in Sichuan, China, was used as samples to study both species and inactivation of baroduric bacteria isolated by HHP treatment under different pressure levels and different pressure holding time. 16S rDNA gene sequence, amplified using genomic DNA of 4 baroduric bacteria from *Pickled Cowpea* as templates, were sequenced and then were identified based on the sequence similarity and homology analysis, as *B. licheniformis*, *B. subtilis*, *B. sonorensis* and *B. pumilus*. The pressure resistance of the 4 strains are compared under pressure from 300 to 500 MPa with holding time from 3 to 25 min. *B. pumilus* which has higher pressure resistance can be selected as indicator bacteria for applying HHP treatment to *Pickle* production.

Keywords: high hydrostatic pressure, baroduric bacteria, isolation, identification, pickled cowpea

1. Introduction

Fermentation, a centuries food preservation technology, can naturally prolong the shelf life of vegetable and improve its safety, nutrition and sensory quality. *Pickle*, a traditional fermented food in China as one of the 4 most popular fermented vegetable in Sichuan, is produced by salt hypertonic effect and lactic acid fermentation. Because of various flavor and nutriment created during fermentation, *Pickle* has abilities such as regulating intestinal microflora and lowering cholesterol level (Yang et al., 1996). Being popular for unique flavor and nutritional value in China, *Pickled Cowpea* is selected as research material in this study. Generally speaking, *Lactic acid bacteria*, growing during the lactic acid fermentation, can inhibit the growth of other microorganisms. Nevertheless, due to effect of microorganisms and enzymes which including breeding of spoilage bacteria in industrial production, shelf life was greatly shortened while quality was reduced (Song et al., 2004; Cheigh & Park, 1994). Thermal sterilization, especially pasteurization around 80 degrees Celsius, which is widely applied to *Pickle* nowadays for increasing its shelf life (Zhong, Li, & Xu, 2008), has a disadvantage that the quality of *Pickle* will be influenced, because the inter-atomic forces of small molecular compound such as pigment, flavor and vitamin in pickled vegetable will be broken (Wu, Yu, & Li, 1999). Therefore, new ways of sterilization, which both extend shelf life and maintain the original quality as far as possible, are requested by modern industrial production.

High hydrostatic pressure (HHP) technology, one of the emerging non-thermal sterilization techniques, not only inactivate microorganisms and spores but also maintain the original special flavor and nutritional quality, by utilizing isostatic pressure to affect cell membranes of microbes, nucleic acid of microbes, and the protein conformations (Meyer et al., 2000; Trujillo et al., 2000; Hartmann, Mathmann, & Delgado, 2006). Although applications of HHP have been lucubrated on kinds of food like juice and jam, even HHP-treated juice has already faced to market; research on HHP technology of fermented vegetables is lack at present. Generally speaking, HHP technology can effectively improve safety and quality of fermented vegetable in industrial production, but due to differences in raw materials, fermented methods and regionalism, inactivation of

microorganisms caused by HHP treatment are very different.

Kuribayashi et al. (1996) reported that microorganisms in *Nozawana-zuke* (a fermented vegetable from Japan) were obviously killed at pressure level between 300 MPa and 400 MPa for 10 min holding time, so that shelf life was extended without affecting the texture, color and flavor at the same time. By reducing quantity of *Lactobacillus plantarum* by 6 log units at 400 MPa for 10 min, the *Kimchi* samples can be stored for 4 weeks at 20 °C. Furthermore, the excessive acidification of *Kimchi* during storage can be restrained by over 400 MPa HHP treatment (Sohn & Lee, 1998). Peñas et al. (2010) reported that the magnitude of microorganisms in *sauerkraut* was reduced by 4 to 5 log₁₀ CFU/g when HHP-treated, in the same time colony counts of *mesophilic bacterium* and *Lactobacillus* were significantly decreased.

Sterilizing effect of *Pickled Cowpea* caused by HHP treatment has not been studied, where the key problem is to confirm target bacterium when applying HHP technology to *Pickled Cowpea*. In this thesis, the residuary baroduric bacteria and inactivation on different pressure levels and holding time is investigated, in order to establish basic instructions for HHP researches on *Pickle*.

2. Materials and Methods

2.1 HHP on Pickled Cowpea Samples

Pickled Cowpea samples for the experiment were provided by Liji Pickles and Condiment Co. Ltd., a famous commercial pickled vegetable producer, located in Sichuan, China. The samples, with pH 3.8-4.0 and 1.3% salt content, were selected randomly from two parallel batches, which were desalted and sliced on the production line after mature fermented in the fermentation pits, without mixing condiment or heating for pasteurization, and then were vacuum-packed promptly by polyethylene bags (25 g/package, -0.1 MPa vacuum degree) for being stored at 4 °C.

The equipment in our experiment is a hydrostatic pressurization unit (RL-003, WenzhouBeinuo Co., Ltd., Zhejiang, China), which use distilled water as the pressure-transmitting fluid. Packaged samples were exposed to 600 MPa hydrostatic pressure for 25 min at room temperature (20 °C), to kill common bacteria except baroduric bacteria. The rising rate of pressure is approximately 150 MPa/min, and the pressure relief process is nearly instantaneous (≤ 2 s).

2.2 Isolation and Purification of Baroduric Bacteria

Each HHP-treated sample was homogenized in 225 mL of sterile 0.85% NaCl solution for 2 min, in order to use aerobic plate count method to detect viable bacteria. Samples were serially diluted with sterile 0.85% NaCl solution to appropriate dilution ratio, and then 1.0 ml diluted sample was sucked and injected into plate count agar (PCA, composition: tryptone 5.0 g/L, yeast extract 2.5 g/L, glucose 1.0 g/L, agar 15.0 g/L, pH 7.0±0.2, autoclaving at 121 °C for 20 min) to detect the viable counts of aerobic plate count. After incubating the plates at 37 °C for 48±2 h, the typical colonies were picked out. Isolation of bacteria was implemented by repeatedly plate streaking on nutrient agar (NA, composition: peptone 10.0 g/L, beef extract 3.0 g/L, NaCl 5.0 g/L, agar 17.0 g/L, pH 7.2±0.2, autoclaving at 121 °C for 20 min) until pure cultures of single strain were obtained. The nutrient agar was also used for preserving strains.

2.3 Identification of Baroduric Bacteria

Gram Stain and microscopic examination were used in preliminary observation of bacterial stains, and 16S rDNA sequence analysis was used to identify residuary baroduric bacteria. Genomic DNA of strains were extracted as template, by genomic DNA extraction reagent kit of bacteria (SK8225, Sangon Biotech (Shanghai) Co. Ltd., China), then the 16S rDNA sequences were performed by employing universal primer of bacteria, including forward primer P1 (5'-AGAG TTT GAT CCT GG TCA GA ACG CT-3', 40 pmol) and reverse primer P6 (5'-T ACG GCT ACC TTG TTA CGA CTT CAC CCC-3', 40 pmol) for polymerase chain reaction (PCR) amplification, according to Jin et al. (2011). PCR reactions were performed in a total volume of 50 µL using a final concentration of 25 µL 2 × Taq PCR MasterMix, 2.0 µL P1, 2.0 µL P6, 2.0 µL DNA templates (50 ng/mL) and 19 µL double distilled water. P1 and P6 were synthesized by Sangon Biotech (Shanghai) Co. Ltd. while 2×Taq PCR MasterMix (KT201-01) was made by Tiangen Biotech (Beijing) Co. Ltd. PCR conditions were: 92 °C for 3 min; 94 °C for 1 min, 58 °C for 1 min, 72 °C for 2 min, 30 cycle process; 72 °C for 8 min; 4 °C for 30 min. The PCR products, which were electrophoresed with EB on 1% agarose gel whose each hole was filled by 5 µL sample and 1 µL loading buffer, run at 100 V for 30 min and visualize DNA fragments with UV light. Then, the PCR products were purified and sequenced by Invitrogen (in Shanghai China). To determine the closest relatives of 16S rDNA partial sequence, the alignment search was performed with BLAST and GenBank databases to carry out sequence homology analysis. Phylogenetic tree was constructed by

Neighbor-Joining method in MEGA 4.0 software (Tamura et al., 2007). Consensus sequence from each strain was generated by Sequencher program (version 4.7), using sequences of 29 *Bacillus* (GenBank Database) as reference strains and 1 *Paenibacillus barcinonensis* (DQ363432, GenBank Database) as comparison, to build the phylogenetic tree. Consensus sequences were aligned with *Bacillus* type strains using Clustal W (Thompson, Plewniak, & Poch, 1999) from MEGA to construct the phylogenetic tree, by running Neighbor-Joining program with the Kimura (2007) correction and boot strapping for 1000 times.

2.4 HHP on Bacterial Suspension of *Bacillus*

Each identified *Bacillus* was inoculated to NA liquid culture medium (NA, composition: peptone 10.0 g/L, beef extract 3.0 g/L, NaCl 5.0 g/L, pH 7.2±0.2, autoclaving at 121 °C for 20 min), to being incubated at 37 °C for 48±2 h, in order to maintain the ratio between vegetative mass and spores of *Bacillus* in 1:1. Then, precipitates gathered by centrifugation at 3000 r/min for 15 min, were added to phosphate buffer (0.03 mol/L, pH 7.2) in order to suspend.

Adjusting the initial concentration to 10^7 - 10^8 CFU/mL, bacterial suspension of *Bacillus* was dispensed and vacuum-packed by heat-sealed sterile polyethylene bags (10 ml/package, -0.1 MPa vacuum degree) under aseptic condition. Each bag of samples was HHP-treated at pressure level in 300-600 MPa for 3-25 min respectively, at room temperature.

2.5 Enumeration of Viable Count

1.0 mL of each HHP-treated sample was immediately sucked into 9.0 mL sterile 0.85% NaCl solution for dilution. Further serial dilutions were made for using pour plate technique to determine the viable count. Total aerobic mesophilic bacteria were enumerated on nutrient agar (NA) after incubation at 37 °C for 48 h.

The inactivation effect of *Bacillus* was assessed by taking LRV (Logarithmic Reduction Value, $-\log(N/N_0)$) as index, where colony counts of HHP-treated samples were recorded as N, while the initial counts of microorganisms of untreated samples were recorded as N_0 .

2.6 Statistical Analysis

All data presented were means ± standard value and the measure ments were done with three replicates for statistical validity. One-way analysis of variance (ANOVA) was performed to check the variability of data and validity of the results. The data were analyzed with the software Statistical Package for the Social Sciences (SPSS, Chicago, IL, USA) version 13.0 and the results were considered significant if $P < 0.05$.

3. Result and Discussion

3.1 16S rDNA Amplification and Sequencing

Four bacterial strains were isolated from samples of *Pickled Cowpea*, were named MJJ-1, MJJ-7, MJJ-9 and MJJ-18. Under the condition of 1% agarose gel electrophoresis, their 16S rDNA amplified and purified products were showed in electrophoresis images (Figure 1), which indicated that the specific bands of amplified products were about 1500 bp according to the Marker band. Then the positive products which fragment lengths were about 1500 bp, were sent to Invitrogen (Shanghai) Co. Ltd. for the full length genome sequencing of 16S rDNA gene.

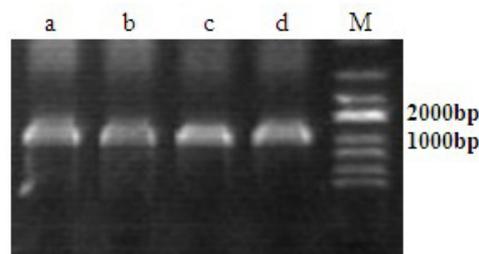


Figure 1. The electrophoresis images of 16s rDNA amplified and purified products. (a): MJJ1; (b): MJJ7; (c): MJJ9; (d): MJJ18; (M): DL2000 Marker

The full length genome sequences of the 4 strains had been submitted to NCBI/GenBank database. The accession number of strains MJJ-1, MJJ-7, MJJ-9 and MJJ-18 in GenBank were JQ837269, JQ837270, JQ837272 and JQ837271 respectively. The results of comparison indicated that all of them were *Bacillus*.

Specifically, the sequence similarity threshold between MJJ-1 and *Bacillus licheniformis* reached 100%. Meanwhile, the sequences of MJJ-7 and *Bacillus subtilis*, MJJ-9 and *Bacillus sonorensis* also showed 100% similar degree, and the sequence similarity threshold between MJJ-18 and *Bacillus pumilus* was exceed 99%.

3.2 Analysis of Phylogenetic Tree

Phylogenetic tree illuminated in Figure 2 showed the phylogenetic analysis on target strains' consensus sequences and reference sequences from 34 *Bacillus* and 1 *Paenibacillus*. With specific *Bacillus* reference strains, each target strain clusters into distinct species according to sequence similarity and homology analysis. MJJ-1 is classified as *B. licheniformis*, while MJJ-7 as *B. subtilis*, MJJ-9 as *B. sonorensis*, and MJJ-18 as *B. pumilus*.

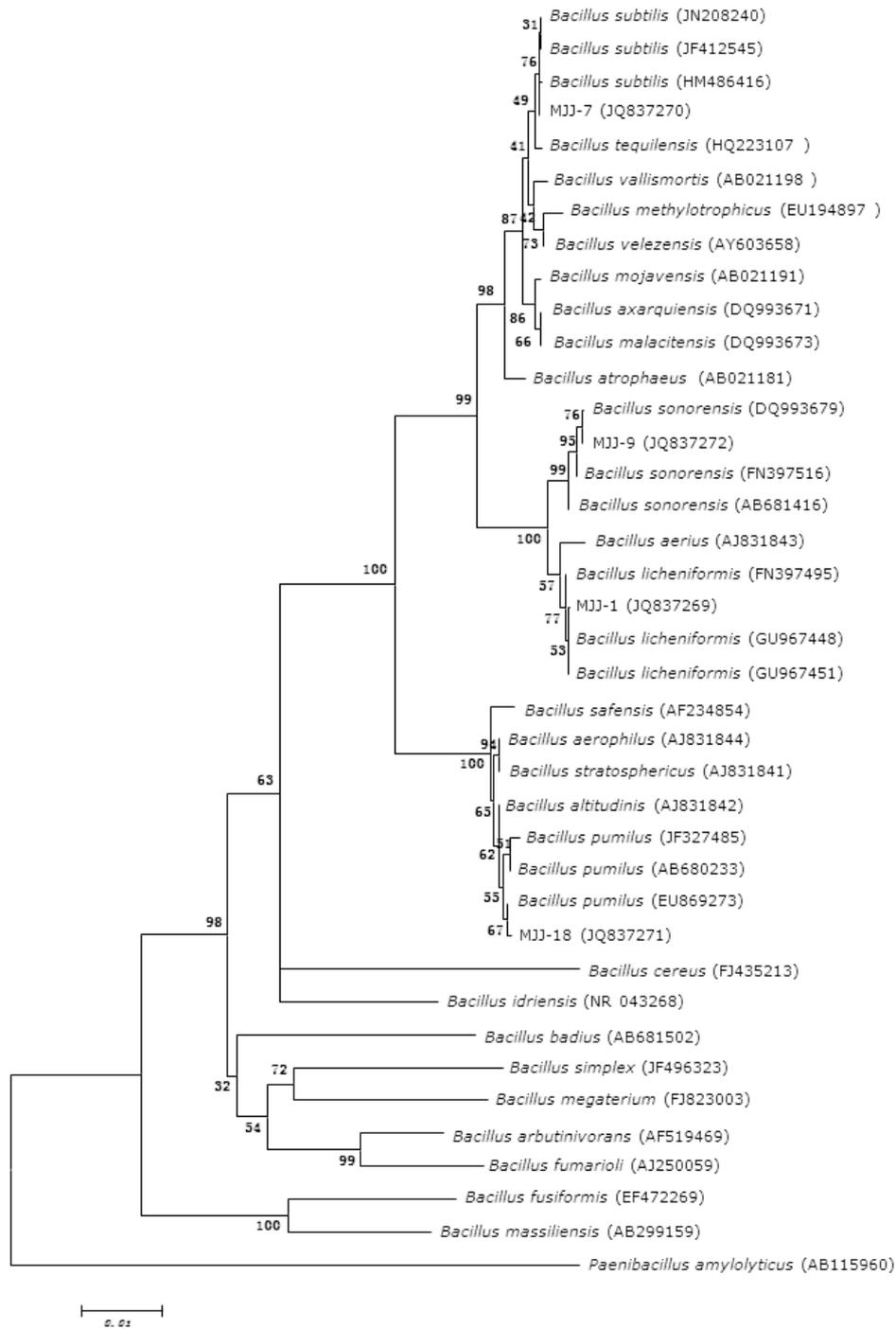


Figure 2. Phylogenetic tree of the strains isolated from *Pickled cowpea* by Neighbor-joining method of 16S rDNA gene sequences

Residuary baroduric bacteria, isolated from *Pickled Cowpea* in high pressure condition, and classified as *Bacillus* by 16S rDNA sequence analysis. The result showed that the bacterial species isolated at HHP condition 600 MPa/25 min were identified as *Bacillus sp.*, in accord with strains isolated from HHP-treated juice and mushroom (Jiang, Yin, Song, Yu, & Chen, 2010; Hou et al., 2011; Xu et al., 2011). *B. licheniformis*, *B. subtilis* and *B. pumilus* had been isolated from previous studies on fermented products. Under common condition, *B. pumilus* was isolated from *Kimchi* (Yamanaka, Moriyoshi, Ohmoto, Ohe, & Sakai, 2010); *B. licheniformis*, *B. subtilis* and *B. pumilus* were isolated from *Pickle* in Sichuan (Wang, 2010); *B. subtilis* and *B. licheniformis* were isolated from fermented bean products such as *Korean Cheonggukjang*, *Indian Kinema* and *African Soumbala* (Yamanaka et al., 2010; Kwon et al., 2009; Sarkar, Hasenack, & Nout, 2002). Additionally, He et al. (2009) isolated and identified baroduric bacteria including *B. subtilis* and *B. licheniformis* from HHP-treated *Low Acid Canned* under 600 MPa condition, while *B. licheniformis* and *B. pumilus* were identified as pressure-resistant bacteria from *Strawberry Pulp* under the same pressure level (Zhang, 2010). Meanwhile, isolation of *B. sonorensis* from fermented products has not been reported.

3.3 Inactivation of Baroduric Bacteria

Based on inactivation under different pressure levels and different pressure holding time (Table 1), Figure 3 and Figure 4 were extracted with different emphasizes on pressure and time.

Table 1. Effect of pressure levels and pressure holding time on inactivation of pressure treated baroduric bacteria (Log₁₀ CFU/mL). Different letters show significant difference at P<0.05 (±Standard deviation)

Strains	Pressure	Pressure holding time							
		Untreated	3min	5min	8min	10min	15min	20min	25min
<i>B. licheniformis</i>	300MPa	7.62±0.18 ^a	4.95±0.09 ^b	4.53±0.08 ^b	4.47±0.13 ^b	4.32±0.10 ^b	3.76±0.08 ^c	3.51±0.07 ^c	3.51±0.09 ^c
	400MPa	7.62±0.09 ^a	3.85±0.09 ^b	3.57±0.05 ^b	3.54±0.10 ^b	3.45±0.09 ^{bc}	3.22±0.11 ^c	2.96±0.07 ^c	2.94±0.08 ^c
	500MPa	7.70±0.09 ^a	3.80±0.10 ^b	3.23±0.14 ^b	3.20±0.10 ^b	3.10±0.09 ^c	2.91±0.08 ^c	2.87±0.08 ^c	2.85±0.11 ^c
	600MPa	7.70±0.09 ^a	3.65±0.12 ^b	3.07±0.09 ^{bc}	3.03±0.06 ^c	2.93±0.09 ^{cd}	2.73±0.05 ^d	2.71±0.09 ^d	2.68±0.09 ^c
<i>B. subtilis</i>	300MPa	8.70±0.09 ^a	6.10±0.10 ^b	5.71±0.06 ^b	5.64±0.13 ^b	5.54±0.09 ^b	5.20±0.10 ^c	4.81±0.07 ^c	4.80±0.08 ^c
	400MPa	8.70±0.09 ^a	5.40±0.08 ^b	4.93±0.07 ^b	4.60±0.05 ^b	4.46±0.10 ^c	4.41±0.09 ^c	4.33±0.09 ^c	4.27±0.08 ^d
	500MPa	8.76±0.09 ^a	4.28±0.05 ^b	3.91±0.09 ^b	3.74±0.10 ^{bc}	3.54±0.08 ^c	3.37±0.06 ^c	3.33±0.10 ^d	3.32±0.07 ^d
	600MPa	8.62±0.09 ^a	3.72±0.07 ^b	3.32±0.07 ^b	3.28±0.06 ^c	3.16±0.05 ^c	3.11±0.13 ^d	3.09±0.11 ^d	3.06±0.07 ^c
<i>B. pumilus</i>	300MPa	7.78±0.09 ^a	5.98±0.06 ^b	5.77±0.08 ^b	5.75±0.08 ^b	5.72±0.05 ^b	5.46±0.10 ^c	5.20±0.09 ^d	5.19±0.07 ^d
	400MPa	7.84±0.09 ^a	5.22±0.08 ^b	4.92±0.08 ^b	4.78±0.09 ^c	4.60±0.07 ^c	4.37±0.09 ^d	4.03±0.08 ^d	4.01±0.08 ^d
	500MPa	7.84±0.09 ^a	4.17±0.05 ^b	3.80±0.09 ^b	3.72±0.07 ^c	3.62±0.06 ^c	3.27±0.07 ^d	3.23±0.10 ^d	3.21±0.07 ^d
	600MPa	7.73±0.09 ^a	3.75±0.06 ^b	3.13±0.08 ^c	3.07±0.07 ^{cd}	3.03±0.05 ^d	2.85±0.09 ^d	2.82±0.08 ^e	2.80±0.07 ^c
<i>B. sonorensis</i>	300MPa	8.49±0.09 ^a	6.13±0.06 ^b	5.69±0.08 ^c	5.66±0.10 ^c	5.60±0.09 ^c	5.51±0.10 ^c	5.34±0.05 ^d	5.31±0.07 ^d
	400MPa	8.20±0.06 ^a	5.11±0.06 ^b	4.55±0.06 ^c	4.46±0.08 ^c	4.31±0.07 ^{cd}	4.03±0.10 ^d	3.58±0.07 ^d	3.57±0.07 ^d
	500MPa	8.25±0.09 ^a	4.13±0.09 ^b	3.60±0.07 ^b	3.47±0.10 ^c	3.40±0.07 ^c	3.11±0.06 ^d	3.07±0.11 ^d	3.06±0.08 ^d
	600MPa	8.33±0.09 ^a	3.75±0.09 ^b	3.32±0.06 ^c	3.29±0.09 ^c	3.23±0.07 ^d	3.04±0.10 ^e	3.03±0.07 ^c	3.04±0.08 ^e

The effects of pressure levels and pressure holding time on the inactivation of 4 *Bacillus* were presented in Figure 3. The inactivation curves of 4 *Bacillus* are similar, that the increasing rate of each LRV of bacterial colony changes as 4 period: great level in the first few minutes, lower level in the following minutes, then increases for a while, after that falls to 0. During the 1st and 2nd period, at each pressure level, inactivation curves increases probably because of death of the vegetative mass of *Bacillus*. In the 3rd period, increasing rate of LRV increases probably because of death of spores. The assumption is demonstrated by studies. The inactivation curves of vegetative mass of *B. licheniformis* and *B. subtilis* won't change at pressure level from 300 MPa to 500 MPa after 10 min, while inactivation curves of spores grows until 20 min (He et al., 2009). Though mechanism of inactivation is unproved yet, it was strongly supported by effect on microorganism

caused by HHP, including cell rupture, cytoplasm disorder, cytoplasm leak, and denaturation of protein, enzyme and nucleic acid (Zeng et al., 2006; Wuytack & Michiels, 2001; Smelt, 1998). The higher pressure resistance of spore wall restrains inactivation caused by HHP, and extends the time of inactivation of spores. As a result, the inactivation curves display two increasing stages. The vegetative cell of bacteria, mycete and saccharomycetes were inactivated at 200-600 MPa pressure at ambient temperature (Smelt, 1998), while some spores can survive under 1200 MPa pressure (Gould, 2001). Pressure resistance of spore wall could be diminished by auxiliary method, such as mild heat and bacteriostat, to impel germination of spores (Vercammen, Vijijs, Lurquin, & Michiels, 2012; Hang, 2012).

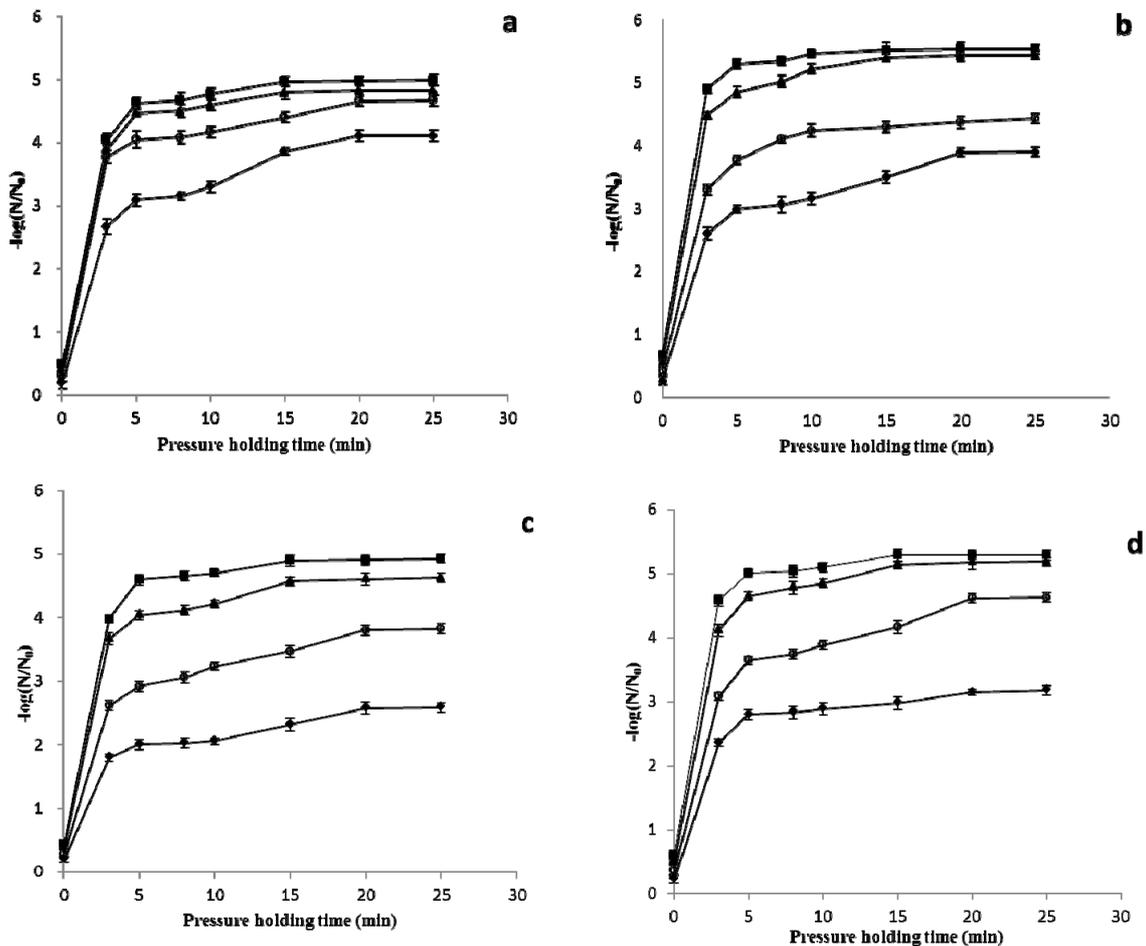


Figure 3. Inactivation curves for 4 *Bacillus* in phosphate buffer, subjected to various pressure (300 to 600 MPa). a: *B. licheniformis*, b: *B. subtilis*, c: *B. pumilus*, d: *B. sonorensis*.; (■) 600 MPa, (▲) 500 MPa, (○) 400 MPa and (◆) 300 MPa

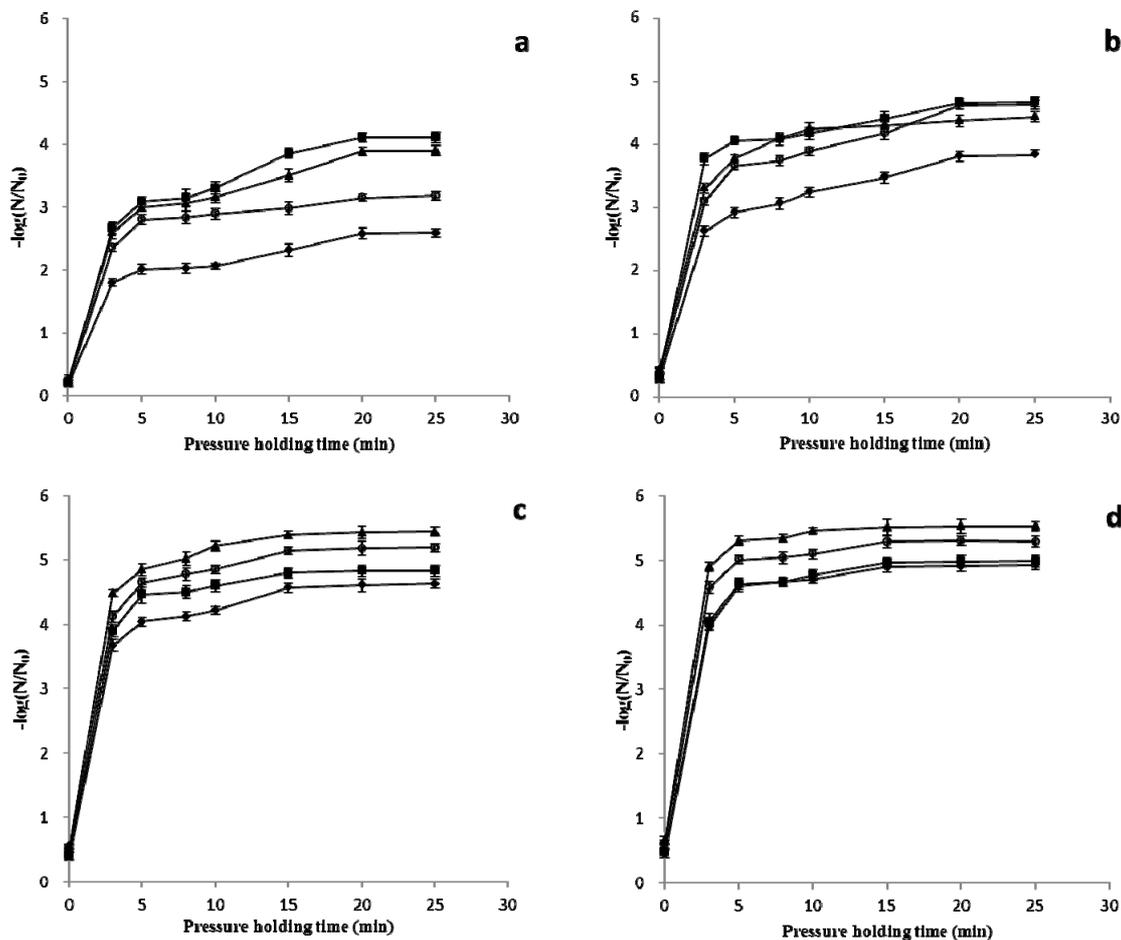


Figure 4. Inactivation curves for 4 *Bacillus* in phosphate buffer, subjected to various processing time (0 to 25 min). a: 300 MPa, b: 400 MPa, c: 500 MPa, d: 600 MPa; (■) *B. licheniformis*, (▲) *B. subtilis*, (◆) *B. pumilus* and (○) *B. sonorensis*

3.4 Influence to Inactivation from Pressure Levels

The inactivation curves of each strain under different pressure conditions were reflected respectively in Figure 3. Within the range of experimental conditions studied, the results showed that survivor counts of *Bacillus* significantly declined along with increasing pressure ($P < 0.05$). The LRVs of bacterial colony of *B. subtilis* at 300 MPa, 400 MPa, 500 MPa and 600 MPa for 15 min were 3.50 log, 4.29 log, 5.39 log and 5.51 log (Figure 3b). Similarly, the LRVs of *B. sonorensis* for 20 min pressure holding time were 3.15 log, 4.62 log, 5.18 log and 5.30 log (Figure 3d). Both of them showed that LRV increased with increasing pressure level.

However, the ratio between increasing rate of LRV and increasing rate of pressure decreased while pressure level was increasing, no matter which *Bacillus* is focused. For instance, under 15 min pressure holding time, the LRV of *B. sonorensis* relatively declined 1.19 log when pressure level increased from 300 MPa to 400 MPa, whereas it relatively dipped 0.97 log when pressure level increased from 400 MPa to 500 MPa, and it relatively decreased 0.15 log when pressure level increased from 500 MPa to 600 MPa (Figure 3d). Therefore, it can infer that the upper bound of LRV exists, when pressure is greater than some threshold, LRV won't increased along with increasing of pressure. Because of the limitation of experimental equipment, the threshold pressure of each *Bacillus* had not been observed yet in this study. Previous research proved that the LRV upper bound and pressure threshold exist under fixed condition, but they changed when different condition was applied, such as different temperature (Reddy et al., 1999).

On the other hand, the differences of LRV of *B. licheniformis* between adjacent pressure levels were minimal among 4 *Bacillus*, while the differences of LRV of *B. pumilus* were maximal. The LRV of *B. licheniformis* at 300 MPa, 400 MPa, 500 MPa and 600 MPa with 10 min pressure holding time were 3.30 log, 4.17 log, 4.60

log and 4.77 log. They increased to 4.11 log, 4.68 log, 4.85 log and 5.02 log when pressure holding time increased to 25 min (Figure 3a). On contrary, the LRV of bacterial colony of *B. pumilus* at pressure level from 300 MPa to 600 MPa with 10 min holding time were 2.06 log, 3.24 log, 4.22 log and 4.70 log, while they were 2.59 log, 3.83 log, 4.63 log and 4.93 log with 25 min pressure holding time (Figure 3c). The probable reason was that sensitivity of vegetative mass and spores of diverse *Bacillus* species were different.

Difference of pressure resistance among 4 *Bacillus* at same pressure level and different processing holding time was described in Figure 4. The result of intuitionistic analysis in Figure 4a and Figure 4b showed that the pressure resistance at 300-400 MPa pressure level was ranked as *B. pumilus*>*B. sonorensis*>*B. subtilis*>*B. licheniformis*. After that, the order was changed to *B. pumilus*>*B. licheniformis*>*B. sonorensis*>*B. subtilis* at 500-600 MPa pressure range (Figure 4c and Figure 4d). Compare with other 3 *Bacillus*, *B. licheniformis* displayed more sensitivity to pressure at 300-400 MPa pressure range and LRV of bacterial colony relatively increased rapidly with pressure holding time prolonging, whereas it showed pressure resistance that was second to *B. pumilus* when pressure level increased to 500 MPa or 600 MPa. Under 300-600 MPa pressure condition for varied holding time, *B. pumilus* always kept the greatest pressure resistance among 4 *Bacillus* identified from *Pickled Cowpea*. Therefore, it could be selected as the indicator bacteria of *Pickled Cowpea* for HHP treatment, and the result could provide the theory basis for research on HHP technology of *Pickle*.

3.5 Influence to Inactivation from Holding Time

Inactivation of HHP-treated bacteria consists of two parts, death caused by instantaneous pressure and subsequent destruction during the pressure holding time (Basak, Ramaswamy, & Piette, 2002). Instantaneous inactivation due to pressurization–depressurization process without holding time while pressure just attains the target value, and its effectiveness depends on pressure levels, the impact obviously increases with increasing pressure.

According to Figure 2, inactivation of *Bacillus* was sensitive to the level of instantaneous pressure. LRV of bacterial colony of *B. subtilis* were 0.26 log, 0.42 log, 0.56 log and 0.67 log on 300 MPa, 400 MPa, 500 MPa and 600 MPa pressure condition, while that of *B. sonorensis* were 0.23 log, 0.36 log, 0.51 log and 0.60 log at the same pressure condition. By contrast, instantaneous inactivation of *B. licheniformis* and *B. pumilus* were relatively small, LRV of bacterial colony were 0.49 log and 0.45 log under 600 MPa pressure respectively. It probably indicated *B. subtilis* and *B. sonorensis* are more sensitive to instantaneous pressure than *B. licheniformis* and *B. pumilus*.

During the whole pressure holding time, the period in which bacterial colony changes is named effective pressure holding time. Figure 4 showed that the length of effective pressure holding time of 4 *Bacillus* was similar, and decreased with increasing pressure level ($P < 0.05$). Bacterial colony no longer changed at 300 MPa after approximate 20 min pressure holding time (Figure 4a). Hence, the effective pressure holding time was 20 min. The rate of inactivation was more rapid at 400 MPa than that at 300 MPa during the same pressure holding time, but effective pressure holding time under both pressure levels was same (Figure 4b). However, Figure 4c and Figure 4d indicated that the effective pressure holding time was reduced to 15 min at 500-600 MPa pressure level. The sterilizing effect cannot be improved through merely extend pressure holding time, when longer than effective pressure holding time (Nakayama & Yano, 1996).

4. Conclusions

Though famous in the world, consumption of *Pickle* is greatly limited by the lack of preservation technology, because its special flavor and nutrient substance are easily destroyed by traditional thermal sterilization methods. In this study, HHP-treated *Pickled Cowpea* was analyzed, from which 4 baroduric bacteria were isolated and all of them were identified as *Bacillus sp* by 16S rDNA gene sequence technology. Inactivation of the 4 baroduric bacteria under different pressure levels and different pressure holding time is studied, to investigate the feasibility of HHP technology on preservation of *Pickle* industry.

The result indicated that pressure level and pressure holding time can significant impact on inactivation of *Bacillus*. Instantaneous pressure affects inactivation of *Bacillus* respectively. *B. subtilis* is more sensitive to instantaneous pressure than others according to LRV curve. Furthermore, during effective pressure holding time, LRV of each bacterial colony increased rapidly at first, which expressed massive death of *Bacillus*, and then the increasing rate of inactivation decreased. The 4 *Bacillus* presented varied pressure resistance under same pressure level. It was ranked as *B. pumilus*>*B. sonorensis*>*B. subtilis*>*B. licheniformis* under 300-400 MPa pressure, while the order changed to *B. pumilus*>*B. licheniformis*>*B. sonorensis*>*B. subtilis* under 500-600 MPa pressure. Since *B. pumilus* has higher pressure resistance in both stages, it is selected as the indicator bacteria for applying HHP treatment to *Pickle* production.

The future work including, firstly, inactivation of vegetative mass and spores of the 4 *Bacillus* by HHP-treated will be investigated respectively to present their inactivate effect under different pressure level and holding time condition, to establish kinetic models of inactivation; secondly, because spores have higher pressure resistance, effect of HHP with assistant methods, such as moderate heat and inartificial bacteriostat, should be studied.

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