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Assessment of *Clostridium perfringens* Spore Response to High Hydrostatic Pressure and Heat with Nisin

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Abstract The elimination of spores from low-acid foods presents food-processing and food-safety challenges to high-pressure processing (HPP) developers as bacterial spores are extremely resistant to pressure. Therefore, the effects of pressure (400–800 MPa), temperature (35–95°C), and nisin (0–496 IU/mL) on the inactivation of *Clostridium perfringens* AS 64701 spores at various pressure-holding times (7.5–17.5 min) were explored. A second-order polynomal equation for HPP- and nisin-induced inactivation of *C. perfringens* spores was constructed with response surface methodology. Experiment results showed that the experimental values were shown to be significantly in agreement with the predicted values because the adjusted determination coefficient (R_{Adj}^2) was 0.9708 and the level of significance was $P<0.0001$. The optimum process parameters (obtained by solving the quadratic polynomal equation) for a six-log cycle reduction of *C. perfringens* AS 64701 spores were pressure of 654 Mpa, temperature of 74°C, pressure-holding time of 13.6 min, and nisin concentration of 328 IU/mL. The validation of the model equation for predicting the optimum response values was verified effectively by ten test points that were not used in the establishment of the model. Compared with conventional HPP techniques, the main process advantages of HPP–nisin combination sterilization in the UHT milk are, lower pressure, temperature, natural preservative (nisin), and in a shorter treatment time. The synergistic inactivation of bacteria by HPP–nisin combination is a promising and natural method to increase the efficiency and safety of high-pressure pasteurization.

Keywords High pressure · Nisin · *Clostridium perfringens* spore · Central composite rotatable design · Response surface model

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Introduction

A human pathogen, enterotoxigenic *Clostridium perfringens*, is an anaerobic endospore forming gram-positive bacterium that is widely distributed in air, water, dust, soil, and food as well as gastrointestinal tract of humans and animals due to the strong resistance of its spores to physical and chemical agents [1]. This wide distribution of the spores has been considered as the main contributing factor for foodborne disease due to this bacterium. *C. perfringens* type A food poisoning currently ranks as the third most commonly reported foodborne illness following *Campylobacter* and *Salmonella* spp. in the U.S. [2]. This food poisoning is caused by a small group (~5%) of type A isolates that produce *C. perfringens* enterotoxin causing a gastrointestinal disease response [2, 3]. It is important to note that *C. perfringens* spores are notably baroresistant [4]. These features make this bacterium a very important target organism to be eliminated from the food chain.

Conventional thermal processing can have detrimental effects on the nutritional value of certain foods. In contrast to thermal processing, high-pressure processing (HPP) technique offers an alternative preservation method for processing foods [5]. Some important advantages in using HPP technology include: significant or total inactivation of vegetative microorganisms, as well as spores [6]; better functional and nutritional retention of ingredients in the processed products [7]; improved food quality parameters [8]; pressure homogeneity of treatment at every point in the product [9]; and significant energy savings in contrast with conventional retorting techniques, because once the desired pressure is reached, it can be maintained without the need for further energy input.

The commercial success of HPP and preservation of foods will depend upon the effective destruction and/or control of growth of foodborne pathogenic and spoilage microorganisms. The vegetative cells of bacteria, molds and yeasts and spores of fungi can be reduced by six log cycles at or below 690 MPa at ambient temperature or by a combination of moderate pressure and temperature. However, a five- to six-log destruction of spores of important foodborne *Bacillus* and *Clostridium* species needs a combination of very high pressure and high temperature [10]. Bacterial spores cannot be inactivated by high pressure alone [11] and can survive at pressure treatments above 1,000 MPa unless pressurization is carried out at temperatures close to 100°C [12]. However, such a drastic treatment can adversely affect the organoleptic attributes and acceptance quality of many foods and thus will have limited commercial application of HPP technology. A possible approach to overcome this important drawback of HPP is the application of hurdle technology, which relies on the synergetic combination of moderate doses of inactivating and or growth-retarding factors [5]. Combinations of high pressure with mild heat treatment and/or preservatives are more promising alternatives [13]. An interesting example of synergetic inactivation is the use of HPP together with the addition of nisin [14]. This synergetic inactivation was observed in many gram-positive bacteria that are sensitive to the natural antimicrobial peptide [15].

Nisin, an antimicrobial peptide with 34 amino acids produced by *Lactococcus lactis* subsp. *lactis*, is highly effective against a broad spectrum of gram-positive bacteria, is on the list of generally recognized as safe products from the United States Food and Drug Administration (USFDA), is approved for use by the World Health Organization [16], and has been used in the food industry as a natural preservative. At present, nisin is allowed in various products in more than 50 countries. Nisin's efficacy in preventing *C. perfringens* spore outgrowth and toxin formation has been demonstrated [17].

First-order kinetics have been used to calculate HPP-induced inactivation of microorganisms previously [18]; however, significant deviations from linearity have been

frequently reported in the literature [19]. A number of empirical models have been proposed to describe these non-linear survival curves [20]. The log-logistic [21], Weibull [22], and modified Gompertz [23] models have been used in describing the non-linear inactivation kinetics of microorganisms under various experimental conditions. Response surface methodology (RSM) is a collection of statistical and mathematical techniques useful for developing, improving, and optimizing processes in which a response of interest is influenced by several variables and the objective is to optimize this response. RSM is preferred in the work because relatively few experimental combinations of the variables are adequate to estimate potentially complex response function.

The objectives of this present study were to investigate the combined effects of high pressure, moderate heat and nisin on the reductions of *C. perfringens* AS 64701 spores; and to develop a response surface model using central composite rotatable design [24] for predicting optimized processing conditions to inactivate *C. perfringens* spores.

Materials and Methods

Bacterial Strain, Culturing Conditions, and Spore Production

The freeze-dried culture of *C. perfringens* AS 64701 type A carrying the gene that encodes the *C. perfringens* enterotoxin on the chromosome was purchased from National Center for Medical Culture Collections (NCMCC; Beijing, China) and stored at −70°C. The culture was hydrated in 10 ml cooked meat medium broth (Difco, BD Diagnostic Systems, Sparks, MD, USA) at 37°C 48 h in anaerobic condition, and then stored at 4°C. The culture stock of *C. perfringens* AS 64701 was inoculated into a tube containing 10 ml of fluid thioglycollate broth (FTB; Difco, BD Diagnostic Systems, Sparks, MD, USA) and incubated for 24 h at 37°C as previously described [25]. The sporulating culture of *C. perfringens* AS 64701 type A was prepared by inoculating 0.2 ml of overnight FTB culture into 10 ml of Duncan–Strong sporulation medium [26], which was incubated for 24 h at 37°C. The presence of spores in DS sporulation medium culture was confirmed by phase-contrast microscopy. The sporulating culture was pasteurized at 85°C for 10 min to kill the remaining vegetative cells. The resultant spore suspension was washed three times by centrifugation at 4,000×*g* for 15 min at 4°C in sterile sodium phosphate buffer (0.1 M, pH 7.0). Finally, the centrifuge-washed spores were re-suspended in the cold sterile distilled water to a concentration of approximately10^9 spores/mL, placed in cryogenic vials (Nalgene, Rochester, NY, USA) and subsequently frozen at −20°C.

Preparation of UHT Milk and Preparation of Spore Suspension in UHT Milk

Ultra-high-temperature (UHT) treated milk was obtained from a local market and stored at 4°C until used. Prior to pressure treatment, the frozen spores of *C. perfringens* AS 64701 from above preparation were thawed. The spores were harvested by centrifugation at 4,000×*g* for 15 min, washed with sterile sodium phosphate buffer (0.1 M, pH 7.0), and re-suspended in UHT milk prepared above to give approximately $(2.4\pm1.7)\times10^9$ spores/mL. One milliliter of un-inoculated UHT milk was transferred onto brain heart infusion (BHI) agar plates (Difco, Detroit, MI, USA), and incubated anaerobically at 37°C for 48 h to verify that the UHT milk sample was sterile. Five-milliliter portions of inoculated milk buffer were transferred into sterile polyethylene plastic micro-test tubes

(5-ml capacity; Nalgene, Rochester, NY), heat-sealed to avoid air enclosure, and stored on ice until pressurized. Pressurization took place within 2 h of sample preparation.

Preparation and Use of Nisin

Nisin (Nisaplin™) was obtained from Aplin and Barrett, Ltd. (Beaminster, Dorset, UK). Stock solutions of 10^6 international units (IU)/g nisin in 0.02 M HCl, were sterilized through a 0.2 μm-pore-size filter and stored at −60°C until use. Fresh nisin stock solutions were prepared weekly. An aliquot of stock solution or appropriate dilution of stock solution was aseptically added to the inoculated UHT milk samples before pressure treatments, yielding working concentrations of 0 to 496 IU/mL. The concentrations of nisin in the inoculated UHT milk samples were prepared depending on the study, as described below.

HPP Inactivation Treatments

Pressurization of samples was treated using a high-pressure unit (200-ml volume; Ke-Fang Food Machinery Inc., Baotou, Neimeng, China) at constant pressure and temperature, in the range of 100–800 MPa and 20–100°C. The bis (2-ethylhexyl) sebacate (Li-Dong Precision Machinery Co., Shenzhen, China) was used as a pressure-transferring liquid. The pressure chamber was heated/cooled to a desired level prior to pressurization using a thermostat jacket connected to a water bath. The temperature of pressure-transferring liquid inside the pressure chamber during pressurization was monitored through a K-type thermocouple (Flow Autoclave System, Inc., Vasteras, Sweden). Pressure level, time, and temperature of pressurization were controlled by a computer program (BTNMC for HPP Control 2.0, pressure value is given to an accuracy of 0.01 MPa, and temperature value is given to an accuracy of 0.1°C). For all experiments in this study, the pressure-holding time reported did not include pressure-increase time and pressure-release time. The pressure-increase rate was 350 MPa/min, and the pressure-release time was almost immediate. The temperature of the pressure-transferring liquid and sample increased during the pressure treatment as a result of adiabatic heating. The temperature increase due to adiabatic heating in the HPP chamber was less than 2°C/200 MPa. Therefore, the initial temperature of pressure-transferring liquid and sample was kept lower than processing temperature at the start of pressurization to have the samples at the processing temperature during treatment. The samples were preheated for 5 min in the pressure chamber prior to HPP treatment to equilibrate the sample temperature. Duplicate samples were used for each test at designed condition.

Measurement of HPP Survivors

According to experimental designs (Table 1), the UHT milk samples were exposed to HPP treatments. Following the release of pressure, surviving spores in the samples were determined immediately by serial dilution in 0.1% sterile peptone (Difco, Detroit, MI, USA) water, plated onto BHI agar plates (Difco), and incubated anaerobically at 37°C for 72 h prior to counting of colonies to allow surviving spores to repair and form visible colonies. The 72 h incubation time was found to be long enough to recover injured spores. Extending the incubation time did not significant ($P>0.05$) increase the plate counts (data not shown). Inactivation was expressed as a logarithmic viability reduction $\log_{10}(N_0/N_t)$,

Table 1 Observed and predicted data for log cycle reductions of *Clostridium perfringens* AS 64701 spores from CCRD matrix of four variables in coded (uncoded) units

Trial number	Variable				Y^a	
	x_1	x_2	x_3	x_4	Observed	Predicted
1	−1(500.0)	−1(50)	−1(10)	−1(124)	2.69(0.11)	2.54
2	1(700.0)	−1(50)	−1(10)	−1(124)	3.67(0.09)	3.52
3	−1(500.0)	1(80)	−1(10)	−1(124)	3.86(0.07)	3.90
4	1(700.0)	1(80)	−1(10)	−1(124)	4.72(0.12)	4.73
5	−1(500.0)	−1(50)	1(15)	−1(124)	3.64(0.07)	3.45
6	1(700.0)	−1(50)	1(15)	−1(124)	4.62(013)	4.38
7	−1(500.0)	1(80)	1(15)	−1(124)	4.48(0.09)	4.34
8	1(700.0)	1(80)	1(15)	−1(124)	5.13(0.05)	5.12
9	−1(500.0)	−1(50)	−1(10)	1(372)	3.72(0.07)	3.64
100	1(700.0)	−1(50)	−1(10)	1(372)	4.79(0.04)	4.86
11	−1(500.0)	1(80)	−1(10)	1(372)	5.73(0.06)	5.90
12	1(700.0)	1(80)	−1(10)	1(372)	6.87(0.05)	6.97
13	−1(500.0)	−1(50)	1(15)	1(372)	5.13(0.16)	5.05
14	1(700.0)	−1(50)	1(15)	1(372)	6.36(0.12)	6.23
15	−1(500.0)	1(80)	1(15)	1(372)	6.79(0.04)	6.84
16	1(700.0)	1(80)	1(15)	1(372)	7.79(.012)	7.87
17	−2(400.0)	0(65)	0(12.5)	0(248)	3.07(0.11)	3.18
18	2(800.0)	0(65)	0(12.5)	0(248)	5.13(0.15)	5.18
19	0(600.0)	−2(35)	0(12.5)	0(248)	3.34(0.11)	3.73
20	0(600.0)	2(95)	0(12.5)	0(248)	6.95(0.09)	6.72
21	0(600.0)	0(65)	−2(7.5)	0(248)	4.82(0.03)	4.73
22	0(600.0)	0(65)	2(17.5)	0(248)	6.29(0.08)	6.54
23	0(600.0)	0(65)	0(12.5)	−2(0)	2.54(0.07)	2.87
24	0(600.0)	0(65)	0(12.5)	2(496)	6.89(0.16)	6.72
25	0(600.0)	0(65)	0(12.5)	0(248)	4.14(0.13)	4.07
26	0(600.0)	0(65)	0(12.5)	0(248)	3.94(0.10)	4.07
27	0(600.0)	0(65)	0(12.5)	0(248)	4.21(0.21)	4.07
28	0(600.0)	0(65)	0(12.5)	0(248)	3.76(0.19)	4.07
29	0(600.0)	0(65)	0(12.5)	0(248)	4.18(0.15)	4.07
30	0(600.0)	0(65)	0(12.5)	0(248)	4.19(0.08)	4.07

[a] Values in parentheses are coefficients of variation of measures (%); $Y=\log_{10}(N_0/N_t)$ (N_0=the initial number of spores (CFU/mL), N_t=the number of survivals after an exposure time t (CFU/mL))

with N_t and N_0, the colony counts after a treatment and in the untreated sample, respectively.

Experimental Design and Methodology

RSM procedure is currently one of the most popular optimization techniques employed in the field of food science [27]. In this study, the four different parameters like pressure, temperature, pressure-holding time, and nisin, were chosen as the main variables and

designated as X_1, X_2, X_3, and X_4, respectively. The low, middle, and high levels of each variable were designated as −2, −1, 0, +1, and +2, respectively, and are given in Table 1. The variables were coded according to Eq. 1:

$$x_{\mathrm{i}} = (X_{\mathrm{i}} - X_0)/\Delta X \tag{1}$$

where x_{i} is the (dimensionless) coded value of the variable X_{i}, X_0 is the value of X_{i} at the center point, and ΔX is the step change. Table 1 shows the actual design of experiments. The behavior of the system was explained by the following second-degree polynomial equation Eq. 2:

$$Y = B_0 + \sum_{i=1}^{n} B_i x_i + \sum_{i<j}^{n} B_{ij} x_i x_j + \sum_{j=1}^{n} B_{jj} x_j^2 \tag{2}$$

where B_0, B_{i}, B_{ij}, and B_{jj} are regression coefficients of the intercept, linear, interactional and quadratic coefficients, respectively, and x_{i} and x_{j} are coded independent variables. Y is predicted response, namely the inactivation parameter, which can be calculated with Eq. 3

$$Y = \log_{10} N_0/N_{\mathrm{t}} \tag{3}$$

For Eq. 2, a total of 30 runs are necessary to estimate the 15 coefficients of the predictive Eq. 2.

Validation of the Experimental Model

The adequacy of the quadratic predictive model was validated, using additional ten conditions (not used for modeling) selected randomly within the range of experimental conditions (Table S1). The bias factor (B_{f}) and accuracy factor (A_{f}) were widely used to evaluate the adequacy of the predictive model [28], which are shown as follows:

$$B_{\mathrm{f}} = 10^{\left(\frac{\sum \log(\mathrm{pred}/\mathrm{Obs})}{n}\right)},$$

$$A_{\mathrm{f}} = 10^{\left(\frac{\sum |\log(\mathrm{pred}/\mathrm{Obs})|}{n}\right)},$$

where Pred is the predicted value, Obs is the observed value of the reductions of *C. Perfringens* spores, and n is the number of observations.

Data Analysis

In the present investigation, Design Expert® software (Version 6.0.5, 2001; Stat-Ease, Minneapolis, MN, USA) was used for regression analysis of the data obtained and for estimation of the coefficients of the regression equation. The SPSS software (Version 16.0, SPSS, Inc. Chicago, USA) was used to analyze the model validation data. The attained fit of the regression model was checked by the adjusted coefficient of determination ($R_{\mathrm{Adj}}{}^2$). The statistical testing of the model was performed in the form of analysis of variance (ANOVA), which is required to test the significance of the model. The two dimensional graphical representation of the system behavior, called the response surface, was used to

describe the individual and cumulative effects of the variables as well as the mutual interactions between the variables on the dependent variable.

Results

Fitting the Model for the Reductions of *C. perfringens* AS 64701 Spores

Under the different experimental conditions (combination of HPP and nisin), log cycle reductions of *C. perfringens* AS 64701 spores are summarized in Table 1. The variability associated with test samples again, was small, as indicated by the coefficients of variation given in the parenthesis. Using RSM procedure, the multiple regression analysis of the experimental data gave the following quadratic polynomial equation:

$$Y = 4.07 + 0.50x_1 + 0.75x_2 + 0.45x_3 + 0.96x_4 + 0.29{x_2}^2 + 0.39{x_3}^2 + 0.18{x_4}^2 + 0.23x_2x_4 + 0.13x_3x_4 \quad (4)$$

A summary of the ANOVA for the selected second-order model is shown in Table 2. The value of ${R_{\mathrm{Adj}}}^2$ (0.9708) for Eq. 4, being close to 1, indicates a high degree of correlation between the observed and predicted values. The value of the ${R_{\mathrm{Adj}}}^2$ (0.9708) suggests that only about 3% of the total variation is not explained by the model. The adequate precision measures the signal to noise ratio, and a ratio greater than 4 is usually desirable [29]. Therefore, the ratio of 32.03 being >4 indicate adequate signal and the quadratic Eq. 4 can be used to navigate the design space. Here, the ANOVA of the regression model demonstrates that the model is highly significant, as is evident from the calculated F value (69.81) and a very low probability value ($P<0.0001$). Moreover, the computed F value is much greater than the tabulated F value ($F_{0.01\ (14,\ 5)}=9.77$) indicating that the treatment differences are highly significant. The lack of fit of the model is not significant relative to the pure error, as is evident from the lower calculated F value (2.03) than the tabulated F value ($F_{0.05\ (14,\ 10)}=2.86$), even at 0.05 level. The coefficient values of Eq. 4 were calculated and tested for their significance using Design Expert®, and are listed (Table S2). The P value is used as a tool to check the significance of each coefficient, which in turn may indicate the pattern of the interactions between the variables. The smaller is the value of P, the more significant is the corresponding coefficient. It can be seen from this table that all the linear coefficients, three quadratic terms are significant (${x_2}^2$, ${x_3}^2$, ${x_4}^2$), and two cross-product terms are significant (x_2x_4, x_3x_4), the P values being small ($P<0.05$). The coefficient for cross-product (x_2x_3) may be slightly significant ($P=0.0654$). The other term coefficients (${x_1}^2$, x_1x_2, x_1x_3, x_1x_4) are not significant at $P>0.05$.

Table 2 ANOVA results of the quadratic polynomial model established for the reductions of *Clostridium perfringens* AS 64701spores

Source	Sum of squares	*df*	Mean square	*F* value	Prob>F
Model	54.04	14	3.86	69.81	< 0.0001
Lack of fit	0.67	10	0.067	2.03	0.2251
Pure error	0.16	5	0.033		
Corrected total	54.87	29			

R^2 =0.9849; ${R_{\mathrm{Adj}}}^2$ =0.9708; adequate precision=32.03

Validation of the Predictive Model

Figure 1 demonstrates that the experimental points were evenly distributed around the diagonal of horizontal and vertical axis, indicating an excellent predictive model performance for the reductions of *C. perfringens* spores in milk under different pressure–temperature–time–nisin (HPP–nisin) combinations of process parameters. The determination coefficient (R^2) between the experimental and predicted values is 0.9996, with a statistical significant level of $P<0.0001$. B_f is a measure of the extent of under- or over-prediction by the fitted model. Perfect agreement between predictions and observations will lead to a B_f of 1. When B_f is below 1, it indicates that the predicted valves are higher than the observed, and the model is fail-safe. Conversely, a value of above 1 means the model is fail-dangerous in practice. Ross [30] proposed the following interpretation of B_f when used for model performance evaluations involving pathogens: 0.90–1.05 can be considered good. According to this standard, 0.9854 of B_f in this work was within the good range. A_f value provides indication of the average accuracy of estimates. An A_f value of 1 indicates that the model produces a perfect fit to the observed data. The larger the A_f value, the less accurate the predictions [31]. The average estimate 1.0182 of A_f in this study was within the acceptable ranges. Thus, the results of analysis indicated that the model of Eq. 4 was satisfactory and accurate.

Localization of Optimum Condition

A few viable pathogenic bacteria in some foods can cause severe foodborne illness, and the naturally present spoilage and pathogenic microorganisms in safe food products for consumers are normally less than 10^6 CFU/g or 10^6 CFU/mL [9, 32]; therefore, a six-log cycle reduction of *C. perfringens* AS 64701 spores was used as a target level of inactivation by combined treatments of HPP and nisin in the work. The process parameters for a six-log cycle reduction of *C. perfringens* AS 64701 spores were to be optimized in this investigation.

The graphical representations of the regression Eq. 4, called the response surfaces and the contour plots, are presented in Fig. 2. The graphical representation visualizes the relationship between the response and experimental levels of each variable.

Figure 2a displays the effects of pressure and temperature on the reductions of *C. perfringens* AS 64701 spores by keeping pressure-holding time and concentration of nisin at the optimum values (13.6 min, +0.44 level; 328 IU/mL, +0.65 level). From Fig. 2a, it can

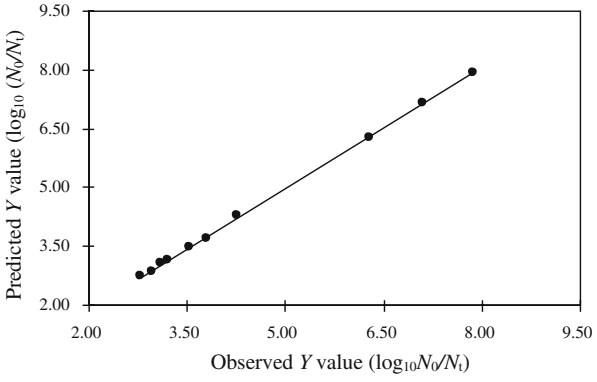

Fig. 1 Predicted vs. observed plot for validation of the regression model

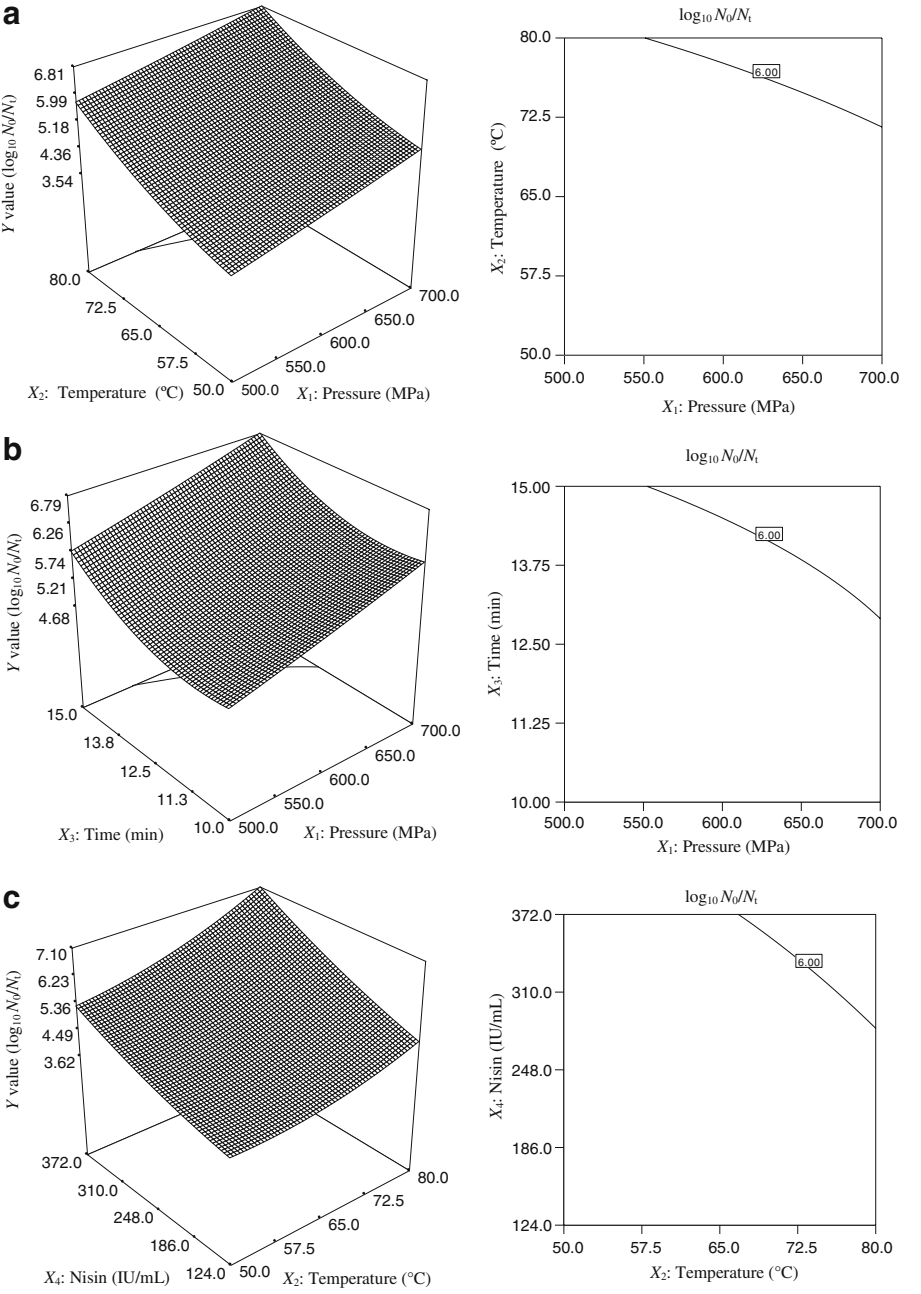

Fig. 2 Response surface and corresponding contour plots showing the reductions of *Clostridium perfringens* AS 64701 spores affected by **a** pressure and tempertaure with constant values of pressure-holding time (13.6 min) and concentration of nisin (328 IU/mL), **b** pressure and pressure-holding time with constant values of temperature (74°C) and concentration of nisin (328 IU/mL) and **c** temperature and concentration of nisin with constant values of pressure (654 MPa) and pressure-holding time (13.6 min); the data on the contour maps are $\log_{10}N_0/N_t$-value

be seen that the reductions of *C. perfringens* AS 64701 spores increased with increase in both pressure and temperature, reaching a six-log cycle reduction at pressures of 550 to 700 MPa and temperatures of 71 to 80°C. The optimum value was obtained by the derivation of Eq. 4 and by solving its inverse matrix, fixing the optimization level of six-log cycle reductions of *C. perfringens* AS 64701 spores at a pressure of 654 MPa and a temperature of 74°C.

Figure 2b shows how the reductions of *C. perfringens* AS 64701 spores vary with pressure and pressure-holding time at fixed temperature of 74°C (+0.60 level) and nisin concentration of 328 IU/mL (+0.65 level). In the range of pressures from 551 to 700 MPa, and pressure-holding times from 12.5 to 15 min, the reductions of spores of *C. perfringens* AS 64701 occur more than six-log cycles. Therefore, one can optimize the level of a six-log cycle reduction of spores of *C. perfringens* AS 64701 at a pressure of 654 MPa and a pressure-holding time of 13.6 min using the model.

The contour plot in Fig. 2c, which gives the reductions of spores of *C. perfringens* AS 64701 as a function of temperature and nisin concentration at a fixed pressure of 654 MPa (+0.54 level), and a pressure-holding time of 13.6 min (+0.44 level), shows that the reductions of *C. perfringens* AS 64701 spores increase with increasing temperature and nisin concentration, reaching a six-log cycle reduction of *C. perfringens* AS 64701 spores at about temperatures of 66 to 80°C and nisin concentrations of 279 to 372 IU/mL. The optimization level of a six-log cycle reduction of *C. perfringens* AS 64701 spores is reached at a temperature of 74°C and a nisin concentration of 328 IU/mL.

Discussion

For minimally processed foods, a deliberate combination of various hurdles can be combined to control food quality and microbial safety. By analyzing the pressure-, temperature-, time-, and nisin-plots, the significant synergistic effects are observed among the four parameters. As the level of one of the four variables rises during pressurization, there is a decrease in the levels of the other three variables. For example, increasing concentration of nisin in a certain treatment gives the opportunity to decrease the treatment pressure, temperature and pressure-holding time. Inversely, an increase of the pressure applied seems to give the opportunity to decrease the treatment temperature, the processing time and nisin concentration.

According to Paredes-Sabja et al. [33] at 75°C and 650 MPa, only about 2.8-log cycle reductions of *C. perfringens* spores were achieved after 15 min of pressure-holding time in citric acid buffer (pH 4.75). Akhtar et al. [34] demonstrated that the spores of *C. perfringens* can be inactivated by approximately 4.0-log cycles with shorter germination period (55°C for 15 min) when spore contaminated-meat was treated with pressure-assisted thermal processing (568 MPa, 73°C, 10 min). Preliminary experiments revealed that spores of *C. perfringens* AS 64701 were inactivated by about 0.71-log cycles at 800 MPa and 25°C for 35 min (data not shown). In summary, it seems that the strains of *C. perfringens* are more resistant to HPP.

By contrast, however, the values of the three process parameters (pressure, temperature, and time) for a six-log cycle reduction of *C. perfringens* AS 64701 spores were greatly decreased in the present study, since the use of HPP combined with the addition of nisin played an important role. It is clear that a marked advantage using Design Expert® to optimize the inactivation condition is the optimized HPP–nisin process at a lower pressure, temperature and concentration of nisin in a shorter treatment times. As the pressure

increases, the costs of the high-pressure vessel and processing costs rise. A specific HPP–nisin combination has appeal in that this treatment must be lower in energy use. This means that less costly apparatus can then be used in many facilities, rather than only in a limit number of specialized laboratories and factories. A food processor will finally choose this combination that will give them the best results under the most economical and beneficial condition. As a maximum volume of product must be treated within an hour to minimize treatment costs, treatment time will be one of most decisive parameters. Processing times in HPP–nisin must be short to make this process cost effective. Therefore, it is necessary to find a treatment that is synergistic with HPP to inactivate endospore forming bacteria at a minimal concentration of nisin. It may be expected that the milder heat treatment has a positive effect on product characteristics which are more susceptible to heat. The main process advantages of HPP–nisin combination sterilization of food products compared to conventional techniques are, therefore, shorter treatment times, lower temperatures, pressures and natural preservative (nisin). This HPP–nisin treatment must be low in energy use. The optimized sterilization process is commercially the most interesting. The synergistic inactivation of bacteria by HPP–nisin combination is a promising and natural method to increase the efficiency and safety of high-pressure pasteurization.

Conclusions

In this communication, a six-log inactivation of *C. perfringens* spores of process condition has been optimized during HPP–nisin in milk. The results can contribute significantly to the safety of minimally processed foods with respect to *C. perfringens* spores, and open perspectives for controlling other sporeforming food spoilage and pathogenic bacteria in other foods. In addition, the data presented here were carried out in UHT milk buffer and extensive experiments for application in various foods are required to give insight into what may happen in other food system. Further study also would be necessary to explore whether the optimum HPP–nisin process conditions in the work are applicable to other organisms.

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