Research Note

Inactivation of *Escherichia coli* K-12 Exposed to Pressures in Excess of 300 MPa in a High-Pressure Homogenizer

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ABSTRACT

Homogenization is used widely in the dairy industry to improve product stability and quality. High-pressure homogenization (HPH) of fluid foods up to pressures of 300 MPa has demonstrated excellent potential for microbial inactivation. Microbial inactivation can be enhanced during HPH with the inclusion of antimicrobial compounds. *Escherichia coli* K-12 cells, grown statically or in chemostat, were exposed to HPH processing pressures of 50 to 350 MPa in the absence or presence of the antimicrobial nisin. Valve temperature was regulated by a water bath and pressure, and temperature data were recorded continuously after process initiation. Survivors were enumerated via plating on nonselective growth media. Pressure and temperature at the valve outlet port exhibited a quadratic relationship ($R^2 = 0.9617$, $P < 0.05$). Significant HPH-induced inactivation of the gram-negative microorganism was observed in the range of 100 to 250 MPa. Above 300 MPa, heat was the main factor promoting microbial inactivation, regardless of whether cells were grown in chemostat or statically. Chemostat-grown cells were significantly ($P < 0.05$) more resistant to HPH processing than were statically grown cells. Data indicate potential synergistic effects of nisin and HPH on the inactivation of bacterial contaminants. This study represents the first report of inactivation of a bacterium with HPH pressures in excess of 300 MPa in the presence and absence of an antimicrobial.

Homogenization has been used widely in the dairy industry for many years to produce monodispersed emulsions and avoid phase separation. The advent of high-pressure homogenization (HPH) has allowed the opportunity to evaluate this technology as a means to inactivate microbial contaminants in fluid foods under conditions of continuous processing. One of the major advantages of using HPH in the food industry is the reduction of potential negative side effects and limitations associated with the thermal processes used for microbial inactivation (15). This new application of homogenization came about primarily because of advances in homogenizer design whereby processing pressures in excess of 100 MPa were made possible (9, 12). Several studies have since detailed the utility of HPH for the inactivation of microorganisms. Early reports used processing pressures approximating 50 MPa and were focused on the inactivation of various yeasts (6, 10). A number of microorganisms have been shown to be inactivated by HPH at pressures of 100 to 300 MPa including *Listeria innocua*, *Micrococcus luteus*, and *Pseudomonas fluorescens* in raw whole milk (11). Other pathogens that have been tested include *Staphylococcus aureus*, *Yersinia enterocolitica*, *Escherichia coli*, and *Salmonella enterica* serovar Typhimurium (3, 5, 19, 20). Researchers have generally agreed that gram-negative pathogens such as *E. coli* are more susceptible to processing, potentially resulting from decreased peptidoglycan content in their outer cell membranes versus their gram-positive counterparts (19).

Keshavarz Moore et al. (10) theorized that impingement of cells through the homogenizer might constitute the primary mechanism of microbial inactivation, based on studies utilizing impact rings of differing diameters and observation of varying levels of inactivation. As opposed to high hydrostatic pressure that has been reported to cause protein denaturation and structural and functional damage to the microbial cell membrane, HPH has been theorized to cause cell lysis and/or inactivation through combined effects of pressure, cavitation, turbulence, high temperature, and shear stress (8, 10, 13, 14).

Microbial inactivation can be enhanced during HPH with the inclusion of antimicrobial compounds. Destabilization of the microbial membrane and conformational modifications of enzyme structures via HPH are two accepted theories explaining increased antimicrobial efficacy (16, 17). The instantaneous loss of viability in selected gram-negative and gram-positive bacteria inoculated in skim milk has provided evidence for the possible existence of synergism between HPH and the antimicrobials lysozyme and the lactoperoxidase system (17). In contrast, Diels et al. (4) determined that pressures exceeding 150 MPa during HPH are necessary for true synergism but are also dependent on antimicrobial concentration.

The objective of this study was to evaluate HPH alone and in combination with the lantibiotic nisin on inactivation of *E. coli* K-12 (biotype 1). The HPH process used was...
unique in that it was capable of achieving 350 MPa, a higher pressure than was achieved in earlier studies. Additionally, the potential differences in inactivation of batch culture cells (stationary phase) with chemostat-cultured mid-log-phase cells were evaluated at 350 MPa.

**MATERIALS AND METHODS**

**Indicator strain and growth conditions.** *E. coli* K-12 was obtained from University of Tennessee Food Science and Technology culture collection and grown aerobically at 35°C prior to processing. Cells were grown either statically in tryptic soy broth (Becton Dickinson, Sparks, Md.) for 24 h or in a Bio-Flo fermenter (New Brunswick Scientific, Edison, N.J.) in tryptic soy broth. In the case of continuously grown *E. coli* K-12 cells, growth media input rate was 0.6 ml/min into a steady volume of 260 ml, with an agitator speed of 400 rpm. Air flow into the system was at a rate of 166.66 vol/vol/min (ml air/ml media/min) standardized, and air was filtered through a 0.2-μm Millex-FG filter (Millipore Co., Billerica, Mass.) prior to mixing with cells.

**Bacteriocin preparation.** The lantibiotic nisin was obtained from Sigma-Aldrich (St. Louis, Mo.) in powdered form with a concentration of 2.5% actual nisin. A stock solution of 0.1 g/10 ml 20 mM hydrochloric acid (Fisher Scientific, Fairlawn, N.J.) was generated and boiled at 100°C for 4 min to inactivate extraneous protein according to previously published methods (18). The concentration of nisin in the stock was 10,000 IU/ml. Nisin was mixed with the experimental diluent (0.9% sodium chloride water; Fisher Scientific) to a concentration of 10.0 IU/ml just prior to addition of target cells.

**Bacterial inactivation assay.** *E. coli* K-12 cells were immediately transferred directly from each growth source and diluted in sterile 0.9% sodium chloride water to give a cell stock (~log 7 to 8 CFU/ml). Cells were mixed with vigorous stirring for at least 1 to 2 min prior to homogenization through an FPG 12500 bench-top high-pressure homogenizer (Stansted Fluid Power, Ltd., Essex, UK). The operating structure consists of a bench-top unit that provides synchronized homogenization through two hydraulic intensifiers. Using the supervisory control and data acquisition software package Lookout, version 5.1, and Labview, version 7.1 (National Instruments, Austin, Tex.), actual chamber pressure and temperature were continuously recorded during experimentation. The data logger was initiated immediately prior to processing. Samples were collected every 50 MPa (50 to 350 MPa) and placed on ice immediately following collection. Valve temperature was regulated by a water bath at a constant temperature of 5°C. After sample collection was completed, samples were serially diluted in sterile 0.1% peptone water (Fisher Scientific) and plated on tryptic soy agar (Becton Dickinson) and incubated at 35°C for up to 36 h to enumerate survivors.

**D-value determination.** Culture from both preparation methods (traditional and chemostat) was diluted 1:10 and placed into closed screw-cap glass vials (12 by 35 mm). Initial population control counts were made on this dilution. The total volume added to each vial was 2.1 ml. The buffer pH was ~7 for both cell types. A circulating water bath was used to immerse and maintain two perforated aluminum baskets containing the test vials at a constant temperature prior to extraction at preset intervals. Tests were performed at 58°C. Water bath temperature was confirmed with a mercury-in-glass thermometer. Two vials of each culture preparation were extracted from the bath at each time point. The initial time point was 1 min, followed by 3-min and 3-min intervals for a total time of 33 min. Upon removal, sample vials were immediately cooled in an ice water bath to stop thermal inactivation. After the vials were adequately cooled, samples were taken. The earlier time points were serially diluted as needed to achieve readable plates for enumeration. D-value was calculated as the time required for a 1-log (90%) reduction from the straight-line regression of log survivors versus time in minutes.

**Statistical analysis.** Results were analyzed as a factorial experiment of two factors (antimicrobial at two levels and pressure at seven levels) in a completely randomized design with two replications for chemostat grown cells and three replications for statically grown cells. In cases where the interaction between antimicrobial and processing pressure was significant (*P* < 0.05), experimental units containing nisin were analyzed as independent experiments from nisin-free experimental units. The Student’s *t* distribution was used to generate 95% confidence intervals for the mean, in cases where analyses of variance showed significant differences. Data were analyzed using SAS software, version 9.1 (SAS Institute, Cary, N.C.).

**RESULTS AND DISCUSSION**

Bacterial inactivation because of HPH is thought to result via both thermal and nonthermal processes. Figure 1 shows the empirical relationship between pressure and temperature for four independent experiments, as homogenizing pressure was increased from atmospheric pressure up to 350 MPa. Although heat was removed from the valve surroundings using a controlled temperature water bath (5°C) connected to the valve casing, pressure and temperature at the valve outlet port exhibited a quadratic relationship ($R^2 = 0.9617; P < 0.05$) (Fig. 1). As homogenizing pressure approached 200 MPa, temperature reached ~60°C, known to promote *E. coli* K-12 inactivation having $D_{60°C} = 1.7$ and 0.94 min for statically and chemostat-grown cells, respectively (unpublished data). Despite the HPH-induced temperature increase in the samples, thermal inactivation effects were relatively minor for pressure below 250 MPa due to the short residence time (20-s maximum, defined as the time between sample pressurization and sample cooling).

Population reduction of statically grown *E. coli* K-12 cells after exposure to HPH or exposure to nisin at 10 IU/
FIGURE 2. Inactivation of statically grown E. coli K-12 cells after high-pressure homogenization in absence (0 IU/ml nisin) and presence (10 IU/ml nisin) of nisin. Symbols represent means of triplicate replications, with error representing 95% confidence intervals for the mean. Equivalent Thermal Treatment is the predicted thermal inactivation by shear-induced heat generated at the corresponding pressure (D58 = 4.04 min, z = 5.4°C, 20-s residence time).

ml, followed by HPH is shown in Figure 2. As the homogenizing pressure increased from 0 to 200 MPa, inactivation of the E. coli K-12 population increased, reaching a maximum 7-log reduction, the limit of the plating assay sensitivity, at pressures above ~200 MPa for nisin-treated samples and ~225 MPa for nisin-free samples. Statically grown E. coli K-12 cells have a D58C = 4.04 min (unpublished data), and based on z = 5.4°C (2) and a 20-s maximum residence time (20-s equivalent thermal treatment), thermal-related inactivation was negligible between 50 to 200 MPa. Above 300 MPa, however, heat itself was the main factor promoting the strong decrease in microbial populations (Fig. 2). A significant pressure-antimicrobial interaction (P < 0.05), evidence of a synergistic effect of nisin on homogenizer-modulated inactivation, was observed with pressure in the 0- to ~200-MPa range. While nisin-free and nisin-treated samples exhibited no significant difference in population at atmospheric pressure (0 MPa), a gradual nisin-induced inactivation increment was observed as the homogenizing pressure was increased, reaching a maximum 2- to 3-log cycle inactivation difference in nisin-treated versus nisin-free samples pressurized at ~200 MPa. Above 225 MPa pressure, shear-induced thermal effects became increasingly important in determining microbial inactivation (Fig. 2).

Chemostat-grown cells demonstrated rates of inactivation after exposure to the bacteriocin and HPH or HPH alone that were dramatically slower than static-grown cells (Fig. 3). Significant HPH-induced inactivation of the gram-negative organism was observed within the 100- to 250-MPa range. Nevertheless, inactivation of chemostat-grown E. coli K-12 cells to the same level as the statically grown cells (ca. 7 log) was observed only above 250 MPa, where thermal effects were a major contributing effect on the microbial inactivation (Fig. 3). There was no significant difference between the HPH inactivation with or without nisin present. The results with mid-log-phase cells were somewhat unexpected in that the literature consistently reports cells in this phase to be more sensitive to stresses than are stationary-phase cells that were represented by the statically grown cells (1, 4). While chemostat cells are more sensitive to elevated temperature (D58C = 2.2 min, z = 5.4°C; unpublished data), a limited additive effect of nisin on pressure-induced inactivation was observed at pressure ~250 MPa, where temperature was the main factor determining microbial inactivation.

Other researchers have reported inactivation of bacterial and fungal species at processing pressures similar to those reported here (3, 5–7, 10, 20). While it is currently unknown as to the exact combination of microbial disruption and inactivation mechanisms, it is likely that enhanced HPH either removes (via shear) or permeabilizes the outer membrane of gram-negative bacteria, thereby increasing sensitivity to the bacteriocin (3). The results of the present study indicate that sensitivity to inactivation processes other than heat may not be easily predicted by growth phase. Data indicated that, while mid-log (chemostat-grown) E. coli K-12 cells were more sensitive to inactivation by elevated temperatures, stationary or statically grown cells were more resistant to inactivation by HPH. This may be evidence that different mechanisms are responsible for cellular inactivation.

In addition to the need for further research into the exact mechanism by which bacterial cells are inactivated by HPH, the exact nature of the interaction between nisin and HPH needs to be fully elucidated. This is the first report of HPH-induced inactivation of a bacterium at processing pressures exceeding 300 MPa, both in the absence and presence of a naturally occurring GRAS (generally recognized as safe) status antimicrobial. Our results demonstrated the potential for HPH processing of fluid foods, not only to increase product stability and quality, but also to reduce the level of microbial contamination of these types of products.

REFERENCES


