Determination of Thermal Inactivation Kinetics of Microorganisms with a Continuous Microflow Apparatus

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ABSTRACT

Use of a continuous microflow submerged microcoil (CSMC) apparatus was compared with the capillary tube (CT) method for measuring the thermal inactivation kinetics of Pseudomonas fluorescens at 61°C for 3 to 29 s. Inocula were continuously pumped through a microbore (<0.0762 cm inside diameter) thin-walled stainless steel capillary tube submerged in a heated oil bath. The heating time was set by changing the flow rate, tube dimensions, or both. With the use of microthermocouples, the time for the inocula to reach within 1°C of the set temperature was <3 s, and shorter than that with capillary tubes or vials. Inactivation curves (61°C) for P. fluorescens prepared by the CSMC method were not different from curves prepared by the CT method, as determined by analysis of variance (P > 0.05). Inactivation of Bacillus cereus spores (105°C) and native microflora found in raw milk (72°C) over heating times of 3 to 42 s were determined by CSMC. CSMC can measure thermal inactivation kinetics of microorganisms efficiently and simply at high temperatures and in short times. Survivors can be enumerated in 1-ml volumes of heat-treated samples, making it useful for determining inactivation kinetics of low numbers of microorganisms, such as those found in high-quality raw milk. Inactivation kinetics were generally more accurately described by the Weibull function (R² ≥ 0.97) than the linear kinetic model.

Several methods have been developed to estimate thermal inactivation of microorganisms, and each has advantages and disadvantages. The most common approach is to heat small volumes of liquid foods or media containing high concentrations of bacterial inocula in small vials or capillary tubes and enumerate the survivors over increasing durations of exposure. Glass vials containing 2 ml of inocula (5) and capillary tubes containing either 0.05 (16) or 0.01 ml (21) are commonly used. Recently Al-Holy et al. (1) described a method that used an aluminum tube to heat 1 g of liquid or semisolid samples by submerging in a heated bath. The time to reach set temperature was 90 s in a 65°C bath, nearly half the time required to reach temperature compared with that in glass tubes. The time required to reach set temperature and the required dilution factor can result in uncertainties when short time periods (<1 min) or low initial counts are used (4).

An alternative method involves heating large volumes of food, buffer, or media, inoculating with a small volume of a high concentration of bacteria, and sampling over time (20, 24). This method significantly reduces the time to reach set temperature, but mixing can be a limitation (11). An alternative method uses a commercially available apparatus based on a stainless steel (SS) coil holding approximately 9.5 ml of inoculum, which is submerged in a heated water bath. Inoculated medium is injected rapidly into the coil, and a solenoid valve system displaces the heated inoculum with sterile water at precise time intervals (3). Pilot plant scale equipment has also been used to generate inactivation curves (6). The use of pilot plant equipment assures that the heat treatment is comparable to commercial practice but is not practical, especially when working with pathogens.

A flow-injection system has been described (16) that pumps small volumes of inocula through glass tubing submerged in a heated water bath. The number of thermal survivors obtained with the flow injection system was consistently greater than that obtained with the capillary tube (CT) method. This result might be attributable to the large time required to reach set temperature because of the large tube inside diameter (i.d.; 3 mm) and the relatively poor thermal conductivity of glass.

In raw milk, vegetative cells and spores are present at approximately 10⁴ and 10² CFU/ml, respectively (2). The 10² dilution factor (0.01 ml inoculum) inherent in the CT method prevents the measurement of inactivation kinetics for these low numbers of bacteria found in milk. These limitations of the CT method prevent accurate measurement of thermal inactivation kinetics at short times and with the use of realistic numbers of bacteria.

Our work requires determination of inactivation kinetics of low levels of vegetative cells and spores at temperatures of 50 to 140°C over short heating times (3 to 60 s). To accomplish this, we needed a method in which the time to reach set temperature was short compared with the exposure time and in which samples of at least 1 ml could be directly plated.

MATERIALS AND METHODS

Description of continuous microflow submerged microcoil. The continuous microflow submerged microcoil (CSMC) ap-

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paratus consisted of a reservoir containing the inoculum, a high-pressure pump (Mini-Pump, Milton Roy Company, Riviera, Fla.), submersed microcoil, and heated oil bath (Fig. 1). Raw milk, in-\culated media, or buffer was pumped from the reservoir. Holding time was adjusted by changing the pump speed, dimensions (length, inside diameter), or both of the microcoil. The inoculum was pumped through thin-walled SS microcoils (Small Parts Inc., Miami Lakes, Fla.) of 0.0254, 0.045, or 0.076 cm i.d. The microcoil was submersed in an insulated bath (Virtis Research Equipment, Gardiner, N.Y.) filled with oil (Fisher High Temperature Oil, Fairlawn, N.J.), heated by a circulator (Thermomix 1480, Braun, Vorwerk, Germany), and agitated with an overhead stirrer (Wheaton Instruments, Mellville, N.J.). Immediately after the oil bath, the coil was connected to a SS tube (0.076 cm i.d., 0.16 cm outside diameter (o.d.), 25 cm length) held in an ice water–flooded flask. The heated and cooled inocula were immediately collected in a sterile vial. Back pressure was regulated by an adjustable screw clamp valve fixed to a section of flexible tubing at the outlet end, allowing the use of temperatures of more than 100°C. System pressure was measured by a 316 SS pressure gauge. Heating times were determined by dividing the coil volume by the flow rate as measured gravimetrically (weight/time). Oil bath temperature was measured with a calibrated mercury thermometer.

**Empirical measurements of time to reach set temperature.** The time required for the test liquid to equilibrate with the set bath temperature was measured for different coiled SS tubes, glass capillary tubes, and vials.

For CSMC, the time required for skim milk (22°C) to reach within 1°C of the set temperature (71.3°C) was measured in two coils of different sizes (0.076 cm i.d. and 0.0254 cm i.d. by 0.1588 cm o.d. by 30 cm, volumes 0.14 and 0.015 ml respectively), with a microthermocouple (model 872 recorder, 0.00762 cm diameter, type J, response time = 0.09 s, Omega, Stamford, Conn.).

Stainless steel tubing similar to that used by Cole and Jones (3) (0.24 cm i.d. by 0.3175 cm o.d. by 208 cm, volume 9.6 ml) was also evaluated in a 71.3°C oil bath. The microthermocouple was inserted 2 cm into the submerged tube from the outlet end. The heated tube was rapidly filled with 22°C skim milk, and the time required to reach 70.3°C was recorded.

Come-up time was also measured for 0.01 g of milk in a glass capillary tube (Kymax-51 no. 34500; 1.5 to 1.8 mm diameter by 50 mm). The thermocouple was placed in the geometric center of the tube and sealed with epoxy after filling. Tubes were lowered into a 71.3°C bath, and the time to reach 70.3°C was recorded. Come-up time for 2 ml of milk in a 2-ml glass crimp-top vial (Agilent Technologies, http://www.chem.agilent.com) was measured in a similar manner.

**Calculated temperatures.** The length of SS tubing required to bring flowing skim milk to set temperature was calculated (12).

The volume (ml) of milk in this calculated length of tubing was divided by the flow rate (ml/s) to calculate the time to reach set temperature.

\[
L = \frac{q_r}{(\pi \cdot \text{i.d.} \cdot h_r \cdot \text{LMTD})}
\]

where \(h_r\) is the average heat transfer coefficient (W/m² K) calculated from the empirical data collected with tube lengths of 30 and 50 cm and flow rates ranging from 3.7 to 9.8 × 10⁻⁵ kg/s (average ± SD) \(h_r = 1.0114 ± 86.1 \text{s}^{-1} \text{[m² K]}^{-1}\); LMTD is the log mean temperature difference (K); i.d. is the inner diameter of the SS capillary tube (m); and \(q_r\) is the rate of heat transfer (W).

\[
q_r = c_p M(T_2 - T_1)
\]

where \(c_p\) is the specific heat (J/kg K, estimated to be 4,000 J/kg K over a range of 0 to 100°C (19)), \(M\) is the mass flow rate (kg/s), \(T_2\) is temperature (K) of milk at the inlet, and \(T_1\) is temperature (K) at the outlet.

To determine the time for 9.6 ml of quiescent skim milk to reach set temperature, the rate of heat transfer \((q)\) was divided by the overall heat transfer coefficient \((q')\) (9), where \(q\) is the rate of heat transfer \((-m \cdot r \cdot \Delta T, J)\), \(m\) is the mass of skim milk \((kg)\), \(c_p\) is the specific heat of skim milk \((J/kg K)\), \(\Delta T\) is the starting temperature minus the final temperature \((K)\), and \(q' = (UA/\Delta T, J/s)\) is the overall heat transfer coefficient.

\[
U = \frac{1/(h_i) + [\ln(r_o/r_i)]k_A}{(1/h_o)} + (1/h_o)
\]

where \(h_i\) is the heat transfer coefficient for water (400 W/m² K, substituted for skim milk (8)), \(h_o\) is the heat transfer coefficient for bath oil (75 W/m² K (8)), \(k_A\) is the thermal conductivity of stainless steel (14.8 W/m K (9)), and \(r_o\) and \(r_i\) are the outside and inside radii (m), respectively.

**Comparison of bacterial inactivation with the CSMC and CT methods.** Stock culture (−80°C) of *Pseudomonas fluorescens* R1-232 was streaked onto brain heart infusion agar and incubated at 30°C for 48 h. An isolated colony was inoculated into 5 ml of trypticase soy broth and incubated for 18 h at 30°C with agitation (150 rpm, G-24 Environmental Incubator Shaker, New Brunswick, N.J.). Seventy-five microliters was transferred to 75 ml of Trypticase soy broth and incubated for 18 h at 30°C with agitation. Butterfield’s buffer (BB; 75 ml (15)) was added. This inoculum (1 × 10⁹ CFU/ml) was held at room temperature and used for infection studies with the CSMC and CT methods.

Glass capillary tubes were injected with 0.01 g of the inoculum (measured gravimetrically) and sealed at both ends with a propylene torch.

A SS capillary tube (0.0762 cm i.d. by 0.16 cm o.d. by 56 cm long, volume 0.255 ml) was used in the CSMC. The pump was set to attain heating times ranging from 3 to 29 s at approximately 3-s intervals. Holding time was calculated as described in “Description of continuous microflow submersed microcoil.” The oil bath temperature was 61°C. The *P. fluorescens* inoculum was pumped through the apparatus, and aliquots were collected in sterile tubes. Similar holding times were used for CT tests. Capillary tubes were heated for the corresponding time, cooled in an ice bath for 1 min, submerged in Trichloro-o-cide sanitizer for 1 min, submerged in 95% ethyl alcohol for 1 min, air dried for 1 min, then transferred to BB and crushed with a sterile glass rod. Both CSMC and CT samples were serially diluted in BB.

Aliquots (100 μl) were spread on tryptic soy agar and incubated at 32°C (monitored with a calibrated thermometer) for 48 h, and colonies were enumerated (Quebec Colony Counter, American Optical Corp, Buffalo, N.Y.). Results were expressed as the
log of the ratio of the number of \( P. \) \( \text{fluorescens} \) at time \( t \) (\( N_t \) CFU per milliliter) and the initial number present (\( N_0 \) CFU per milliliter) and are the averages of three independent experiments.

**Inactivation rates \( (72^\circ C) \) of \( B. \) \( \text{cereus} \) \( A1-029 \) spores.** \( B. \) \( \text{cereus} \) \( A1-029 \) was provided by the Food Safety Lab (Cornell University), and high-purity (>95%) spores were prepared as previously described (23). An aliquot of the spore preparation (0.1 ml) was added to 100 ml of BB, held on ice, and used as the inoculum (3.4 \( \times \) 10^8 CFU/ml) for the inactivation rate experiments. Temperature of the oil bath was \( 105^\circ C \). A 0.551-ml SS holding tube (0.0762 cm i.d. by 0.16 cm o.d. by 121 cm long) was used. Holding times were determined as before and ranged from 5.7 to 42 s. Back pressure ranged from 11 psi at the lowest flow rate to 22 psi at the highest flow rate.

Heat-treated and untreated (time 0) spores were vortexed, diluted in BB, plated on standard methods agar (SMA) containing 0.1% soluble starch (14), incubated at 33\( ^\circ C \) for 38 h, and enumerated. The experiment was repeated in triplicate.

**Inactivation rates of indigenous raw milk microorganisms.** Inactivation rates were determined for native flora in raw milk from two different sources. Initially, multifarm, commingled raw whole milk was obtained from Dairy One (Lansing, N.Y.), a milk-testing lab. Fifteen samples, each collected from individual milk tankers 4 or 5 days before the experiment, were commingled and held on ice. Initial standard plate counts (SPCs) were 3 \( \times \) 10^8 CFU/ml. Commingled milk was pumped through a 0.250-ml SS microcoil (0.076 cm i.d. by 0.16 cm o.d. by 55 cm long) submerged in a 72\( ^\circ C \) oil bath. Heat-treated milk was collected in preweighed vials and weighed, and holding times (2.6 to 19.2 s) were determined as before. Heat-treated milk was serially diluted in BB, pour plated (SMA), and incubated at 32\( ^\circ C \) for 48 h, and survivors were enumerated. Results are expressed as the log of the survivor ratio. This experiment was repeated in triplicate.

In a separate experiment, raw skim milk was obtained from the Cornell University Dairy and held at 4\( ^\circ C \) for 24 h before preparing inactivation curves. Inactivation rates for four different bacterial populations were measured simultaneously. Gram-negative bacteria were plated on MacConkey agar. Spores were plated on SMA after the heat-treated inoculum was heated further for 12 min at 80\( ^\circ C \). SPCs were plated on SMA. Plates were incubated at 32\( ^\circ C \) for 48 h, and spores were enumerated. Psychrotrophs were plated on SPCs and incubated at 5\( ^\circ C \), and enumerated after 10 days. Results were expressed as log CFU per milliliter.

**Statistical methods.** The log of the survivor ratios, \( S = N_t / N_0 \), were plotted against time (s). The NLIN procedure of the SAS statistical program (version 8.02, Cary, N.C.) was used to estimate scale (\( b \)) and shape (\( n \)) parameters of the Weibull distribution function \( \log S = -bt^n \). These parameters were substituted back into the Weibull function to generate data plots of predicted survivors over time with Microsoft Excel 97. \( R^2 \) values for both the linear and nonlinear regressions were calculated according to standard statistical methods (17). An analysis of variance from the general linear model (Minitab, version 14.1, Carey, N.C.) was used to compare the rate of microbial inactivation measured by the CSMC and CT methods.

**RESULTS**

**Time to reach set temperature.** The times to reach within 1\( ^\circ C \) of the set temperature for milk in the microcoils were 3 and 0.29 s for 0.076 and 0.025-cm i.d coils, respectively. The calculated time to reach set temperature for both tubes agreed with the measured values to within 0.1 s. A submerged coil similar to that described by Cole and Jones (3) required 25.3 s \( (\pm 0.38 \) s) to reach within 1\( ^\circ C \) of the set temperature, which agreed well with the calculated time of 24.5 s. The glass vials and capillary tubes averaged 148 \( (\pm 5.3) \) and 9 s \( (\pm 0.48) \), respectively, to reach within 1\( ^\circ C \) of the set temperature. Similar times for the CT method have been reported by Stern et al. (21), who found when heating tubes from 26 to 172\( ^\circ C \) that after 4.5 s, the fluid was 8\( ^\circ C \) below that of the heated oil bath. Fairchild et al. (7) reported that it required 2.5 s for inoculated medium in glass capillary tubes to reach set temperature. They used a capillary tube reactor with a high-velocity, heated water flow around the capillary tube, which accounts for the shorter time than we report. The relatively long time required to come to set temperature for the glass vials and the submerged coil methods would not meet our objective of measuring thermal inactivation over short times, and the low volume of the capillary method precluded its use for low initial counts. However, the short time for the CSMC to reach set temperature and the ability to collect large volumes suggested that this method would meet our objectives if the data compared favorably to established methods such as the CT method.

**Comparison of thermal inactivation rates with the CSMC and CT methods.** The time required to reach within 1\( ^\circ C \) of the set temperature for the CT method compared with CSMC suggested that similar survivor ratios would be obtained by the CT method. Inactivation curves prepared for the same inoculum by the two different methods (Fig. 2) were statistically indistinguishable \( (P > 0.05) \) level. Both the CSMC and CT inactivation rate data fit the Weibull function \( R^2 = 0.990 \) and 0.989, respectively) better than the linear function \( R^2 = 0.890 \) and 0.782, respectively). There were fewer numbers of survivors measured at time points before 20 s with the use of CSMC, as might be expected because of the shorter time to reach set temperature compared with the CT method. At longer holding times, the CT method appeared to result in somewhat greater inactivation than for CSMC.

As a negative heat treatment control for CSMC, we determined the effects of passage through the coil at ambient temperatures on the viability of \( P. \) \( \text{fluorescens} \). In both buffer and milk, there was no reduction in viability. These data suggest that CSMC can generate thermal inactivation rate data that is comparable to or better than the CT method. These results agree with those of Fairchild et al. (7), who found a greater survival ratio for \( Listeria \) \( \text{innocua} \) by the CT method compared with the use of a laboratory scale pasteurizer.

**Thermal inactivation of \( B. \) \( \text{cereus} \) spores and raw milk microflora.** With the CSMC method, we determined the thermal inactivation rate of \( B. \) \( \text{cereus} \) \( A1-029 \) spores at 105\( ^\circ C \) (Fig. 3). These data points have a low standard deviation \( (\pm 0.1 \) log \( S \)) over short (5 to 42 s) heating times. The Weibull model described the inactivation kinetics of these spores as accurately as a linear model \( (R^2 = 0.99 \) for both).

Thermal \( (72^\circ C) \) inactivation of the mixed microflora in...
raw whole milk had a steep initial drop during the first 3 s of treatment, presumably because of inactivation of the heat-labile vegetative cells, and a slower inactivation rate from 3 to 20 s, most likely representing more thermally resistant organisms, including spores (Fig. 4). Overall, only a 1-log reduction was achieved, giving survivor counts of $10^3$ CFU/ml. The linear model fit these data poorly ($R^2 = 0.49$), whereas the Weibull model was highly descriptive of the mixed culture ($R^2 = 0.97$). The Weibull function has been used successfully to describe microbial inactivation rates by nonthermal and thermal treatments (18), including milk (13).

The inactivation kinetics of four microbial populations in raw skim milk were determined (Fig. 5). These data demonstrate the utility of CSMC for measuring inactivation kinetics of microorganisms at concentrations similar to those found in raw milk and at times and temperatures that are similar to commercial processing. As would be expected, the thermally resistant spore population was unaffected by the time-temperature conditions comparable to high-temperature, short-time pasteurization. Such data could not be collected easily with the CT method because the initial spore counts would be decreased by the 100-fold dilution inherent in the 0.01-g inoculum. Thermally labile gram-negative organisms were reduced below 1 CFU/ml within the first 3 s of treatment.

FIGURE 2. Comparison of thermal (61°C) inactivation curves for P. fluorescens R1-232 prepared by the CSMC (X, n = 3; x, Weibull model predictions, log S = −0.00169t+0.3530; $R^2 = 0.990$) and CT (O, n = 3; O, Weibull model predictions, log S = $-6.259 \times 10^{-6}t^{1/2}$; $R^2 = 0.989$) methods. Error bars represent ±SD.

FIGURE 3. Thermal (105°C) inactivation of B. cereus A1-029 spores in BB (O, n = 3, except at 42 s, n = 2). ●, Weibull model predictions (log S = $-0.0834t^{1.655}$; $R^2 = 0.999$). Error bars represent ±SD.

FIGURE 4. Thermal (72°C) inactivation of raw milkborne microorganisms (X, n = 3). Dashed line represents the Weibull model predictions (log S = $-0.5683t^{0.1886}$; $R^2 = 0.977$). Error bars represent ±SD.

FIGURE 5. Thermal (72°C) inactivation of bacterial populations in raw milk (X, SPC; O, psychrotrophs; △, spores; □, gram-negative bacteria), n = 1.
DISCUSSION

The time required for a microbial suspension to reach set temperature affects the calculated inactivation rate, even when accounting for the heating and cooling lag times. Thus, an accurate correction is difficult (22). Times to reach set temperature can be too long to allow for measurement of inactivation kinetics over short periods of time (i.e., seconds).

Inactivation kinetics of microorganisms over short heating times have not been widely reported, in part because methodology to determine inactivation without the confounding effects of time to reach experimental temperatures have not been developed. Inactivation rates are estimated by extrapolating from data collected at lower temperatures (7) or by ignoring the short-term data and using only the linear portion of the curve (11, 18). Use of the CSMC method minimizes limitations related to come-up time and dilution factors.

The CSMC method also allows for thermal survival data to be collected at realistic processing temperatures, rather than depending on lower temperatures and long times to model thermal inactivation. In all cases, the Weibull model fit the inactivation data as well as or better than the linear model. When using the linear model, it is the general practice to eliminate data points that cause shoulders (16) and tails, permitting a better fit of the data to the linear model (18). With the use of the CSMC method and the Weibull model, it is not necessary to arbitrarily drop initial nonlinear data points. These data could be used to characterize accurately and precisely the thermal resistance of a range of spores and vegetative cells that are responsible for limiting the shelf life of fluid foods.

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