Prediction of growth of *Pseudomonas fluorescens* in milk during storage under fluctuating temperature

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ABSTRACT

Accurate prediction of growth of undesirable organisms (e.g., *Pseudomonas fluorescens*) in perishable foods (e.g., milk), held under sub-ideal storage conditions, can help ensure the quality and safety of these foods at the point of consumption. In this investigation, we inoculated sterile milk with *P. fluorescens* ($\sim 10^3$ cfu/mL) and monitored inoculum growth behavior at constant and fluctuating storage temperatures. Three storage temperatures, 4°C, 15°C and 29°C, were selected to simulate proper refrigeration conditions (4°C) and temperature abuse, respectively. To simulate temperature fluctuation, milk held at 4°C was subjected to temperature shifts to 15°C or 29°C for 4 to 6 h. A modified logistic model was used to obtain the best-fit curve for the microbial growth under constant storage temperature. The specific growth rates at 4°C, 15°C, and 29°C, obtained from experimental data, were 0.056 ± 0.00 , 0.17 ± 0.05 , and 0.46 ± 0.02 h⁻¹, respectively, and the lag time values were 29.5 ± 4.2 , 12.7 ± 4.4 , and 2.8 ± 0.3 h, respectively. A model predicting bacterial growth under different temperature fluctuations was obtained using the growth parameters extracted from constant temperature experiments. Growth behavior predicted by the fluctuating temperature model and that obtained experimentally were in good agreement. Lag time exhibited a larger variation compared with specific growth rate, suggesting that it depends not only on growth temperature but also on the sample population and temperature gradient. Additionally, experimental data showed that changing the temperature during the lag phase induced an additional lag time before growth; however, no significant lag time was observed under the temperature fluctuation during the exponential phase. The results of this study provide information for precise shelf-life determination

and reduction of food waste, particularly for milk and milk-containing food products.

Key words: *Pseudomonas fluorescens*, shelf life, spoilage, prediction of growth, temperature fluctuation

INTRODUCTION

One of the major challenges facing the food industry in the 21st century is supplying sufficient food for the growing population. The world population is expected to increase rapidly in the foreseeable future, reaching 8.1 billion in 2025 and 9.6 billion in 2050 (United Nations, 2014). Therefore, great efforts are required to increase food production to meet the demand for feeding the world population. However, increasing world food production may not be the only solution when a significant portion of produced food is discarded or lost uneaten. Food supply chain contributes the greatest waste among all major industries, and nearly 40% of food in the United States is wasted at different stages, including production, processing, packaging, distribution, retail, and in the consumer's home (Gunders, 2012). Surprisingly, retail and consumer waste account for a large proportion of the total waste. For instance, 50% of the total waste in grain product, seafood, and milk is incurred by consumers (Gustavsson et al., 2011).

Although there are numerous reasons for food waste, the main cause is food spoilage due to physical damage, chemical reaction, or microbial growth. Among all causes, microbial activity is the most important factor, accounting for a quarter of food loss (in't Veld, 1996). Given the conditions under which food is produced, processed, or stored, it should be possible to accurately predict microbial growth, and hence the final product's shelf life. Processor-defined shelf life may not reflect the actual time of spoilage because it is deliberately underestimated to account for potential abuse during storage and to ensure the quality and safety of the product (Skinner et al., 1994; Kilcast and Subramaniam, 2000). Hence one of the best approaches to reduce wasting food is to predict its actual shelf life.

To determine the actual shelf life of food, the environmental parameters and the response of food to those

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parameters should be known. One such important environmental factor is the storage temperature, which can vary during postharvest processes. It is nearly impossible to maintain a constant temperature at different stages of the food supply chain including transportation and consumers' homes. Therefore, food products may encounter a storage temperature that deviates from their standard and recommended value. This temperature variation during storage and handling could have a huge influence on microbial activity, which is a decisive factor for food spoilage. Hence, it is essential to take into account the time-temperature profile of microbial activities for monitoring food spoilage and establishing a realistic shelf life (Baranyi and Roberts, 1995; Semenov et al., 2007; Gospavic et al., 2008).

Microbial growth often appears sigmoidal, which can be described efficiently by Logistic and Gompertz models. Modifications have been applied to these models to improve their accuracy and suitability (Gibson et al., 1987; Zwietering et al., 1990). To take into consideration the temperature variation during the storage and to better predict shelf life, dynamic models provide ways to describe the influence of variable temperature on microbial growth (Van Impe et al., 1992; Baranyi et al., 1995; Bovill et al., 2000). A 2-step approach is generally used to construct a dynamic model for growth prediction. First, the experimental data are generated under constant temperature and a mathematical model (known as the primary model) is developed to describe the relationship between microbial population in food and storage time. The second step is to evaluate the dependence of parameters used in the model on temperature, and such a function or relationship is considered the secondary model (McKellar and Lu, 2003; Gospavic et al., 2008; Peleg and Corradini, 2011).

The objective of this work was to investigate the feasibility of predicting microbial growth in contaminated food based on storage temperature, particularly when it is fluctuating. *Pseudomonas fluorescens*, a ubiquitous psychrotrophic spoilage bacterium, was tested in this investigation. The bacterium is known to cause spoilage in several foods by producing extracellular enzymes such as lipases and proteases when the bacterial population reaches or exceeds $\sim 10^6$ cfu/g (or cfu/mL) in a food product (Ternström et al., 1993). If milk is contaminated before processing, the extracellular enzymes would remain active after HTST treatment (Chen et al., 2003), causing a reduction in shelf life. Similar risks are expected when pasteurized milk is exposed to the bacterium as a postprocessing contaminant. Therefore, growth of *P. fluorescens* in pasteurized milk exposed to temperature abuse was monitored experimentally and modeled mathematically in this study; the results

should provide fundamental knowledge for applying the concept to other foods and spoilage microorganisms.

MATERIALS AND METHODS

Culture Preparation and Milk Inoculation

Fresh culture of *P. fluorescens* was inoculated into tryptic soy broth (Difco/Becton, Dickinson and Company, Franklin Lakes, NJ) and incubated at 29°C for 24 h. Incubated culture ($\sim 10^9$ cfu/mL *P. fluorescens*) was diluted in 0.1% peptone water (Difco/Becton, Dickinson and Company) and inoculated into UHT-pasteurized low-fat (1%) milk (Horizon Organic, Broomfield, CO) at the rate of approximately 10^3 to 10^4 cfu/mL. Aliquots (0.5 mL) of inoculated milk were dispensed into micro-centrifuge tubes and held at controlled temperature in an incubator (Benchmark Scientific Inc., Edison, NJ). Samples were taken at appropriate time intervals (every 1 h at 29°C, every 3 h at 15°C, and every 12 h at 4°C) and the population of *P. fluorescens* was enumerated as described later.

Temperature Variation

Inoculated milk samples were held under 2 different experimental conditions: constant (4°C, 15°C, and 29°C) and fluctuating temperatures. To mimic ambient temperature in summer, 29°C was selected based on the average high temperature during summer in Columbus, Ohio. For variable temperature experiments, 3 patterns of temperature fluctuation were used, as presented in Figure 1 and Table 1. In the first and second patterns, the single temperature fluctuation happened at 24 or 48 h of storage and lasted 6 h at 15°C and 4 h at 29°C. In the second pattern, the first temperature fluctuation happened at 24 h and the second occurred 24 h after the first was completed. For this set of experiments, the time interval between data points was 12 h at 4°C, 90 min at 15°C, and 60 min at 29°C.

Determination of Population and Growth Curve

One micro-centrifuge tube was taken as a sample at each data point. The sample was then diluted 10-fold in 0.1% peptone water, and 0.1 mL of appropriate dilution was spread onto a tryptic soy agar plate. Inoculated plates were incubated aerobically at 29°C for 48 h, colonies were counted, and population was calculated (cfu/mL).

Mathematical software (Matlab, version: r2014a; The Mathworks Inc., Natick, MA) was used to analyze growth data. Using nonlinear regression, microbial

Figure 1. Patterns of temperature (*T*) fluctuation over storage time (*t*) with (a) 1 fluctuation, and (b) 2 fluctuations.

count data were fitted to the modified logistic function shown in Eq. [1] (Zwietering et al., 1990) to obtain population *N(t)* as a function of initial population (*N*₀), specific growth rate (*μ*) and lag time (*λ*):

$$\text{Log}_{10}N(t) = \text{Log}_{10}N_0 + \frac{A}{\left\{1 + \exp\left[\frac{4\mu}{A}(\lambda - t) + 2\right]\right\}}, \quad [1]$$

where *t* is the storage time in hours, and *A* is the log ratio of maximum and initial populations:

$$A = \text{Log}_{10}\left(\frac{N_{\text{max}}}{N_0}\right). \quad [2]$$

The maximum generation time (**MGT**) was obtained from (Yousef et al., 1991; Delignette-Muller, 1998)

$$\text{MGT} = \left(\frac{\text{Log}_{10}(2)}{\mu}\right). \quad [3]$$

The dependence of specific growth rate, lag time, and time to maximum population on temperature were analyzed as described later.

RESULTS AND DISCUSSION

Growth Curves at Constant Temperature

Incubation of *P. fluorescens* in milk at constant temperatures produced typical growth curves (Figure 2) compared with published data (Gospavic et al., 2008; Chen et al., 2011). To describe the microbial growth as a function of storage time, a modified logistic model (Eq. [1]) was used and growth parameters were directly obtained from nonlinear regression analysis (Table 2). The maximum growth rates for storage at 4°C, 15°C, and 29°C were 0.056, 0.17, and 0.46 h⁻¹, respectively, and the MGT were 6.42, 2.82, and 0.81 h, respectively, which are in agreement with published data for milk (Cox and MacRae, 1988).

To examine the fitness of model to experimental data, a fitted curve for 4°C with 99% confidence limits is presented (Figure 3). As shown in this figure, almost all measured data points were located inside the upper and lower confidence bounds, indicating that the modified logistic model provided a good fit for the growth of *P. fluorescens* at 4°C in low-fat milk. As expected, when incubation temperature increased from 4°C to 29°C, the lag time decreased from 29.5 to 2.8 h and specific growth rate increased from 0.06 to 0.46 h⁻¹. It

Table 1. Temperature (*T*) values and time (*t*) intervals in fluctuation tests illustrated in Figure 1

Test	Temperature (°C)		Time (h)		
	<i>T</i> ₁	<i>T</i> ₂	<i>t</i> ₁	<i>t</i> ₂	<i>t</i> ₃
One fluctuation (Figure 1a)	4	15 or 29	24	4 h at 29°C, 6 h at 15°C	NA ¹
One fluctuation (Figure 1a)	4	15 or 29	48	4 h at 29°C, 6 h at 15°C	NA
Two fluctuations (Figure 1b)	4	15 or 29	24	4 h at 29°C, 6 h at 15°C	24

¹Not applicable.

Figure 2. Experimental data for growth of *Pseudomonas fluorescens* in milk (●) and fitted modified logistic model (solid line) for samples stored at (a) 4°C, (b) 15°C, and (c) 29°C (results averaged from 4 replicates at 4°C or 3 replicates at 15°C and 29°C).

is notable that lag time exhibited a larger variation for each temperature compared with specific growth rate (Table 2). This is believed to be due to the dependence of lag time on the cells' previous history in addition to new environmental conditions (Baranyi et al., 1995; Bovill et al., 2000). Baranyi et al. (1995) suggested that the cells' history could be very different even for the same inoculum population, depending on metabolic activity in the preinoculation environment. Consequently, the physiological state of cells before inoculation might result in a large variation in lag time.

The temperature dependence of specific growth rate is shown in Figure 4a. A linear relationship between square root of specific growth rate, μ , and temperature, T , known as the Ratkowsky square root model is presented as follows (Ratkowsky et al., 1982, 1983; Zwietering et al., 1991):

$$\sqrt{\mu} = c(T - T_0), \quad [4]$$

where c is the slope of regression line and T_0 is a conceptual temperature where the extrapolation of the regression line intersects the T axis, and is considered as a hypothetical minimum growth temperature. The conceptual temperature T_0 is an intrinsic property of the microorganism (Ratkowsky et al., 1982), and its value for the data obtained in this work was 264K (−9.15°C). This is in a good agreement with published data for *Pseudomonas* group when T_0 is reported at the range of 263K to 272K (−10.15°C to −1.15°C; Ratkowsky et al., 1982; Phillips and Griffiths, 1987).

Figure 3. Growth curve fitted by modified logistic model for 4°C with 99% confidence band and 5 sets of measured data points.

Table 2. Initial population, asymptote, maximum specific growth rate (μ), lag time (λ), and maximum generation time (MGT) determined using experimental data and the modified logistic model (means $\pm$ SD)

Temperature (°C)	Initial population (log cfu/mL)	Asymptote (log cfu/mL)	μ (1/h)	λ (h)	MGT (h)
4	2.9 ± 0.2	5.667 ± 0.2	0.056 ± 0.00	29.5 ± 4.2	6.42 ± 0.65
15	4.4 ± 0.1	3.478 ± 0.2	0.17 ± 0.05	12.7 ± 4.4	2.82 ± 0.33
29	3.6 ± 0.7	4.207 ± 0.7	0.46 ± 0.02	2.8 ± 0.3	0.81 ± 0.08

The relation between the reciprocal of specific growth rate and the lag time (Figure 4b) was found to be linear:

$$\lambda = b \left(1/\mu \right) + a, \quad [5]$$

where λ is the lag time, $1/\mu$ is the reciprocal of specific growth rate, and b and a are regression parameters. The regression analysis showed a strong linear relationship between the 2 parameters ($R^2 = 0.9834$). This relation explains how the lag time represents the work necessary for cells to adjust to a new environment (Li and Torres, 1993; Koutsoumanis, 2001; Brown, 2007).

Modeling Growth Under Temperature Fluctuations

Two patterns of temperature change, single and double fluctuations, were used to simulate temperature abuse, and the storage temperature was increased above refrigeration temperature (4°C) in each fluctuation. Single fluctuations occurred during the lag and exponential phases (Figure 1 and Table 1). To predict bacterial growth under temperature fluctuation, the time of entire growth curve was divided into intervals of constant temperature, and the following modified logistic model was used:

$$\begin{aligned} \text{Log}N(t) = & \text{Log}N_i \\ & + \frac{\text{Log}(N_{\max}/\text{Log}N_i)}{\left\{ 1 + \exp \left[\frac{4\mu_i}{(\text{Log}N_{\max} - \text{Log}N_i)} (\lambda_i - t + t_i) + 2 \right] \right\}}, \\ & t_i < t < t_{i+1}, \end{aligned} \quad [6]$$

where t is the storage time in hours, N_i is the microbial population at the beginning of temperature change t_i , and $N_{\max}$ is the maximum population. The temperature is constant for $t_i < t < t_{i+1}$, and λ_i and μ_i are the lag time and specific growth rate for this time interval, respectively (Zwietering et al., 1990).

The value of specific growth rate μ_i corresponding to T_i of each fluctuation interval is calculated using Eq. [4], and the lag time, λ_i , is approximated using Eq. [5]; however, variation of lag time should be taken into consideration. It was reported that the lag time under variable temperature conditions deviates from that obtained under constant temperature (Ng et al., 1962; Zwietering et al., 1994; Bernaerts et al., 2002).

Bacterial growth with one fluctuation and the corresponding mathematical models are shown in Figure 5. The storage temperature (4°C) was increased (29°C

Figure 4. (a) Temperature dependence of the specific growth rate ($\mu^{1/2}$) obtained by modified logistic model; (b) correlation of lag time (λ) and specific growth rate (μ). T_0 is a conceptual temperature where the extrapolation of the regression line intersects the T axis.

Figure 5. Prediction (solid line) and experimental data (●) of *Pseudomonas fluorescens* in milk with 1 temperature fluctuation (a) at 24 h (temperature increased to 29°C for 4 h); (b) at 24 h (temperature increased to 15°C for 6 h); (c) at 48 h (temperature increased to 29°C for 4 h); and (d) at 48 h (temperature increased to 15°C for 6 h). Results were averaged from 2 replicates in both trials.

in Figure 5a, and 15°C in Figure 5b) after 24-h holding; this fluctuation coincided with the lag phase. In response to the temperature increase, the bacterium expressed short or no obvious additional lag time. Zwietering et al. (1994) suggested that in such a case, the length of an additional lag is one-fourth the lag time that normally happens for that temperature. When the temperature decreased to 4°C, a longer lag time was observed. The lag after temperature decrease is similar to the lag observed at the beginning of the growth curve. To further analyze the duration of lag, the changes in population after temperature fluctuation were fitted to a modified logistic model (Eq. [1]) as a new independent growth. The results showed that the new lag times were 17.4 h (Figure 5a) and 15.0 h (Figure 5b), which is close

to half of the lag at constant 4°C ($\lambda = 29.5$ h). In this scenario, an acceptable agreement between prediction and experimental data was obtained by incorporating half a standard lag time in Eq. [5].

When a fluctuation was introduced during the exponential phase—that is, after 48 h of storage at 4°C—a different pattern of lag phase during and after temperature fluctuation was observed (Figures 5c and 5d). Some studies suggest that temperature change during the exponential phase causes no lag time (Zwietering et al., 1994; Kreyenschmidt et al., 2010). Therefore, the specific growth rate changes instantaneously as temperature changes and the additional lag phase is neglected in the model. The prediction afforded by the model provided a good agreement with experimental

Figure 6. Prediction (solid line) and experimental data (●) of *Pseudomonas fluorescens* in milk with 2 temperature fluctuations with (a) temperature increasing to 29°C for 4 h in each fluctuation; and (b) temperature increasing to 15°C for 6 h in each fluctuation. Results were averaged from 2 replicates in both trials.

data, whereas the use of proportional lag may result in underestimated growth.

The prediction of the growth with 2 fluctuations, one in the lag phase and the other in the exponential phase, is displayed in Figure 6. These data were modeled using a combination of assumptions used in Figure 5. Even though prediction after the second temperature fluctuation was overestimated, the model showed good agreement with experimental growth data (Figure 6a). However, the prediction underestimated the growth when temperature was shifted from 4°C to 15°C (Figure 6b). Because no lag time was considered for this region, the fitted data predicted the maximum possible growth based on data obtained from constant temperature.

The modified logistic model (Eq. [1] and [6]) generally provided an acceptable prediction for growth of *P. fluorescens* in milk. The value of lag incorporated into the model played an important role in the accuracy of the prediction. Predictions received a better degree of agreement among the experiments in which temperature changed from 4°C to 29°C, which indicates that the temperature gradient during fluctuation may be a factor affecting the lag. The effects of temperature change on lag have been discussed previously. It is thought that a large and abrupt temperature shift may result in a new adaptation phase (Ng et al., 1962; Bernaerts et al., 2002). Baranyi et al. (1995) and Zwietering et al. (1994) also found that temperature shifts around the minimum growth temperature showed large deviations from the model predictions. Moreover, lag time could be affected by the size of the microbial population, which can be seen from the new and independent growth after fluctuation in Figure 5. Therefore, for further refinement of bacterial growth prediction, additional studies are needed to incorporate the effect of growth history, population, and temperature gradient on lag time.

Effect of Temperature Fluctuation on Shelf Life of Processed Milk

In this study, pasteurized milk with postprocessing contamination was simulated by inoculating commercially sterile milk with a small population of *P. fluorescens*. When this psychrotrophic bacterium is found in milk as a postpasteurization contaminant, it becomes the main reason for termination of product shelf-life. Other psychrotrophic bacteria that survive milk pasteurization also cause spoilage of pasteurized milk (Blackburn, 2006; Momani and Natsheh, 2012). The shift from refrigeration (4°C) to higher temperatures (15°C and 29°C) simulated temperature abuse during storage. This study aimed to illustrate how to predict the detrimental effect of the combination of temperature abuse and presence of psychrotrophic population on product quality and shelf life. It is estimated that a population of 10^6 cfu of milk contaminant/mL of milk is sufficient to cause product spoilage (Momani and Natsheh, 2012).

Temperature increase after 48 h of refrigerated storage to 29°C resulted in an approximately 1-log increase in population of *P. fluorescens* (Figure 7a). It took almost 1.5 d for the control group (stored at refrigeration temperature, 4°C) to reach the same population that the test group had at the end of the fluctuation, meaning that this fluctuation could reduce the shelf-life by 1 d, at least. Similarly, the population increase during temperature fluctuation at 24 h of storage was

Figure 7. Growth of *Pseudomonas fluorescens* in milk when the temperature increases from 4°C to 29°C for 4 h: (a) and (b) show 1 fluctuation after 48 and 24 h, respectively, and (c) 2 fluctuations. Results were averaged from 2 replicates in all trials.

approximately 1 log (cfu/mL) (Figure 7b); however, for the control group stored at 4°C, it took longer (almost 2 d) to reach the same population that the test group reached at the end of fluctuation. These results suggest that if the temperature abuse happens early in storage, the time to reach the maximum population of bacteria

is drastically decreased, leading to shorter shelf life. If the temperature fluctuation occurs later, the time to reach the maximum population is less affected; nonetheless, the bacterial population may reach the spoilage threshold ($\sim 10^6$ cfu/mL) during the fluctuation.

When temperature fluctuations were introduced at 24 h of refrigerated storage and again 24 h after the first one ended, an increase in the population of *P. fluorescens* of approximately 1 log (cfu/mL) was observed during each fluctuation (Figure 7c). When milk was stored at constant temperature, it took about 90 h for *P. fluorescens* to reach a population of 10^6 cfu/mL, which could be considered as the start of spoilage in milk. However, after 2 temperature fluctuations to 29°C, *P. fluorescens* took only 55 h to reach that population size, highlighting the effect of temperature abuse on shelf-life, which could be cut by 40% if the milk is exposed to higher temperature in a short period.

The experimental data presented in this work emphasize the importance of the temperature abuse and its effect on the actual shelf life of a food product. Our data also suggest that if the storage temperature is continuously monitored after production by using the mathematical model presented in Eq. [6], an actual and accurate shelf life of milk could be determined in real time.

In conclusion, we developed a model that can predict the shelf life of pasteurized milk, presumed to contain a small initial population of spoilage microorganisms, based on the temperature profile during storage. The results show that temperature abuse in the early stage of food storage could drastically reduce a product's shelf life.

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