Prediction Of Growth Of *Pseudomonas fluorescens* Under Temperature Fluctuation

Thesis

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By

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ABSTRACT

One of the major challenges the Food industry faces in 21st century is to supply enough food for growing population. Although there is a tremendous effort to increase the food production at the present time, nearly 40 percent of food produced in the United State is wasted. While different stages of food supply chain contribute to the waste, the largest waste happened after processing and packaging, in retail stores or in consumers’ home. One reason for the waste is food spoilage. The main causes of food spoilage could be physical damage, growth of microorganisms, and chemical or microbial reactions. It is estimated that a quarter of food supply is lost through microbial activity alone. Another reason is that people discard the food which has reached the stamped use-by-date. However, pre-defined shelf life may not reflect the actual time of spoilage since it is overestimated to consider potential abuse during the storage and to ensure the quality and safety of the product. Therefore, a study about microbial behavior in food products exposed to uncontrolled storage conditions can provide valuable information for a more precise shelf-life determination and reduction of food waste.

The microbial behavior is largely affected by ambient conditions in food products such as storage temperature. While it is nearly impossible to keep temperature constant in different stages of food supply chain, it would be beneficial to predict microbial growth under variable temperature conditions.

Efforts had been made to model and predict the microbial growth under constant and variable temperature conditions and the results maintained high accuracy.
However, many of them were completed in experimental media instead of actual food products. The complexity of natural microflora in food products and variety of food ingredients affects the accuracy of the modeling for different foods. In order to provide more reliable information, it would be essential to start with studying the behavior of a single microorganism in a specific food product. Therefore, the objectives of this study are (a) to monitor the growth of *Pseudomonas fluorescens* in sterile low fat milk; (b) to test the fitness of using current mathematical model (Modified Logistic) to describe the growth under constant temperature; (c) to provide a prediction of the growth with temperature fluctuations. Additionally, the effect of temperature abuse on shelf life of product is discussed.

The growth of *Pseudomonas fluorescens* in sterilized low fat (1%) milk (Horizon Organic) is monitored under both constant and fluctuating temperatures. *P. fluorescens* is enriched in tryptic soy broth to $\sim 10^9$ CFU/ml, and inoculated in milk at the rate of $10^3$ CFU/ml. For constant temperature conditions, the milk is stored at 4°C (refrigeration), 15°C (representing refrigeration failure) and 29°C (simulating excessive temperature abuse). For variable temperature conditions, two patterns of fluctuations are studied: (i) single fluctuation from 4°C to 15°C and 29°C after 24 hour of storage; (ii) double fluctuations with 24 hours of refrigeration between. The population of *P. fluorescens* is periodically measured by pour-plating and plate-counting on tryptic soy agar.

Growth data from constant temperature are fitted to the Modified Logistic function in nonlinear regression analysis using Matlab® software. The model provides a good fitness to describe the growth of *P. fluorescens* in milk as a function of time, indicating the usefulness of applying Modified Logistic to an actual food product. Growth parameters such as specific growth rate and lag time are obtained from the
model. The lag time exhibits a large variation compared to specific growth rate. A possible explanation is that the lag time may be influenced by the physiological state of cells, which is the cell’s history before inoculation. Additionally, a linear relationship between square root of specific growth rate and temperature is observed, which is consistent with the Ratkowsky square root equation. The Ratkowsky square root equation is further used to calibrate the value of specific growth rate for variable temperature conditions.

Growth data from fluctuating temperature show that changing the temperature during the lag phase induces an additional lag time to the growth; however, no lag time is exhibited under the temperature fluctuation in exponential phase. Based on the observation, the Modified Logistic function is also adopted to provide prediction for growth under temperature fluctuation by incorporating different values of lag and calibrated specific growth rate. Overall, results display good agreement between prediction and actual growth, indicating that Modified Logistic model is able to provide acceptable predictions for growth of \textit{P. fluorescens} in milk. Unlike the specific growth rate which is only a function of storage temperature, the duration of lag highly depends on different parameters such as the history of the bacteria and the temperature gradient during the fluctuation.

In additions, the effect of temperature fluctuation on shelf life is discussed. The results show that temperature abuses could drastically reduce the shelf life up to as much as 40\% when milk is exposed to ambient temperature for only few hours. A temperature abuse at early stage of food storage may have a large impact on shelf life.

In conclusion, the temperature abuses during storage have significant effects on the microbial growth and shelf life of food product. Modified Logistic model displays the usefulness and fitness of predicting the growth of \textit{P. fluorescens} in milk.
As a result, if the storage temperature is continuously monitored after production, mathematical model can be used to estimate the microbial growth in real time, providing valuable information for a precise shelf-life determination and reduction of food waste, particularly milk.
DEDICATION

Dedicated to my beloved father and mother.
ACKNOWLEDGMENTS

I first wish to express my sincere gratitude to my adviser, Dr. Farnaz Maleky, for her support and encouragement during my graduate studies. I feel greatly honored that I can work under her supervision.

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INTRODUCTION

One of the major challenges the food industry faces in 21st century faces is the supply of enough food for growing population. The world population increases by nearly one billion within the next 12 years, reaching 8.1 billion in 2025 and 9.6 billion in 2050 (UN Press, 2013). Therefore, a tremendous effort is required to increase the food production and meet the demand for world population. On contrary, the food supply chain produces the largest wastes among all major industries, and every year nearly 40 percent of food in the United State is wasted in different stages such as production, processing and packaging, distribution, retail and even consumers’ homes (Gunders, 2012). In many food products, waste from retail and consumer account for a large proportion of the total waste. For instance, more than 50 percent of total waste is the consumers’ waste especially for grain product, seafood and milk (Gustavsson et. al, 2011). One reason for the waste is food spoilage. The main causes of food spoilage could be physical damage, growth of microorganisms, and chemical or microbial reactions. It is estimated that a quarter of food supply is lost through microbial activity alone (Huis in’t Veld, 1996). Another reason is that people discard the food which has reached the stamped use-by-date. However, pre-defined shelf life may not reflect the actual time of spoilage since it is overestimated to consider potential abuse during the storage and to ensure the quality and safety of the product (Kilcast and Subramaniam, 2000; Skinner et. al., 1994).

One of the most important environmental factors that affect the shelf life is the
storage conditions after production and during the transportation and storage. For instance, it is nearly impossible to keep the temperature constant at different stages of food supply chain. Therefore, food products may encounter storage temperature which deviates from their standard and recommended storage conditions. This temperature variation during storage and handling could potentially have a huge influence on the microbial activity, which is the main reason of food spoilage and food waste. Hence, it is essential to take time-temperature profile into account for monitoring food spoilage and establishing a realistic shelf-life (Baranyi and Roberts, 1995; Gospavic et. al. 2008; Semenov et al., 2007). Efforts have been made to develop mathematical models to predict the microbial growth under constant temperature condition. Sigmoidal curves such as Logistic and Gompertz are mostly used to describe the increase of population with respect to storage time because of their similarity to microbial growth curves. Modifications have been applied to these models to improve their accuracy and suitability (Gibson, et. al., 1987; Zwietering et al., 1990; McKellar and Lu, 2004). In order to take into consideration the temperature variation during the storage and have a better prediction of shelf-life, dynamic models provide ways to describe the influence of variable temperature on microbial growth (Baranyi and Roberts, 1995; Bovill et. al., 2000; Van Impe et al., 1992). In general, a two-steps approach is used to construct dynamic model for growth prediction. In first step experimental data is generated under constant temperature, and the mathematical model is developed to describe the relationship between population and time as the primary model. The second step is to evaluate the dependence of parameters used in the model on temperature, and such a function or relationship is considered as the secondary model (Gospavic et. al. 2008; Peleg and Corradini, 2011). Even though many research works resulted in high accuracy, many of them were completed in experimental media instead of actual food products.
(Li and Torres, 1993; Zwietering et al., 1994; Baranyi and Roberts, 1995). However, the complexity of natural microflora in food products and variety of food ingredients affect the accuracy of the model for different foods. In order to provide more reliable information, several studies have been done to evaluate the prediction of microbial growth in actual food products (Koutsoumanis, 2001; Mataragas et al, 2006; Gospavic et al, 2008; Kreyenschmidt et al., 2010). To achieve a widely application of prediction, it would be essential to start with studying the behavior of a single microorganism in a specific food product.

*Pseudomonas fluorescens* is a ubiquitous spoilage bacterium that can grow under refrigeration temperature, and is known to cause spoilage in a number of food by producing extracellular enzymes such as lipases and proteases (Ternström, et al., 1993). Especially for milk, the extracellular enzymes would remain after the UHT heat treatment (Chen et al., 2003). Therefore, to monitor the growth of *P. fluorescens* in pasteurized milk under refrigerating storage temperature and possible temperature abuses, has significant meaning in assessing the risks of shelf life.

In this paper, the growth of *Pseudomonas fluorescens* in milk under temperature fluctuation is studied. The objectives of this study include (a) to monitor the growth of *Pseudomonas fluorescens* in sterile low fat milk under both constant and variable temperature conditions; (b) to test the fitness of using current mathematical model (Modified Logistic) to describe the growth under constant temperature; (c) to provide a prediction of the growth with temperature fluctuations; (d) to discuss the effect of temperature abuse on shelf life.
CHAPTER 1

LITERATURE REVIEW

Food industry in 21st century faces different challenges, one of which is to supply enough food for growing population. The world population is increasing by almost one billion within the next 12 years, reaching 8.1 billion in 2025 and 9.6 billion in 2050 (UN Press, 2013). Therefore, a tremendous effort is required to increase the food production and meet the demand for such a large population. On contrary, the food supply chain produces the largest wastes and every year nearly 40 percent of food in the United State is wasted in different stages such as production, processing and packaging, distribution, retail and even consumers’ homes (Gunders, 2012). The largest waste happens after processing and packaging, in retail stores or in consumers’ home, mainly because the food has reached its shelf-life. For instance, more than 50 percent of total waste especially for grain product, seafood and milk is the consumers’ waste (Gustavsson et. al, 2011). Food shelf-life may be influenced by intrinsic or extrinsic factors. Intrinsic factors are defined by components and structure of the raw materials, while extrinsic factors are those factors the food products might encounter through the food supply chain such as time-temperature profile, relative humidity during processing and storage, packaging and consumer handling (Kilcast and Subramaniam, 2000). Since extrinsic factors may vary from one product to another, pre-defined shelf life may not reflect the actual time of spoilage since it is overestimated to consider a margin that
assures the quality and safety of the product (Kilcast and Subramaniam, 2000, and Skinner et. al., 1994). This results in waste of food products that reach their use-by-date but have maintained their quality.

Spoilage also causes food waste by physical damage, growth of microorganisms, and chemical or microbial reactions, and it is estimated that a quarter of food supply is lost through microbial activity alone (Huis in’t Veld, 1996). Since food quality is highly related with microorganisms, many aspects of microbiology have been applied to food science. One aspect is to predict the behavior of microorganisms in food products, including microbial growth and inactivation. Prediction of microbial behavior provides useful information to food safety and food preservation. Understanding the growth mechanism of pathogenic microorganisms helps reducing the risk of foodborne diseases and outbreaks, and study of spoilage microorganisms helps to establish a more precise shelf-life of food products.

**Factors of microbial growth**

Stability of a product directly relates to the microbial population, which makes it important to predict the growth of microorganisms. Microbial growth is influenced by many physiological and chemical factors, including water activity, pH, nutrition and temperature (Blackburn, 2006). Microorganisms, especially bacteria, are fastidious to pH and it is demonstrated that the optimal pH value for most microorganisms is around 7.0 (6.6-7.5), while few microorganisms prefer values below 4.0 (Jay, 2005). Approximate pH growth ranges for some foodborne microorganisms are shown in figure 1.1. When ambient pH deviates from the optimal pH, the specific growth rate of bacteria will be affected in various way: the specific growth rate increases or decreases with pH (figure 1.2a and 1.2b); the specific growth rate reaches a maximum number
but comes to a smaller constant value at low pH or high pH (figure 1.2c and 1.2d); the specific rate reaches zero both at low and high pH (figure 1.2e and 1.2f) (Tan, Wang and Marshall, 1998).

![Figure 1.1 Approximate pH growth ranges for some foodborne organisms. The pH ranges for *Listeria monocytogenes* and *Staphylococcus aureus* are similar. (Jay, 2005)](image)
Figure 1.2 Specific growth rate of bacteria affected by pH value in various way: the specific growth rate increases or decreases with pH (a and b); the specific growth rate reaches a maximum number but comes to a smaller constant value at low pH or high pH (c and d); the specific rate reaches zero both at low and high pH (e and f) (Tan, Wang and Marshall, 1998).

Another parameter that influences the reproduction and metabolism of microorganisms is the water activity. As water activity decreases, the microbial growth and activity reduced. In general, molds and yeasts are more tolerant to a decreased water activity than bacteria. For most bacteria including *Pseudomonas* spp., *Escherichia coli* and *Clostridium* spp., a minimum water activity of 0.90 is required for growth, whereas
some molds and yeasts such as *Saccharomyces bailii* can grow when the water activity is 0.80 (Beuchat 1981; Jay, 2005). However, it is reported that some pathogens such as *Staphylococcus aureus* with high-salt tolerance can stand much lower water activity situation (Medvedova, Valik and Studeničová, 2009). Table 1.1 displays the minimum water activity required for growth of some microorganisms.

<table>
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<th>Range of water activity</th>
<th>Microorganisms inhibited by lowest water activity in this range</th>
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| 1.00-0.95               | *Pseudomonas*, *Escherichia*, *Proteus*, *Shigella*, *Klebsiella*,  
|                         | *Bacillus*, *Clostridium perfringens*, *C. botulinum* E, G,  
|                         | some yeasts                                                   |
| 0.95-0.91               | *Salmonella*, *Vibrio parahaemolyticus*,  
|                         | *Clostridium botulinum* A, B, *Listeria monocytogenes*,  
|                         | *Bacillus cereus*                                             |
| 0.91-0.87               | *Staphylococcus aureus* (aerobic), many yeasts (Candida,  
|                         | *Torulopsis*, *Hansenula*), *Micrococcus*                     |
| 0.87-0.80               | Most molds (mycotoxigenic penicillia), *Staphylococcus  
|                         | *aureus*, most *Saccharomyces* (bailii) spp., *Debaryomyces*  |
| 0.80-0.75               | Most halophilic bacteria, mycotoxigenic aspergilli           |
| 0.75-0.65               | Xerophilic molds (*Aspergillus chevalieri*, *A. candidus*,  
|                         | *Wallemia sebi*), *Saccharomyces bisporus*                    |
| 0.65-0.61               | Osmophilic yeasts (*Saccharomyces rouxii*), a few molds ( 
|                         | *Aspergillus echinulatus*, *Monascus bisporus*)              |
| <0.61                   | No microbial proliferation                                    |


One of the most important factors affecting microbial growth is the ambient
temperature. Daud et. al. (1978) observed that the spoilage rate of fresh poultry products at 15°C will be approximately three times faster than that at 5°C, and that at 10°C will be approximately twice than that at 5°C. The result clearly indicated that temperature is a major and determinant factor for microbial activity in poultry products. Since microbial activity is one of a key parameter in food quality and shelf-life establishment, studies about microbial behavior with respect to temperature has significant meaning to food industry.

There are three important temperature values for microbial growth: the minimum growth temperature ($T_{\text{min}}$), the optimum growth temperature ($T_{\text{opt}}$) and maximum growth temperature ($T_{\text{max}}$). Microorganisms don’t grow or may die when ambient temperature is below minimum temperature or above maximum temperature. The rate of their growth will reach a maximum value at optimum temperature, and decrease as temperature increases or decrease (Huang, 2011). In general, microorganisms can be categorized into three groups based on their best growing temperature range: psychrotrophs are those microorganisms that grow well below 5°C and have their $T_{\text{opt}}$ above 20°C; mesophiles are those microorganisms that grow well between 20°C and 45°C with $T_{\text{opt}}$ between 30°C and 40°C; thermophiles are those microorganisms that grow well above 45°C with $T_{\text{opt}}$ between 55°C and 65°C (Kasana, 2010, Jay, 2005). The first group, Psychrotrophs, is particularly a concern because of their ability to grow causing spoilage at refrigerator temperature.

Storage temperature is a critical parameter in maintaining quality and nutrition values of a wide range of food products, including fresh produce, dairy, meat and sea food. Many food products should be kept under constant temperature; however, it is nearly impossible to maintain a constant temperature during transportation, retail storage, and in consumers’ hands. Moreover, refrigerated foods have become an
inevitable part of consumers’ diet resulting in additional concern about the effect of temperature-abuse on food safety (Skinner et. al., 1994). Therefore, it is important to study the behavior of microorganisms under variable temperature in order to have a more accurate estimation of the shelf life. As demonstrate in the literature, the change of ambient temperature can influence the growth rate of microbial growth. Moreover, when environmental factors change, the microorganisms need time to adjust their physiological state to adapt to new conditions (Buchanan and Cygnarowicz, 1990).

Ambient temperature in which a food product is kept may easily vary, and up to date there have been numerous research work investigating the growth of microorganisms under certain temperature. Many studies have successfully established mathematical models to predict the temperature dependent microbial growth. Modifications are made continuously to improve the accuracy of the prediction by taking into consideration different influencing factors. However, there is no single model that can predict the growth of all microorganisms at any given temperature and media composition (Li and Torres, 1993).

**Mathematical modeling of microbial growth**

A typical curve of microbial growth is shown in figure 1.3. The growth usually can be divided into three phases, which are the lag phase, exponential phase and stationary phase. Also, when the population is described as a function of time by using a mathematical model, growth parameters, such as lag time, specific growth rate and maximum population are introduced. Several mathematical models have been used to describe the microbial growth under constant temperatures. In general, investigating the effect of temperature on microbial growth includes two steps. The first step is to fit a mathematical model to experimental microbial growth under a given temperature that
describes the relationship between microbial population and time. This mathematical model is also known as “primary model”. Once parameters of primary model are determined, the second step is to evaluate the dependence of parameters on temperature. The parameters dependence is mathematically described by what is known as “secondary model” (Peleg and Corradini, 2011).

Figure 1.3 A typical curve of microbial growth, shown as population versus time, with growth parameters

There are several ways to establish primary model. In early studies, regression analysis of experimental data was used to generate models including different factors in addition to the temperature. For instance, Lindroth and Genigeorgis (1986) used regression analysis to predict the probability of Clostridium botulinum spore to initiate growth and produce toxin in rockfish under different temperatures. Even though good agreement between prediction and experimental data was shown in most regression
studies, the major shortcoming was their lack of universality, because the polynomial forms resulted from curve fitting and remained unique (Davey, 1989).

Different from simply curve fitting by polynomial functions, sigmoidal curves have been used to describe microbial growth over time because of its unique similarity to microbiological growth curve (Gibson, et. al., 1987). There are two major sigmoidal curves: Gompertz (Eq. 1.1) and logistic functions (Eq. 1.2). The main difference between these two models is that the logistic model is symmetric about the point where the slope reaches its maximum, while Gompertz is not (Gibson, et. al., 1987).

\[ \log_{10} N(t) = A + Ce^{-e^{[-B(t-M)]}} \]  \hspace{0.5cm} (1.1)

where \( t \) is the time, \( N(t) \) is the population at \( t \), \( A \) is the initial population in logarithmic scale, \( C \) is the asymptotic logarithmic growth ratio which is \( N(t)/N(0) \) when \( t \) is infinite, \( B \) is the relative growth rate at \( M \), and \( M \) is the time when the growth rate reaches maximum (McKellar and Lu, 2004).

\[ \log_{10} N(t) = A + \frac{C}{1+e^{[-B(t-M)]}} \]  \hspace{0.5cm} (1.2)

where parameters have the same meanings as Gompertz model (Eq. 1.1).

Gibson et. al. (1987) used these two basic sigmoidal curves to fit the data of growth of \textit{Clostridium botulinum} under several certain conditions in pork slurry. Lag times, growth rates, generation times and time to maximum growth rates were derived and calculated by fitting the models to data. As they suggested for their experimental data Gompertz is a better fit than logistic model. Moreover, they have discussed the
accuracy of curve fitting of the experimental data. An adequate number of data points is important for improving accuracy, especially for the earlier growth phase such as lag and acceleration phases.

Based on these two fundamental sigmoidal functions, some modified forms were obtained. Schnute (1981) reported a versatile growth model which presents not only sigmoidal growths (such as Gompertz and logistic), but also linear or exponential growth. The Schnute model was designed for growth of fish population, but its usefulness could be introduced to other species including microorganisms (Skinner et al., 1994). However, it was observed that in some cases the Schnute model overestimates the growth rate (Zwietering, 1990). Stannard et. al. (1985) modified the logistic model into a new form, known as Stannard model, to describe the growth of some psychrotrophic bacteria at chill temperatures, displaying a good agreement with experimental data. Subsequently, Phillips and Griffiths (1987) successfully applied Stannard model to predict the growth of several psychrotrophic bacteria in dairy products.

Even though modified forms of sigmoidal models provided good fitness to describe microbial growth under multiple situations, they were not written in biological terms. Zwietering et. al. (1990) compared several sigmoidal models, including the modified forms (logistic, Gompertz, Schnute, Richards, and Stannard) by applying them to a large set of growth data. Instead of using mathematical parameters, they interpreted models with biological meanings (Table 1.2). Mathematical parameters were reformulated and substituted by lag time, specific growth rate and time to reach maximum population. It is easier to compare those models if biological parameters are obtained directly from the equations. Comparison of fitness among models indicated that the basic logistic and Gompertz models fit well with experimental data. Moreover,
the Gompertz model was accepted in a high confidence level, concluding that Gompertz model seemed to be the most suitable one to fit with growth curve. Therefore, if sufficient to describe the data, three-parameter models (such as Gompertz and logistic) are preferred over four-parameter models (such as Schnute) because of their simplicity and ease of use.

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<td>Logistic</td>
<td>( y = \frac{a}{1 + \exp(b - cx)} )</td>
<td>( y = \frac{A}{1 + \exp(\frac{4\mu_{\text{max}}}{\lambda} (x - \eta) + 1)} )</td>
</tr>
<tr>
<td>Gompertz</td>
<td>( y = a \exp[-\exp(b - cx)] )</td>
<td>( y = A \exp[-\exp(\frac{\lambda c}{A} (x - \eta) + 1)] )</td>
</tr>
<tr>
<td>Richards</td>
<td>( y = a {1 + v \exp \left[ (x - \lambda)^{1 - \alpha} \right] }^{1 - \alpha} )</td>
<td>( y = A \left[1 + v \exp(1 + v) \exp \left(\frac{4\mu_{\text{max}}}{\lambda} (x - \eta) \right)^{1 - \alpha}\right] )</td>
</tr>
<tr>
<td>Stannard</td>
<td>( y = a \left[1 + \exp \left(-\alpha (x - \lambda)\right)\right]^{-\alpha} )</td>
<td>( y = A \left[1 + v \exp(1 + v) \exp \left(\frac{4\mu_{\text{max}}}{\lambda} (x - \eta) \right)^{1 - \alpha}\right] )</td>
</tr>
<tr>
<td>Schnute</td>
<td>( y = \left[\frac{\lambda}{\gamma_1} + \left(\frac{\lambda}{\gamma_2} - \gamma_1\right) \right] \frac{1 - \exp(-a(t - \eta))}{1 - \exp(-a(t - \eta))} )</td>
<td>( y = \left[\frac{(1 - b)}{a} \right] \frac{1 - \exp(a \lambda + 1 - b - \omega)}{1 - b} )</td>
</tr>
</tbody>
</table>

Table 1.2 Sigmoidal models and their biological modified forms (Zwietering, 1990)

a, b, c are mathematical parameters, A is the asymptote of growth curve when population reaches maximum, \( \mu_{\text{max}} \) is the maximum of specific growth rate, \( \lambda \) is the lag time.

Investigating the dependence of biological properties on temperature is pivotal to the prediction of microbial growth. In early studies, Arrhenius based models were used to describe dependence of specific growth rate on temperature, because of its usefulness in analyzing reaction rates of chemical reaction. A characteristic of Arrhenius equation is that there is a linear relationship between temperature and reaction rate, which is also known as Arrhenius plot (Figure 1.4a). However, Ratkowsky (1982) plotted six sets of growth data from five different microorganisms, and observed that the Arrhenius plot resulted in a curve instead of a straight line (Figure
1.4b). As a conclusion, it is suggested that the Arrhenius equation doesn’t describe the effect of temperature on specific growth rate well.

![Arrhenius plots](image)

Figure 1.4 The characteristic Arrhenius plot (a) and Arrhenius plot applied to microbial specific growth rate (b) (Ratkowsky, 1982)

Based on their observation, Ratkowsky (1982) provided an alternative equation, known as the square root model (Eq. 1.3), to describe relationship between temperature and specific growth rate. The model presents a linear relationship between square root of specific growth rate and temperature in a temperature range from minimum to optimum growth temperatures, as shown in figure 1.5.

\[
\sqrt{\mu} = A(T - T_0) \quad (1.3)
\]

where \( \mu \) is the specific growth rate, \( T \) is the temperature, \( A \) is a regression coefficient and \( T_0 \) is the lowest temperature for growth.
Figure 1.5 Typical plot of the Ratkowsky square root model, square root of specific growth rate versus temperature

It should be noticed that the intersection of regression line and temperature axis is defined as a hypothetical minimum growth temperature. It is suggested that \( T_0 \) is an intrinsic property of the microorganism and the value of \( T_0 \) doesn’t change as environmental factors. Based on this finding, referring the value of \( T_0 \) is also an alternative way to categorize microorganisms as psychrophile, psychrotroph, mesophile or thermophile.

The model does not work well outside this temperature range, but Ratkowsky tested its accuracy with 65 sets of growth data and received high correlation coefficients between \( \sqrt{\mu} \) and \( T \ (>0.97) \). Phillips and Griffiths (1987) compared the performances of the Arrhenius equation and the square root model, using growth data from psychrotrophic bacteria in dairy product. They found that the square root model provided more accurate predictions than Arrhenius equation for microbial growth regardless of growth medium.

The basic square root model was further modified and extended to cover a wider
temperature range (Ratkowsky, 1983; Zwietering et al, 1991), as shown follow:

\[ \sqrt{\mu} = A(T - T_{min})\{1 - \exp\left[B(T - T_{max})\right]\} \quad (1.4) \]

where \( \mu \) is the specific growth rate, \( T \) is the temperature, \( A \) and \( B \) are regression coefficients, \( T_{min} \) is the lowest temperature for growth and \( T_{max} \) is the maximum growth temperature.

It is suggested that the modified forms of square root model seemed to be most suitable for growth rate analysis (Zwietering, 1991). However, the major criticism to square root model is that it is a purely empirical model, because it doesn’t have any biological basis (Zwietering et al, 1991; Huang, 2011).

Even though empirical models are widely used, a less empirical growth model which can reflect the mechanism of temperature effect on microbial growth is still needed (Baranyi and Roberts, 1993). Previous studies, such as those determining the parameters from sigmoidal models, used to describe the lag phase separated from the exponential phase. In order to eliminate the problem of separating modeling the dependence of the specific growth rate and the lag, Baranyi and Roberts (1993; 1994) introduced a new growth model. The model (Eq. 1.4 and 1.5), generated from previous models including logistic model, is able to describe how the environmental factors define the specific growth rate and lag phase together.

\[ \frac{dN(t)}{dt} = \frac{q(t)}{1+q(t)} \mu_{max} N(t) \left[1 - \left(\frac{N(t)}{N_{max}}\right)^m\right] \quad (1.4) \]
\[ \frac{dq(t)}{dt} = \mu_{max} q(t) \quad (1.5) \]
where $t$ is the time, $N(t)$ is the population at $t$, $N_{\text{max}}$ is the maximum population, $\mu_{\text{max}}$ is the specific growth rate, $q(t)$ is the parameter defining physiological state, $q(t) /[1 + q(t)]$ is the adjustment function associated with lag time (McKellar and Lu, 2004).

There are two features of this model: (i) the definition of lag is independent of the shape of the growth curve, and (ii) the effects of the previous and the present environment are separated. It is suggested that the duration of lag depends not only on current environmental condition, but also on previous cells’ history. Therefore, an adjustment function ($q(t) /[1 + q(t)]$) is used to describe the process of adjustment of previous environment. The adjustment function varies from zero to one representing no growth because of lagging and total adjustment. The model demonstrated that if the new environment is close to previous one, then the population should carry on growing without a lag period. It successfully described the growth better than Gompertz model (Baranyi and Roberts, 1993).

**Prediction of microbial growth under variable temperature condition**

Temperature is one of the environmental parameters affecting the microbial growth that often changes during food storage, transportation and consumer handling. The experimental analysis of the temperature oscillation and its effect on the microbial survival show that behavior of foodborne microorganisms experiencing oscillating temperature is not the same as those exposed to a the constant temperature (Semenov et al., 2007). Semenov demonstrated that it is not proper to use a mean temperature to predict microbial behavior under oscillating temperature. Since fluctuating ambient temperature affects the shelf life of food products, it is critical to take variable temperature conditions into consideration to be able to precisely determine the shelf life.
and reduce the food waste.

As mentioned above, the lag phase depends not only on current condition, but also on previous cells’ history. The cells adapt themselves to new environment during the lag time. It is suggested that the lag time is related to the ratio between the amount of work that cells need to do for adaptation and the rate of doing that work, as indicated follow (Robinson et. al, 1998; Koutsoumanis, 2001):

\[ t_{lag} = \frac{W}{R} \quad (1.7) \]

where \( t_{lag} \) is the duration of lag phase, \( W \) is the work needed for adaption from previous to current environmental condition, and \( R \) is the rate of doing the work. It has been reported that the rate \( (R) \) equals to the maximum specific growth rate (Robinson et. al, 1998). Therefore, when the reciprocal of specific growth rate is plotted against the lag time, a linear relationship crossing origin point is expected (figure 1.6). This relationship was observed in many studies (Li and Torres, 1993; Baranyi and Roberts, 1994; Koutsoumanis, 2001; Brown, 2007).
Figure 1.6 Correlation of lag time and specific growth rate (Koutsoumanis, 2001)

Baranyi and Roberts (1995) introduced the Baranyi model to include variable temperature. Growth of *Brochothrix thermosphacta* under both gradual and sudden temperature changes were investigated. Overall, the dynamic model showed good predictions with a certain temperature range, but the fitness didn’t hold when temperature decreased below 3°C and additional lag was observed. Similar result was observed with *Lactobacillus plantarum* (Zwietering et al., 1994). The prediction of growth showed very large deviation when the temperature fluctuated around the minimum growth temperature. A possible explanation was that the low temperature shock altered the physiological state of organism, or that the low temperature resulted in death of cells.

It is reported that the lag time under variable temperature conditions is very different from the normal lag obtained under constant temperature, as shown in figure 1.7 (Ng et al., 1962; Zwietering et al., 1994; Bernaerts et al., 2001). Zwietering et al.
(1994) applied the modified Gompertz equation to model the growth of *Lactobacillus plantarum* with fluctuating temperatures, and investigated the effect of temperature shifts on the duration of lag. They assumed that temperature shifts could result in an additional lag phase which is proportional (1/4) to the lag time normally found. Prediction made with this assumption provided acceptable agreement with actual growth. They also indicated that the additional lag time could be neglected, which means that a simplified prediction of growth under temperature fluctuation could be made by using linear equations.

![Figure 1.7 An intermediate lag caused by temperature change on growth of *Escherichia coli* (Bernaerts et al., 2001)](image)

Several studies have applied the prediction from pure media to actual food
products (Koutsoumanis, 2001; Mataragas et al, 2006; Gospavic et al, 2008; Kreyenschmidt et al., 2010). In general, primary models such as logistic and Gompertz, were modified then fitted with experimental data to determine growth parameters. Subsequently, parameters dependence on temperature was evaluated. Usually it is assumed that the specific growth rate changes immediately as temperature changes. Predictions of the growth of microorganisms under different temperature shifts were made after parameters finally fixed. The successful predictions presented in these studies provide a suitable method to construct model for predicting microbial growth under dynamic temperature conditions and establishing the shelf life of food products.

However, there are still some factors limiting the accuracy of prediction models. Bovill et. al. (2000) examined the accuracy of using Baranyi model to predict the growth of *Listeria monocytogenes* and *Salmonella* spp. under temperature fluctuation. They suggested the prediction of lag time is less accurate than growth rate. This situation can be also explained by the complexity of lag time. Since the duration of lag may be influenced by previous and current environmental condition (the cell’s history), it makes the lag of a cell hard to be predict from others. Moreover, the competition effect between microorganisms may also cause large deviation. Even though most studies on growth prediction provided acceptable results, the current models are still unable to take the competition effect into account.

Large progresses of predicting microbial growth under temperature fluctuation have been made in the past. Both mechanical and empirical models are established and successfully predict growth parameters, and made it possible to describe microbial growth under variable temperature conditions. The good fitness of models shows that the prediction is highly practical when models are applied to actual situations.

 Nonetheless, there are still some limitations, including differences between
experimental environment and actual food products, factors influencing lag time other than temperature, and lack of a universal model. The complexity of natural microflora in food products and variety of food ingredients lead to different levels of accuracy for different foods. Even though many research works conducted under a range of experimental environments resulted in high accuracy, it should be noted that there is no single model that can predict the microbial growth for all microorganisms at any given temperature and media composition. Since the demand of more precise models is increasing, a further study with certain food products is still needed.

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CHAPTER 2

MATERIALS AND METHODS

Enrichment and transition of *Pseudomonas fluorescens*

An inoculating loop of fresh culture of *Pseudomonas fluorescens* is inoculated into 5ml of 3% tryptic soy broth (BD Difco™). The broth is well-homogenized then incubated at 29°C in an incubator. After 24 hours, the broth is re-suspended and the population of *Pseudomonas fluorescens* is determined. Results from replicates show that the population will reach approximately ~10^9 CFU/ml, meaning that the growth of *Pseudomonas fluorescens* will stay in stationary phase after overnight incubation. This enrichment step can maintain a similarity of initial population between milk samples.

Sample preparation

The enriched broth is 10-fold diluted to ~10^5 CFU/ml by using 0.1% peptone water (BD Difco™). Further dilution is made with fresh milk. Sterilized low fat (1%) milk (Horizon Organic, CO) is obtained from local grocery (Kroger, Columbus OH). The dilution with ~10^5 CFU/ml *P. fluorescens* and sterile milk are mixed in a ratio of 1:99 to reach a population of 10^3-10^4 CFU/ml in the mixture. The mixture is well-homogenized for one minute by vortex mixer. Then, the mixture is dispensed into several micro-centrifuge tubes (Fisherbrand™) with 0.5ml in each. More than three sets of replicate are prepared for constant temperature. Two sets of replicates are prepared
for temperature fluctuation. The samples were stored under controlled temperature in an incubator (Benchmark Scientific, Inc.). Samples are periodically taken by appropriate time intervals, and the population of *P. fluorescens* is measured.

**Temperature variations of incubation condition**

Experiments under three constant temperatures are done at 4°C, 15°C and 29°C. The constant temperature conditions are simulating refrigerating temperature (4°C) and possible temperature abuse during storage (15°C and 29°C). For variable temperature conditions, two patterns of temperature fluctuation over storage time are introduced as presented in figure 2.1 and table 2.1.

When the temperature fluctuation happens, the duration is 6 hours for 15°C and 4 hours for 29°C. For experiment with one temperature fluctuation, temperature fluctuates at 24th hour, simulating the temperature abuse happening at early-phase of storage. For experiments with two temperature fluctuations, first temperature fluctuation happens at 24th, and second one happens 24 hours after the first one is done.

<table>
<thead>
<tr>
<th></th>
<th>Temperature (°C)</th>
<th>Time (h)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>T₁</td>
<td>T₂</td>
</tr>
<tr>
<td>One fluctuation</td>
<td></td>
<td></td>
</tr>
<tr>
<td>(Figure 2.1a)</td>
<td>4</td>
<td>15 or 29</td>
</tr>
<tr>
<td>Two fluctuations</td>
<td></td>
<td></td>
</tr>
<tr>
<td>(Figure 2.1b)</td>
<td>4</td>
<td>15 or 29</td>
</tr>
</tbody>
</table>

Table 2.1 Values of temperature (T₁ and T₂) and time intervals (t₁ and t₂) in fluctuation tests, as presented in figure 2.1
Figure 2.1 Patterns of temperature fluctuation over storage time: (a) one fluctuation, (b) two fluctuations. Values of temperature ($T_1$ and $T_2$) and time intervals ($t_1$ and $t_2$) are illustrated in table 2.1.

**Determination of microbial population in milk samples**

One micro-centrifuge tube of sample is taken each time for generating growth data. The sample is homogenized by vortex mixer first. Serial 10-fold dilutions of sample are made by using 0.1% peptone water (BD Difco$^{TM}$). Dilutions are homogenized for 15 seconds before doing next diluting. Two dilutions at appropriate concentration are plated on tryptic soy agar (3% tryptic soy broth, 1.5% granulated agar powder, BD Difco$^{TM}$). For each concentration, 0.1 ml of the dilution is spread on two
plates separately. Then the plates are incubated aerobically at 29°C for 48 hours in the incubator. Plates within 20 ~ 200 colonies are counted and used for determination of microbial population. The microbial population is calculated in CFU/ml as follow:

\[
Population = \frac{Average \ of \ colonies \ on \ two \ plates}{0.1 \times dilute \ factor} \ CFU/ml \quad (2.1)
\]
CHAPTER 3

EXPERIMENTAL DATA AND DISCUSSIONS

Introduction

The importance of studying psychrotrophic microorganisms is that, those bacteria can grow at refrigerating temperature regardless of their optimal growth temperature (Morita, 1975). They are known to cause widely spoilage of daily products by producing extracellular enzymes such as lipases and proteases (Ternström, et al., 1993). Lipases and proteases are able to degrade milk fat and protein, which would cause rancid, bitter off-flavors and gelation of milk. Even though UHT (Ultra-high-temperature processing) is an effective way to inactive psychrotrophic microorganisms, the enzymes would remain after the heat treatment (Chen et al., 2003). Consequently, psychrotrophic microorganisms is believed to be a major hazard to refrigerating food products.

*Pseudomonas fluorescens* belongs to *Pseudomonas* spp., a group of Gram-negative microorganisms. In addition of some members of *Aeromonas*, *Listeria*, *Staphylococcus*, and *Enterobacteriaceae*, pseudomonads constitutes the predominant microbiota in raw milk (Adams et al., 1975). The contamination of *P. fluorescens* in refrigerating milk may come from filling procedure, exposure to surrounding air and
incompletely-cleaning surfaces in fill machine (Eneroth, et al., 1998; Eneroth, et al., 2000). Usually, spoilage of milk would begin when population of *P. fluorescens* reaches $10^6 \sim 10^7$ CFU/ml (Matselis and Roussis, 1998). They cause spoilage and gelation in pasteurized milk by producing thermoresistant extracellular lipases and proteases (De Jonghe et al., 2010). Therefore, to monitor the growth of *P. fluorescens* in pasteurized milk under refrigerating storage temperature and possible temperature abuses, has significant meaning in assessing the risks of shelf life.

3.1 Experimental growth data of *Pseudomonas fluorescens* in milk

The growth data for bacteria stored under three different temperatures, 4°C, 15°C and 29°C, were obtained, which are displayed in figure 3.1 to 3.3. 4°C represents the refrigerating temperature of storage, while 15°C and 29°C represent the ambient temperature in spring and autumn separately. As displayed in the figures, all of the growth curves display nice sigmoidal shape, which can be divided into three phases: lag phase, exponential phase and stationary phase. In the stationary phase, the maximum population of *P. fluorescens* in low fat milk is approximately obtained as 8 log (CFU/ml), even though the values for initial population of each set of data are not perfectly maintained as the same. The result of maximum population is consistent with the report on behavior of *P. fluorescens* in UHT milk at refrigerating temperature (Chen, 2011).

These growth data provide a roughly and basic look of behavior of *P. fluorescens* in milk. As expected, it takes approximately 16 hours to reach maximum population under optimal growth temperature (29°C), while the time increases by
nearly tenfold when temperature decreases to 4°C. It should be noticed that spoilage usually begins when population reaches $10^6 \sim 10^7$ CFU/ml ((Matselis and Roussis, 1998), however, to study the complete growth cycle and continue the mathematical analysis, all growth tests are continued to reach the maximum population.

Figure 3.1 Growth of *Pseudomonas fluorescens* in sterilized milk under 29°C, sampling each one hour, presented in three replicates.
Figure 3.2 Growth of *Pseudomonas fluorescens* in sterilized milk under 15°C, sampling each six hours, presented in three replicates.

Figure 3.3 Growth of *Pseudomonas fluorescens* in sterilized milk under 4°C, sampling each 12 hours, presented in four replicates.
Two patterns of temperature fluctuation were introduced to experiment. The temperature fluctuations from refrigerated temperature (4°C) to higher temperature (15°C and 29°C) simulates the temperature abuse of food products during storage. The duration of temperature fluctuation was designed according to results from constant temperature. One third of the time needed to complete the exponential phase was selected to simulate the temperature abuse. As seen in figure 3.1 and 3.3, the exponential phase of growth of *P. fluorescens* in milk is approximately 12 hours at 29°C and 20 hours at 15°C. Therefore, the duration of temperature fluctuation was set to four hours for 29°C and six hours for 15°C.

The growth data for bacteria stored under variable temperatures are obtained as well, as shown in figure 3.4 to 3.5. As seen in the figures, *P. fluorescens* corresponds to temperature change immediately. When temperature increases, the growth speeds up; on the contrary, when temperature decreases, the growth slows down. Consequently, an obvious increment on growth is observed within the temperature fluctuation. There is no doubt that the raise of temperature results in a faster microbial growth, which means that a temperature abuse out of refrigeration has a potential effect on accelerating microbial growth in food product. As a result, the shelf life may be shortened because of a larger amount of microbial population. A detailed discussion about the effect of temperature fluctuation on microbial growth and shelf life is presented in the next section.
Figure 3.4 Growth of *Pseudomonas fluorescens* in sterilized milk with one temperature fluctuation at 24\textsuperscript{th} h (a) temperature increased to 29°C for 4h; (b) temperature increased to 15°C for 6h, presented in two replicates.
Figure 3.5 Growth of *Pseudomonas fluorescens* in sterilized milk with two temperature fluctuations, while the first fluctuation happens at 24\(^{th}\) h and the second fluctuation happens 24h after the first one is done, (a) temperature increased to 29°C for 4h in each fluctuation; (b) temperature increased to 15°C for 6h in each fluctuation, presented in two replicates.
3.2 Effect of temperature fluctuation on shelf life

The growth of *P. fluorescens* in milk with one and two temperature fluctuations is investigated. The temperature fluctuations represent the possible temperature abuses during transport and storage, and the experimental settings of temperature fluctuations are explained in the previous section. As mentioned, temperature fluctuations accelerate the growth of *P. fluorescens* in milk, which is a potential hazard to shelf life. Therefore, the idea of this section is to make a comparison between average data of growth under constant and variable temperature, and to discuss the effect of temperature fluctuation on shelf life.

Growth data are displayed as figure 3.6 to 3.7. In figure 3.6, the temperature increases after 24 h, simulating the temperature abuse in early phase of storage. As shown, there is approximately one log (CFU/ml) increment of population of *P. fluorescens* during the time the milk is exposed to higher temperature; however, for the control group stored at 4°C, it takes near two days to reach the same population that the test group reach at the end of fluctuation. These results suggest that if the temperature abuse happens in early phase of storage, it drastically decreases the time to reach the maximum population, leading to shorter shelf life.
Figure 3.6 Comparison between growth of *P. fluorescens* in milk under constant temperature (4°C) and with one temperature fluctuation at 24th h (a) temperature increased to 29°C for 4h; (b) temperature increased to 15°C for 6h, presented in average data
To further investigate the effect of temperature abuse, samples exposed to two temperature fluctuations are studied. Figure 3.7 shows the growth of *P. fluorescens* in milk under this condition. The first temperature fluctuation happened after 24 h, and the second one happened 24 h after the first one is done. As shown in figure 3.7, an approximately one log (CFU/ml) increment of population was observed during each of the temperature fluctuation, which is similar to what mentioned before. What’s more, the effect of temperature abuses on shortening shelf life is clear to see in these figures. As displayed, when the milk was stored at constant temperature (4°C), it took about 90 hours 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 two temperature fluctuation to 29°C, *P. fluorescens* took only 55 h to reach that population. If we consider the time that *P. fluorescens* needs to reach $10^6$ CFU/ml as the shelf life, the shelf life of milk is cut as much as 40% after temperature abuses. This result emphasizes the significant influence of temperature abuse on shelf life. The milk is exposed to higher temperature for only a short period of time, but the shelf life is greatly shortened.
Figure 3.7 Comparison between growth of *P. fluorescens* in milk under constant temperature (4°C) and with two temperature fluctuation (a) temperature increases to (a) temperature increased to 29°C; (b) temperature increased to 15°C, presented in average data
In conclusion, the results of temperature fluctuation and its effect on the microbial growth emphasize the significance of the temperature abuse and its effect on the actual shelf life of a food product. It should pay attention to every temperature abuse even though the duration is only few hours. If the microbial growth with temperature abuse is predicted, a real time shelf life can be obtained so that it reminds the consumers to utilize products before spoilage happens.

References


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CHAPTER 4

MODELING OF GROWTH OF PSEUDOMONAS FLUORESCENS IN MILK

Introduction

The temperature variation during storage and handling could potentially have a huge influence on the microbial activity, and it is essential to take time-temperature profile into account for monitoring food spoilage and establishing a realistic shelf-life (Baranyi and Roberts, 1995; Gospavic et. al. 2008; Semenov et al., 2007). Efforts have been made to develop mathematical models to predict the microbial growth under constant temperature condition. In general, a two-steps approach is used to construct dynamic model for growth prediction. In first step experimental data is generated under constant temperature, and the mathematical model is developed to describe the relationship between population and time as the primary model. The second step is to evaluate the dependence of parameters used in the model on temperature, and such a function or relationship is considered as the secondary model (Gospavic et. al. 2008; Peleg and Corradini, 2011).

Sigmoidal curves have been used to describe microbial growth over time because of its unique similarity to microbiological growth curve (Gibson, et. al., 1987). There are two major sigmoidal curves: Gompertz and Logistic functions, as shown below:
$Log_{10}N(t) = A + Ce^{-e^{-B(t-M)}}$ (4.1)

where $t$ is the time, $N(t)$ is the population at $t$, $A$ is the initial population in logarithmic scale, $C$ is the asymptotic logarithmic growth ratio which is $N(t)/N(0)$ when $t$ is infinite, $B$ is the relative growth rate at $M$, and $M$ is the time when the growth rate reaches maximum (McKellar and Lu, 2004).

$Log_{10}N(t) = A + \frac{C}{1+e^{-B(t-M)}}$ (4.2)

where parameters have the same meanings as Gompertz model (Eq. 4.1).

As shown in figure 3.1 to 3.3, the growth of *P. fluorescens* under constant temperature displays a typical sigmoidal growth pattern. Therefore, a sigmoidal model will be suitable to model these growth data. In this study, Modified Logistic function was chosen to fit the growth curve for two reasons. First, its simplicity makes it easy to use. Second, it is well-modified with biological meanings. Growth parameters can be generated directly by non-linear regression analysis.

### 4.1 Mathematical model and non-linear regression analysis

By using the nonlinear regression in Matlab® software, microbial population data are fitted to the modified logistic function as follows (Zwietering, 1990):

$Log_{10}N(t) = Log_{10}N_0 + \frac{A}{1 + \exp\left[\frac{4\mu}{A} (\lambda - t) + 2\right]}$ (4.3)
where $N_0$ is the initial population, $t$ is the storage time in hour, $u$ is the specific growth rate, and $\lambda$ is the lag time. $A$ is defined at the log ratio of maximum and initial populations:

$$A = \log_{10}\left(\frac{N_{\text{max}}}{N_0}\right)$$ (4.4)

The best fit line was generated by non-linear regression analysis, and the average of experimental data and the nonlinear growth curves for each temperature are shown in figure 4.1. A good fitness is observed between the experimental data and the interpolation of Modified Logistic model. It could be seen that the best fit line describes the actual growth with time very well.

![Figure 4.1](image-url)

Figure 4.1 Average of experimental data for growth of *P. fluorescens* in milk (dots) and fitted modified logistic model (solid line) for samples stored at: (a) 4°C; (b) 15°C; (c) 29°C.
To further analyze the fitness of the function, fitted curves with 99% confidence band were generated, which is shown in figure 4.2. As displayed in the figure, nearly
all measured data points are located inside the upper and lower confidence bounds. This result shows that the best fit line not only describes the average data, but also represents all of the data points very well. It demonstrates the fitness of using Modified Logistic to describe the growth data.

Consequently, it could be concluded that the Modified Logistic model is suitable to describe the growth of \textit{P. fluorescens} in milk as a function of time. Such a conclusion shows the usefulness of applying Modified Logistic to an actual food product.

Figure 4.2 Growth curve (solid line) fitted by modified logistic model with 99% confidence band (dashed line) and measured data points (dots) for (a) 4°C; (b) 15°C; (c) 29°C.
Figure 4.2 continued
4.2 Temperature dependence of growth parameters

Growth parameters for samples kept under constant temperature are obtained from Modified Logistic model, equation 4.3. These parameters, including initial population, asymptote, maximum specific growth rate and lag time, are presented in table 4.1. Asymptote represents the range between initial population and maximum population, instead of maximum population reached, because all of the samples have initial population of \(N_0\) in the Modified Logistic equation. The sum of initial population and asymptote is close to 8 CFU/ml, which is consistent to the discussion about maximum population in the previous chapter. As expected, a higher temperature will result in a higher specific growth rate and a longer lag time. When temperature increases from 4°C to 29°C, the lag time decreases from 29.512 h to 2.808 h and the specific growth rate increases from 0.057 /h to 0.462 /h.

If the shelf life is considered as the time required by pseudomonad growth to reach \(10^6 \sim 10^7\) CFU/ml, it can be seen that the lag time is approximately one-fourth of the shelf life. Therefore, it is very important to accurately predict lag time for determination of the shelf life. However, compared to specific growth rate, the lag time exhibits a larger variation, for all of three temperatures. This is believed due to the dependence of lag time on the cells’ previous history in addition to new environmental conditions (Baranyi and Roberts, 1995; Bovill et. al., 2000). Baranyi et al. (1995) suggested that cells’ history could be very different even for the same inoculum population, depending on metabolic activity in the pre-inoculation environment. If the cells maintained a high metabolic activity in the pre-inoculation environment, the lag would be shorter after inoculation (Baranyi and Roberts, 1994). There are two
difficulties to maintain the consistency of lag: first, as seen, it is hard to maintain the same initial population for milk samples; second, it is nearly impossible to maintain the cells’ metabolic activity in pre-inoculation environment. Consequently, because of the cells’ history before inoculation, when the cells were transferred to milk samples, there was a larger variation in the values of lag between replicates.

<table>
<thead>
<tr>
<th>Temperature(°C)</th>
<th>Initial population (logCFU/ml)</th>
<th>Asymptote (logCFU/ml)</th>
<th>( \mu ) (1/h)</th>
<th>( \lambda ) (h)</th>
</tr>
</thead>
<tbody>
<tr>
<td>4</td>
<td>2.9 ± 0.15</td>
<td>5.667 ± 0.242</td>
<td>0.057 ± 0.002</td>
<td>29.512 ± 4.240</td>
</tr>
<tr>
<td>15</td>
<td>4.4 ± 0.05</td>
<td>3.478 ± 0.168</td>
<td>0.168 ± 0.048</td>
<td>12.662 ± 4.385</td>
</tr>
<tr>
<td>29</td>
<td>3.6 ± 0.68</td>
<td>4.207 ± 0.662</td>
<td>0.462 ± 0.024</td>
<td>2.808 ± 0.305</td>
</tr>
</tbody>
</table>

Table 4.1 Initial population, asymptote, maximum specific growth rate and lag time determined using experimental data and the modified logistic model, presented in three replicates for 15°C and 29°C; four replicates for 4°C

The temperature dependence of specific growth rate is shown in figure 4.3. A linear relationship between square root of specific growth rate, \( \mu^{1/2} \), and temperature, known as the Ratkowsky square root model (Ratkowsky et al., 1982; Ratkowsky et al., 1983; Zwietering et al. 1991), is presented below:

\[
\sqrt{\mu} = c (T - T_0)
\] (4.5)

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.

Figure 4.3 Regression analysis of the relationship between temperature ($T$) and square root of maximum specific growth rate ($\mu^{1/2}$)

The Ratkowsky square root (equation 4.5) model has been demonstrated to work well for the temperature range between minimum and optimal. In this study, the values of specific growth rate from $4^\circ$C to $29^\circ$C fit in the equation very well, indicating that Modified Logistic model provides a good and accurate estimation for specific growth rate of $P. fluorescens$ in milk samples. Additionally, $T_0$ value for the set of data obtained in this work is 264K, which is in a good agreement with published data for $Pseudomonas$ group (Ratkowsky et al., 1982; Phillips and Griffiths, 1987). $T_0$ is believed to be an intrinsic characteristic of a microorganism, and its value doesn’t change with media. Such a good agreement further demonstrates the accuracy of calculating specific growth rate in our case.
The relation between the reciprocal of specific growth rate and the lag time is plotted in figure 4.4. In our case, the regression analysis shows a strong linear relationship between the two parameters ($R^2 = 0.9834$). In fact, this relation is mentioned by other studies (Li and Torres, 1993; Baranyi and Roberts, 1994; Koutsoumanis, 2001; Brown, 2007).

\[
\lambda = b \left( \frac{1}{\mu} \right) + a
\]  \hspace{1cm} (4.6)

where $\lambda$ is the lag, $1/\mu$ is the reciprocal of specific growth rate, $b$ and $a$ are regression parameters.

Figure 4.4 Regression analysis of the relationship between lag time ($\lambda$) and reciprocal of maximum specific growth rate ($1/\mu$)

As mentioned, the lag time depends not only on the current environmental
condition, but also on previous state. The lag represents a period in which the cells adapt themselves to new environment. It is suggested that the lag time is related to the ratio between the amount of work that cells need to do for adaptation and the rate of doing that work, and the rate is reported to equal to maximum growth rate, as shown in equation 4.6 (Koutsoumanis, 2001; Robinson et. al, 1998).

\[ t_{lag} = \frac{Work}{Rate} \quad (4.7) \]

Therefore, when the lag time is plotted against the reciprocal of specific growth rate, a straight line crossing point (0, 0) is expected. The offset obtained in this study, \( a = 0.6 \), displays a negligible deviation from origin point (0, 0). Considering the large variation in lag, the regression result in this experiment is consistent with published literatures.

In this section, the temperature dependence of specific growth rate and lag time is discussed. The relationships presented here are consistent with published literatures. To some extents, we could say that the Modified Logistic provide a good estimation of the growth parameters. Also, the result further demonstrates the fitness of applying this model to our case.

**4.3 Modeling growth of Pseudomonas fluorescens under temperature fluctuations**

In the previous sections, the fitness of using Modified Logistic model to describe the growth of P. fluorescens in milk has been discussed. The results indicates that the function is suitable to conduct the modeling for variable temperature conditions.
In this study, to predict the bacterial growth under temperature fluctuation, the storage time is divided into different intervals of constant temperature, and the following modified logistic model is used:

\[
\log N(t) = \log N_i + \frac{\log N_{\text{max}} - \log N_i}{1 + \exp\left(\frac{4\mu_i}{(\log N_{\text{max}} - \log N_i)(\lambda_i - t - t_i) + 2}\right)}
\]

\[
t_i < t < t_{i+1}
\]

where \( t \) is the storage time in hour, \( N_i \) is the microbial population at the beginning of temperature change \( t_i \) and \( N_{\text{max}} \) is the maximum population. The temperature is constant for \( t_i < t < t_{i+1} \), and \( \lambda_i \) and \( \mu_i \) are the lag time and specific growth rate for this time interval, respectively.

The values of specific growth rate \( \mu_i \) corresponding to \( T_i \) of each fluctuation intervals are calculated using equation (4.8). In order to provide prediction of the growth, the next step is to determine the value of lag time, \( \lambda_i \). The relationship presented in figure 4.4 and equation 4.6 is used to estimate the lag from specific growth rate (and storage temperature), however, the variation of lag time due to different history of bacteria before change of temperature should be taken into consideration. It is reported that the lag time under variable temperature conditions deviates from the lag time obtained under constant temperature (Bernaerts et al., 2001; Ng et al., 1962; Zwietering et al., 1994). Therefore, different values of lag were incorporated into equation 4.8 to generate predictions.

The bacterial growth with one fluctuation and their mathematical models are
displayed in figure 4.5. In figure 4.5a and 4.5b, the temperature increased to higher temperatures (15°C and 29°C) after 24 h. Based on the data presented in table 4.1, the temperature change happened during the lag phase of the growth at original refrigeration temperature (4°C). As seen from the experimental data, the bacteria responded quickly to the temperature increase showing small 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 (1/4) of the lag time that normally happens for that temperature. However, a longer duration of lag was observed when temperature decreased to 4°C, showing a new and independent growth after temperature fluctuation. In this case, the change from higher to lower temperature displays the similar effect observed at the beginning of the growth curve, where the microorganisms are transferred from 29°C to 4°C and their growth displays a full lag. To further analyze the duration of lag, the growth after temperature fluctuation was fitted to Modified Logistic as a new and independent growth, which is shown in figure 4.6. The results showed that the new values of lag were 17.40 in figure 4.6a and 15.01 in figure 4.6b, which is close to one half (1/2) of the normal lag (29.51). In this scenario, an acceptable agreement between prediction and experimental data is obtained by incorporating 1/2 lag in equation 4.8 when the storage temperature decreases.
Figure 4.5 Prediction (solid line) and experimental data (dot) of *P. fluorescens* in milk with one temperature fluctuation at 24\textsuperscript{th} h (a) temperature increased to 29°C for 4h; (b) temperature increased to 15°C for 6h.
Figure 4.6 Fitted Modified Logistic model (solid line) for growth data (dots) after temperature fluctuation in figure 4.3.1

The prediction of the growth with two fluctuations was displayed in figure 4.7. Two fluctuations were located in lag phase and exponential phase separately. The model
adopted the assumption used in figure 4.5, which is to incorporate a proportional lag to predict growth in the first fluctuation. In the second fluctuation, the experimental data displayed a different pattern of lag phase during and after temperature fluctuation. Some studies suggest that in exponential phase temperature change causes no lag time (Kreyenschmidt et al., 2010; Zwietering et al., 1994). Therefore, the specific growth rate changes instantaneously as temperature changes and the additional lag phase was neglected in the model. Even though prediction after second temperature fluctuation is over-estimated, the model displays a good agreement with experimental growth data in figure 4.7a. However, the prediction under-estimated the growth in figure 4.7b, where temperature varied between 4°C and 15°C.

Even though acceptable agreements were obtained in figures, it could be noticed that the value of lag incorporated into the model plays a significant role in affecting the accuracy of the prediction. As seen in both figures, predictions for growth with temperature fluctuating to 29°C received better agreement than that to 15°C. As mentioned, the duration of lag also depends on cell’ history. A possible explanation for this result is that temperature gradient may affect the cells’ metabolic activity. It is suggested that a large and abrupt temperature shift may result in a new adaptation phase (Bernaerts et al., 2001; Ng et al., 1962). Zwietering (1994) and Baranyi (1995) also found that temperature shifts around the minimum growth temperature showed large deviations from the model predictions. Therefore, it is possible that temperature gradient is a factor that should be incorporated into the equation in order to provide a better prediction. This is a potential direction of future studies. What’s more, the microbial population reaches a relative high level at the end of second temperature fluctuation, getting close to the maximum population, which means that the growth is probably entering the stationary phase. It is obvious that the growth will slow down
near the stationary phase. Therefore, the microbial population is also a potential factor that affects the accuracy of prediction in this case.

Figure 4.7 Prediction (solid line) and experimental data (dot) of *P. fluorescens* in milk with two temperature fluctuations (a) temperature increased to 29°C for 4h in each fluctuation; (b) temperature increased to 15°C for 6h in each fluctuation.
In general, modified logistic model provides an acceptable prediction for growth of *P. fluorescens* in milk. It can be noticed that the accuracy of prediction largely depends on whether an appropriate value of lag is used or not. We can say that the determination for the value of lag is the key in our prediction. However, the lag is a very sensitive parameter. As mentioned above, unlike the specific growth rate which is only a function of storage temperature, the lag phase highly depends on different parameters such as the history of the bacteria and the temperature gradient during the fluctuation. In addition to these parameters, the lag time could be also affected by the size of microbial population. Consequently, future studies can focus on comprehensively understanding the lag time. For accurate estimation of bacterial growth.
growth, equation (4.8) should be modified to incorporate the effect of these parameters into consideration.

REFERENCES


CHAPTER 5

CONCLUSION

In this work, the growth of *P. fluorescens* in low fat milk was studied. Growth data were obtained from both constant and variable temperature conditions. Modified Logistic model was used to describe the growth of *P. fluorescens* in milk under constant temperature as a function of time. A good fitness is observed between the experimental data and the interpolation of Modified Logistic model, indicating the usefulness of applying Modified Logistic to an actual food product.

Growth parameters such as lag time, specific grow rate and time to reach the maximum growth were determined from the model as well. Compared to specific growth rate, the lag time exhibits a larger variation. A possible explanation for this result is that the lag time depends not only on the new environmental conditions, but also on the cells’ previous history.

Additionally, predictions for growth under temperature fluctuations were generated by Modified Logistic model. In general, Modified Logistic model provides an acceptable prediction for growth of *P. fluorescens* in milk. In addition to storage temperature, the lag time highly depends on different parameters such as the magnitude of temperature shift, previous history and potentially the microbial population. To develop a precise mathematical model, it is critical to understand microbial behavior during the fluctuation and characterize their behavior. Consequently, further study
focusing on those effects is still needed in order to provide a better understanding about lag and a more accurate prediction from the model.

The effects of temperature fluctuation on shelf life were also discussed. Temperature abuse at early phase of storage has a large impact on reducing shelf life. Moreover, temperature abuse could drastically reduce the shelf life up as much as 40% when milk is exposed to ambient temperature for only few hours. If the microbial growth with temperature abuse is predicted, a real time shelf life can be obtained so that it reminds the consumers to utilize products before spoilage happens. Therefore, a good prediction for microbial growth from mathematical model will provide valuable information for a more precise shelf-life determination and reduction of food waste.
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