Dynamic predictive model for the growth of *Salmonella* spp. in liquid whole egg and risk evaluation of egg white hydrolysates manufacturing process for spore formers: *Bacillus cereus* and *Clostridium perfringens*.

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DYNAMIC PREDICTIVE MODEL FOR THE GROWTH OF SALMONELLA SPP. IN LIQUID WHOLE EGG AND RISK EVALUATION OF EGG WHITE HYDROLYSATES MANUFACTURING PROCESS FOR SPORE FORMERS: BACILLUS CEREUS AND CLOSTRIDIUM PERFRINGENS

By

Aikansh Singh

A THESIS

Presented to the Faculty of
The Graduate College at the University of Nebraska
In Partial Fulfillment of the Requirements
For the Degree of Master of Science

Major: Food Science and Technology

Under the supervision of Professor Harshavardhan Thippareddi

Lincoln, Nebraska

May, 2010
DYNAMIC PREDICTIVE MODEL FOR THE GROWTH OF SALMONELLA SPP. IN LIQUID WHOLE EGG AND RISK EVALUATION OF EGG WHITE HYDROLYSATES MANUFACTURING PROCESS FOR SPORE FORMERS: BACILLUS CEREUS AND CLOSTRIDIUM PERFRINGENS

Aikansh Singh, MS
University of Nebraska, 2010

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A dynamic model for the growth of Salmonella spp. in liquid whole egg under continuously varying temperature was developed and validated. A primary model (Baranyi model) was fitted to each temperature growth data and corresponding maximum growth rates were estimated. Pseudo-R$^2$ values were greater than 0.97 for primary models. The maximum growth rates obtained from each primary model were then plotted against temperature and modeled using the modified Ratkowsky model. The pseudo-R$^2$ and root mean square error were 0.99 and 0.06 h$^{-1}$, respectively for the secondary model.

A dynamic model for the prediction of Salmonella spp. growth under varying temperature conditions was developed by solving a combination of primary and secondary models using fourth-order Runga-Kutta method. The developed dynamic model was validated for two sinusoidal temperature profiles, 5-15$^\circ$C and 10-40$^\circ$C with corresponding RMSE values of 0.28 and 0.23 log CFU/ml, respectively between predicted and observed values. The developed dynamic model can be used to predict the growth of Salmonella spp. in liquid whole egg under varying temperature conditions.

The germination and outgrowth of B. cereus and C. perfringens spores during the egg white hydrolysate (EWH) manufacturing process was evaluated. EWH was prepared by enzymatic hydrolysis of the inoculated egg white solution and maintained at optimum pH
for enzymes (trypsin and chymotrypsin) for 24 h at 37°C. *B. cereus* population was increased by > 3.5 log CFU/ml during the EWH preparation using chymotrypsin, while minimal increase was observed during trypsin hydrolysis. *C. perfringens* populations were reduced during EWH preparation irrespective of the enzyme used. Hydrolysis of major egg proteins by chymotrypsin was completed within 4 h of incubation, while minimal hydrolysis of egg white proteins was observed with trypsin subsequent to 24 h incubation. New enzymes other than trypsin and chymotrypsin should be exploited for manufacturing egg white hydrolysates.

**Keywords:** *Salmonella, B. cereus, C. perfringens, egg, hydrolysates, predictive modeling*
ACKNOWLEDGEMENTS

I would like to express my thanks to my committee members for their support throughout my period of study. I am thankful to Dr. Thippareddi for his constant support, encouragement, opportunities and his patience in guiding me. He has been a great mentor, senior, guardian and friend throughout the period of my degree. Thanks to Dr. Subbiah for helping me with the concepts of modeling. I am also grateful to Dr. Froning for serving as a member on my committee. I also thank Dr. Rao for making me understand the real meaning of research along with his valuable assistance in my project work and guidance in preparation of thesis.

I want to express my thanks to all with whom I have worked in Reddi’s lab for their constant support. I would like to thank my family members (Parents, Mausaji, Mausi, Goldy and Vicky) for everything which I have achieved till now. I strongly believe that without their blessings it would not have been possible for me to come at this stage and feels grateful for their unrelenting love and encouragement. They definitely made my wishes come true. Special thanks go to my friends in India for their constant touch and encouragement.
THESIS FORMAT

This thesis consists of four chapters. Chapter 1 is the introduction and literature review for *Salmonella* spp. in liquid whole egg along with the information about models used in predictive microbiology. Chapter 2 is written as a technical journal article for publication and describes a predictive model for the growth of *Salmonella* spp. in liquid whole egg under non-isothermal conditions. Chapter 3 is the introduction and literature review of egg white hydrolysates manufacturing process and information regarding bacterial spores. It also discuss about different peptides obtained from food protein hydrolysis. Chapter 4 again is written as a technical journal article for publication and provides a risk evaluation of egg white hydrolysates manufacturing process.
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CHAPTER I
INTRODUCTION

Foodborne illnesses affect people and society economically around the world (WHO, 2002). The United States Centers for Disease Control and Prevention (CDC) estimated 76 million cases, 300,000 hospitalizations and 5,000 mortalities from foodborne illnesses annually (Mead et al., 1999). There is a need to systematically evaluate the risk of food manufacturing processes to control, reduce and/or eliminate the food safety hazards for improving the safety of food supply. Egg and egg products are used widely in food preparation and approximately 900 food products contain eggs or egg components (AEB, 2007). Eggs can be contaminated with Salmonella spp. before laying as well as subsequent to laying. Potential sources of egg contamination include air, wash water, soil, equipment, cross contamination, and the food handlers (AEB, 2007). A majority (> 90%) of the foodborne illnesses are caused by improper storage, handling, or cooking of food (FSIS, 2006). In most cases, temperature abuse of contaminated raw materials and absence of a step in the food chain which can reduce and/or eliminate the microorganisms can result in proliferation of the foodborne pathogens, increasing the potential risk of foodborne illness. Salmonella spp. is the most commonly associated pathogen with egg and egg products. It is estimated that salmonellosis affects 1.3 million people in the United States annually (CDC, 2003). Temperature abuse of shell eggs during collection, storage and processing may create favorable conditions for the growth of Salmonella spp. The USDA Food Safety and Inspection Service (FSIS) published a risk assessment of Salmonella spp. in shell eggs and liquid egg products (FSIS, 2005) and identified several research needs to improve the existing risk assessment model. The overall objective of
this study was to develop models to predict the growth of *Salmonella* spp. in liquid whole egg under non-isothermal conditions. The specific objective of the study was to develop and validate a dynamic model to predict the growth of *Salmonella* spp. in liquid whole egg under non-isothermal conditions.

**LITERATURE REVIEW**

**Microorganism**

*Salmonella* spp.

*Salmonella* is a rod-shaped, Gram negative, motile (few non-motile exceptions), non spore forming, facultatively anaerobic bacterium. The prevalence of *Salmonella* spp. is high in animals and animal products, especially poultry and swine. Sources of contamination include water, soil, insects, kitchen surfaces, cross contamination, animal feces, raw meats, raw poultry including eggs, and raw sea foods (FDA-CFSAN, 2006). *Salmonella* spp. can grow at temperatures ranging from 5.3°C to 46.2°C (Matches and Liston, 1968), with an optimum between 35°C and 37°C. *Salmonella* spp. is not salt tolerant and salt (NaCl) concentrations of 3-4% are inhibitory to its growth. *Salmonella* can grow between pH 4.5 to 9.0 with an optimum pH of 6.5-7.5.

Over 2,500 *Salmonella* serovars have been identified and are categorized into two species; *Salmonella enterica* and *Salmonella bongori* (Janda, 1998). Majority of serovars belong to *Salmonella enterica* and are further sub–categorized into five subtypes. The O antigen is used to classify the serovars into groups A, B, C1, C2, D, and E1, based on the similarities and differences of specific O antigens.
Salmonellosis

The symptoms of salmonellosis include diarrhea, abdominal cramps, nausea, vomiting, fever, and headache. The onset of symptoms begins within 6 to 72 h after the consumption of food and can last from 4 to 7 days. The symptoms are usually resolved without antibiotic treatment in healthy individuals. However, the bacteria can enter the blood stream, leading to a severely fatal illness. The invasive, life-threatening form of the disease is more likely to occur in highly susceptible populations, including children, elderly, and the immunocompromised (Gordon, 2008). *Salmonella enterica* serovar Enteritidis (SE) was found to cause greater proportion of foodborne illnesses compared to other serotypes (CDC, 1996). SE related outbreaks between the years 1985 and 1995 accounted for 24,058 cases of illness, 2,290 hospitalizations, and 70 deaths (MMWR, 1996). A gradual increase of SE infections occurred from 1976 to 1995, with slight reductions by 1999. However, the number of illnesses and outbreaks remained stable since 2001 (MMWR, 2003), with an estimated number of cases alone involving human SE is 200,000 to 1 million annually in the U.S. (Morales and McDowell, 1999).

**Salmonella Entry into the Eggs**

Eggs internally contaminated with SE were the leading cause of foodborne illness during past two decades (MMWR, 2003). A farm-to-table risk assessment of shell eggs and egg products by FSIS, FDA and CDC estimated that 2.3 million shell eggs in the U.S. are contaminated with SE when laid (USDA-FSIS, 1998). Undercooked and raw shell eggs and their products are the most common sources of *Salmonella* spp. infection (MMWR,
SE introduced in chicken either naturally or experimentally resulted in contamination of the egg’s liquid interior with a few hundred SE cells in the freshly laid eggs (Humphrey et al., 1991; Gast and Holt, 2000a; Chen et al., 2002). Refrigeration of eggs to an internal temperature of \(\leq 7.2^\circ C\) can inhibit the growth of SE and lower the potential for human exposure and thus, the risk of illness. The location of *Salmonella* spp. in egg contents is an important factor for the potential growth of organism. *Salmonella* spp. can colonize the reproductive tract of the hen, resulting in an entrapment of SE in the egg albumen or yolk during formation of the egg (Humphrey et al., 1989; Gast and Beard, 1990; Humphrey et al., 1991; Gast and Holt, 2000b). Gast and Holt (2001) reported that contamination of *Salmonella* spp. is mostly on the yolk membrane, rather than the interior of the yolk. *Salmonella* spp. can survive, but cannot grow in albumen due to higher pH and other inhibitory factors in albumen (Baron et al., 1997; Gast and Holt, 2000b). On the contrary, yolk is a nutrient rich medium, and rapid growth of *Salmonella* spp. is possible at storage temperatures above \(20^\circ C\) (Bradshaw et al., 1990; Humphrey and Whitehead, 1993; Braun and Fehlhaber, 1995; Gast and Holt, 2000b). Gast and Holt (2001) reported that *Salmonella* spp. can penetrate the yolk through the yolk membrane and then multiply. Rapid refrigeration is necessary to prevent bacterial penetration into the yolk and further proliferation. Jones et al. (2004) reported that SE population in the interior egg contents increased during storage of SE inoculated eggs at room temperature for 5 weeks and demonstrated that SE growth can take place in the yolk during cooling of the egg subsequent to lay.

**Egg Tempering Step**
The egg tempering step is usually involved before breaking of eggs. The objective of tempering the eggs is fine separation of egg yolk and egg white from shell eggs. During this step the eggs are stored in rooms having temperatures $\geq 7.2^\circ$C where *Salmonella* spp. present in egg products can proliferate and increase to higher numbers. The tempering of shell eggs can be for 2 to 3 days in some cases which can result in substantial amount of increase in *Salmonella* counts if present initially.

**Regulatory Issues**

The CDC reported a decrease in *Salmonella* prevalence by 9%, probably due to the decrease in incidences of *S. Typhimurium* (which decreased 42% in 2005 compared to 1996-1998). The estimated prevalence of SE (25%), *S. Heidelberg* (25%) and *S. Javiana* (82%) have increased significantly during the same time period (CDC, 2006). The CDC reported that *Salmonella* serotypes Typhimurium, Enteritidis, Newport, Heidelberg and Javiana accounted for 61% of the total isolates and SE as the second leading serotype causing human illnesses (CDC, 2006). The risk assessment for SE in eggs and egg products indicated that multiple interventions are required in farm-to-table chain to reduce the risk of illnesses from SE (FDA, 1997). The FDA proposed transportation and storage of eggs $\leq 7.2^\circ$C as mandatory temperature to prevent the rapid multiplication of SE in contaminated eggs. Subsequently, FSIS (1998) mandated transportation and storage of eggs at $\leq 7.2^\circ$C. However, cooling of eggs can be slow and may require several days to reach this specific internal egg temperature to restrict bacterial growth in the commercial processing operations (Curtis et al., 1995; Thompson et al., 2000).
Predictive Microbiology

Predictive Modeling

Growth of microorganisms in foods is affected by both intrinsic and extrinsic factors such as temperature, pH, sodium nitrite, sodium chloride, water activity, antimicrobials, and modified atmosphere packaging conditions (McKeller, 1997; Juneja, 2003; Malakar et al., 2003; Schultze et al., 2006). Understanding of parameters governing growth of pathogens in food can be useful in determining their survival, growth and behavior in foods and in controlling their proliferation. Factors responsible for microbial growth can be effectively studied with the help of predictive models (Francois et al., 2006; Schultze et al., 2006). Predictive microbiology use a combination of mathematical, statistical and microbiological principles to quantitatively predict the behavior of microbial populations in foods (Ross et al., 2000). A number of mathematical functions have been proposed to describe the sigmoid curves, and have been used to model the batch growth of microbial cultures (McMeekin et al., 2008). The Pathogen Modeling Program (PMP), developed by USDA Agriculture Research Service has growth models for Salmonella spp., Listeria monocytogenes, Escherichia coli O157:H7, Bacillus cereus, Staphylococcus aureus, C. perfringens, and Shigella flexneri under different environmental conditions. The predictive models can be used by food industry to evaluate the risk of pathogen growth or control to minimize the risk of foodborne illness. Food processing facilities use variable temperatures during processing which in turn affect the microbial growth. Dynamic models that can predict microbial growth under variable temperature conditions are
developed by integrating primary and secondary models. Primary models can be obtained by growth data of pathogens under a constant environment such as temperature over time. Secondary models describe the effect of environmental conditions on the parameters of primary models. Finally, both primary and secondary models can be integrated to develop dynamic models.

**Primary Models**

The microbial growth curve can be divided into four phases viz. lag, logarithmic, stationary and death. The first three phases are of much interest for food microbiologists. Under isothermal conditions, researchers use sigmoid functions to fit the growth curve of microorganisms (Amézquita, 2004). Common primary models are:

(i) **Gompertz Model**

\[ N(t) = C + A^* \exp \left( -B \, t - M \right) \] \hspace{1cm} (1.1)

where, \( C \) represents the value of the lower asymptote with units of \( \log_{10} \) (CFU/g or ml), the parameter \( A^* \) is the asymptotic term (\( \log_{10} \)), the parameter \( M \) corresponds to the time at which the slope of the sigmoidal growth reaches a maximum value and the parameter \( B \) is the maximum growth rate relative to the amount of growth at time (t).

(ii) **Modified Gompertz and Logistic models** (Gibson et al., 1987, 1988)

Modified Gompertz model is given by equation

\[ L(t) = A + (B - A) \times \exp \left[ - \exp(-b(t - m)) \right] \] \hspace{1cm} (1.2)

and the logistic equation (Gibson et al., 1987, 1988)
The modified Gompertz and logistic models contain the same parameters: \( L(t) \) is the log (CFU/mL) of cell concentration; \( A \) and \( B \) are the initial and final cell concentrations; \( t \) the growth time; \( b = \left( \frac{e \times r_{\text{max}}}{B - A} \right) \) (h\(^{-1}\)); and \( m \), the inflection point of the slope (h), and \( r_{\text{max}} \) is the maximum specific growth rate (h\(^{-1}\)).

(iii) Baranyi Model

A model developed by Baranyi et al. (1993, 1994, 1995a, 1995b) is being frequently used for modeling microbial growth in temperature varying conditions. Baranyi et al. (1993, 1994, 1995a, 1995b) have used the term “bottleneck substance” meaning a critical substance \( q(t) \) that the cells needs in order to adjust to their new environment and reach the exponential phase. The development of the critical substance, \( q \), follows first order kinetics and has a constant specific rate of \( v \), which is assumed to be equal to \( \mu_{\text{max}} \) of the bacterial culture. The process of adjustment of the cells to their new environment can be described by

\[
\alpha(t) = \frac{q(t)}{1 + q(t)} \tag{1.4}
\]

This process of adjustment (lag period) is characterized by the gradual increase of \( \alpha(t) \) from a low value towards 1 (Baranyi and Roberts, 1994). Another transformation:

\[
\mathcal{H}(t) = \ln \left( 1 + \frac{1}{q(t)} \right) = -\ln[\alpha(t)] \tag{1.5}
\]

is calculated by the product of the lag period, \( \alpha(0) \), and the maximum specific growth rate. \( \mathcal{H} \) is constant for the different growth curves provided that the physiological state of the cells at inoculation is identical., Generally, the lag period and growth rates are
modeled independently despite the high correlation between them. The models of Baranyi et al. (1993, 1994, 1995a, 1995b) allow these two parameters to be modeled together and the model is made applicable to a time-dependent environment.

Because of the transition from the exponential to the stationary phase, a logistic-type limiting function is applied to the cell concentration (Baranyi et al., 1995):

$$\frac{dx}{dt} = \left(\frac{q}{1+q}\right)\mu_{max}\left(1 - \frac{x}{x_{max}}\right)x$$  \hspace{1cm} (1.6)

with initial values of $x = x_o$ and $t = 0$, where $x$ is the cell concentration, and

$$\frac{dq}{dt} = vq = \mu_{max}q$$  \hspace{1cm} (1.7)

with initial conditions of $q = q_o$ and $t = 0$.

If $y(t)$ is the natural logarithm of the cell concentration and if in a constant environment (isothermal conditions), the solution to the above system is

$$y(t) = y_o + \mu_{max}A(t) - \log_e\left(1 + \frac{e^{\mu_{max}A(t)} - 1}{e^{y_{max} - y_o}}\right)$$  \hspace{1cm} (1.8)

where $y_o = \ln(x_o)$, $y_{max} = \ln(x_{max})$, and

$$A(t) = t + \frac{1}{v}\log_e(e^{-vt} + e^{-h_o} - e^{-(vt-h_o)})$$  \hspace{1cm} (1.9)

Under the assumption that the critical substance grows at the same specific rate as the cells in the exponential phase, $v = \mu_{max}$, resulting in four parameters in Baranyi’s model: $y_o$, $y_{max}$, $\mu_{max}$, and $h_o$. Figure 1.1 shows these parameters and how they are calculated from the growth profiles.

This primary model can be used to predict the growth of microorganisms under isothermal conditions. $y_o$ is a function of time, while $\mu_{max}$ is a function of temperature which is also a function of time.
Figure 1.1. Parameters associated with Baranyi’s growth model. Parameters $y_o$ and $y_{max}$ are the initial and maximum growth, respectively (log$_e$CFU/mL), $\mu_{max}$ is the maximum specific growth rate (h$^{-1}$) and $h_o = \lambda \times \mu_{max}$.

Secondary Models

Secondary models are used to model the impact of environmental conditions, such as temperature, pH, water activity and factors responsible for bacteria growth on the values of the parameters of a primary model (McKellar, 1997). Maximum specific growth rate $\mu_{max}$ and the lag time $\lambda$ are two most important parameters in modeling of bacteria.

(i) Square-Root Models

Ratkowsky et al. (1983) proposed a square root relationship between temperature and maximum specific growth rate.
\[ \sqrt{\mu_{\text{max}}} = b \ (T_{k} - T_{\text{min}}) \] \hspace{1cm} (1.10)

where, \( b \) is a regression coefficient and is equivalent to the slope of the regression line with units of \( \text{K}^{-1} \cdot \text{h}^{-1/2} \), and the parameter \( T_{\text{min}} \) is the theoretical minimum temperature for growth, with units of K. The parameter \( T_{\text{min}} \) is the intercept of the predicted function and the temperature axis.

(ii) Modified Ratkowsky’s equation and Gamma equation

The modified Ratkowsky’s equation (Zwietering et al., 1991),

\[ \mu_{\text{max}} = a(T - T_{\text{min}})^2[1 - \exp(b(T - T_{\text{max}}))] \] \hspace{1cm} (1.11)

in which \( c \) (with units of \( \text{K}^{-1} \)) and \( T_{\text{max}} \) (with units of K), are parameters to be estimated, \( b \) and \( c \) are Ratkowsky parameters.

Gamma equation (Rosso et al., 1993),

\[ \mu_{\text{max}}(T) = \mu_{\text{opt}} \times \tau(T) \] \hspace{1cm} (1.12)

where, \( \tau(T) = \frac{(T - T_{max})(T - T_{\text{min}})^2}{(T_{opt} - T_{\text{min}})(T_{opt} - T_{\text{max}})(T_{opt} + T_{\text{min}} - 2T)} \) \hspace{1cm} (1.13)

each contain four parameters: \( \mu_{\text{opt}} \), optimum maximum specific growth rate (\( \text{h}^{-1} \)); \( T_{\text{opt}} \), optimum temperature (\( ^\circ \text{C} \)); \( T_{\text{min}} \), theoretical minimum temperature (\( ^\circ \text{C} \)); and \( T_{\text{max}} \), theoretical maximum temperature (\( ^\circ \text{C} \)).

Dynamic models
The differential forms of the primary models which allow for prediction under non-isothermal conditions can be solved using numerical methods. A common method to solve first order differential equations in the form

\[ \frac{dy}{dx} = f(x, y), y(0) = y_0 \]  

is the fourth order Runge-Kutta method (Kam, 2006). This algorithm discretizes the time domain as

\[ t_n = t_0 + \Delta t \cdot n, \quad \text{for} \ n = 0, 1, 2, \ldots \]  

and evaluates the function \( f(t, y(t)) \) at the beginning, end, and midpoint of each time interval (Amézquita, 2004). All the Runge-Kutta methods have algorithms of the form

\[ y_{i+1} = y_i + h(x_i, y_i, h) \]  

with \( h \) as the increment function that is a chosen approximation to \( f(x, y) \) on the interval \( x_i \leq x \leq x_{i+1} \) (Carnahan et al., 1969). Letting \( h \) be a weighted average of four derivative evaluations \( k_1, k_2, k_3, \) and \( k_4 \) on the interval \( x_i \leq x \leq x_{i+1} \) results in the Runge-Kutta 4th order algorithm

\[ y_{i+1} = y_i + h(a_1 k_1 + a_2 k_2 + a_3 k_3 + a_4 k_4) \]  

Knowing the value of \( y = y_i \) at \( x_i \), the value of \( y = y_{i+1} \) at \( x_{i+1} \) and \( h = x_{i+1} - x_i \) can be found and Equation (1.14) is equated to the first five terms of Taylor series (Kam, 2006).
Knowing that \( \frac{dy}{dx} = f(x, y) \) and \( x_{i+1} - x = \Delta t \),

\[
y_{i+1} = y_i + f(x_i, y_i) \Delta t + \frac{1}{2} f'(x_i, y_i) \Delta t^2 + \frac{1}{6} f''(x_i, y_i) \Delta t^3 + \frac{1}{24} f'''(x_i, y_i) \Delta t^4. 
\]

(1.19)

and a popular solution to Equations (1.15) and (1.16) is

\[
y_{n+1} = y_n + \frac{1}{6} (k_1 + 2k_2 + 2k_3 + k_4). 
\]

(1.20)

\[
k_1 = \Delta t \cdot f(x_n, y_n). 
\]

(1.21)

\[
k_2 = \Delta t \cdot f\left(t_n + \frac{1}{2} \Delta t, y_n + \frac{1}{2} k_1\right). 
\]

(1.22)

\[
k_3 = \Delta t \cdot f\left(t_n + \frac{1}{2} \Delta t, y_n + \frac{1}{2} k_2\right). 
\]

(1.23)

\[
k_4 = \Delta t \cdot f(t_n + \Delta t, y_n + k_3). 
\]

(1.24)

(Amézquita, 2004 and Kam, 2006).

The differential equations of Baranyi, Gompertz, and Logistic models can be solved using the Runge-Kutta 4\(^{th}\) order algorithm. These differential equations are integrated with a specific secondary model and then can be used to predict growth over time for non-isothermal profiles.

*Salmonella* spp. growth in egg
Egg shells can often be contaminated with *Salmonella* spp. and other bacteria. The surface contamination can translocate to the interior of eggs. Grijspeerdt (2001) described a model for the penetration of surface contamination to the interior of eggs. The movement of motile bacteria inside egg was found to be based on diffusion and a first-order linear movement. Other study involved the effect of immunization of hens on the growth of SE in yolk (Gurtler and Fehlhaber, 2004) where hens were immunized with SE. The eggs were collected and the SE populations were determined. Schoeni et al. (1995) reported that regardless of the initial inoculum size, $10^2$ or $10^4$ CFU/g in yolk or albumen, populations of all *Salmonella* serovars increased by $\geq 3$ logs in 24 h at 25°C. Gast and Holt (2000b) reported that rapid growth of SE at 25°C when the *Salmonella* spp. reached closer to the yolk with storage (2 or 3 days). Lower inoculum size, shorter storage resulted in a slower growth of SE. The growth of *Salmonella* spp. or SE in albumen was either slower or resulted in reduced cell population (Schoeni et al., 1995; Gast and Holt, 2000b; Messens et al., 2004; Grijspeerdt et al., 2005). Whiting and Buchanan (1997) reported that pasteurization provided sufficient consumer protection from SE contamination of eggs. However, certain cases of inadequate pasteurization and/or temperature abuse during storage can lead to a hazardous, contaminated product. Gumudavelli et al. (2007) developed a dynamic model for the growth of SE in egg yolk using Barayni model and modified Ratkowsky’s equation for fitting primary and secondary models, respectively. The current USDA-FSIS guidelines for pasteurization of liquid whole egg product (3.5 min at 60°C) results in an estimated 5.9 log CFU/g reduction in *Salmonella* spp. (USDA-FSIS, 2006). The cumulative prevalence rates (from
1995 through 2008) per 100 g of whole eggs or yolks (with < 2% of added ingredients other than salt or sugar), whole eggs with added yolks or whole egg blends (with >2% salt or sugar added) were 0.28, 0.27, and 0.62%, respectively (USDA-FSIS, 2009). The prevalence rates for the same products in 2008 were 0.45, 0.00, and 0.29%, respectively. These data indicate that either the product is recontaminated subsequent to pasteurization or the levels of *Salmonella* spp. prior to pasteurization were higher than the performance standard required for pasteurization. Regardless, the prevalence of *Salmonella* spp. subsequent to pasteurization requires the processors to refrigerate the product subsequent to pasteurization to minimize its growth. While the egg product processors can set critical limits for the temperature of storage of these products, it is possible that process deviations can occur. In such cases, the processors need a tool to evaluate the potential risk of *Salmonella* spp. growth and the subsequent risk of foodborne illness. The developed dynamic model for *Salmonella* spp. growth in liquid whole egg will be useful for the processors to set critical limits within their HACCP plans or to evaluate the potential risk of *Salmonella* spp. growth during process deviations. The objective of the current study was the development of dynamic model that can predict the growth of *Salmonella* spp. under varying temperature conditions and hence can be used to mitigate *Salmonella* spp. risk from egg and egg products.

**REFERENCES**


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CHAPTER II

Dynamic Predictive Model for the Growth of *Salmonella* spp. in Liquid Whole Egg
ABSTRACT

A dynamic model for the growth of *Salmonella* spp. in liquid whole egg under continuously varying temperature was developed and validated. Liquid whole egg was inoculated with ca. 2.5-3.0 log CFU/ml of *Salmonella* spp. to obtain the growth data at several isothermal conditions (5, 7, 10, 15, 20, 25, 30, 35, 37, 39, 41, 43, 45 and 47°C). A primary model (Baranyi model) was fitted for each temperature growth data and corresponding maximum growth rates were estimated. Pseudo-$R^2$ values were greater than 0.97 for primary models. The maximum growth rates obtained from each primary model were then plotted against temperature and modeled using the modified Ratkowsky
model. The pseudo-$R^2$ and root mean square error was 0.99 and 0.06 log CFU/g, respectively for the secondary model. A dynamic model for the prediction of *Salmonella* spp. growth under varying temperature conditions was developed by solving a combination of primary and secondary models using fourth-order Runge-Kutta method. The developed dynamic model was validated for two sinusoidal temperature profiles, 5-15°C and 10-40°C with corresponding RMSE values of 0.28 and 0.23 h$^{-1}$, respectively between predicted and observed values. The developed dynamic model can be used to predict the growth of *Salmonella* spp. in liquid whole egg under varying temperature conditions.

**Keywords:** predictive model, *Salmonella*, whole egg, dynamic

**INTRODUCTION**

Egg and egg products are used extensively as ingredients in food products and approximately 45% of the eggs are further processed (United Egg Producers, 2009). An estimated 209 million cases of eggs are produced in the U.S. with a per capita consumption of 249 eggs (United Egg Producers, 2009). The eggs are collected and stored for transportation, subsequently increasing the time between collection and processing. In most cases, shell eggs are transferred from the laying house to the egg packaging plant by conveyers or manually. The eggs are subsequently cleaned and packed in cartons and palletized. The pallets are placed in refrigerated rooms set at 7°C (Keener et al., 2000). The USDA-FSIS proposed 7.2°C as a mandatory temperature for transportation and storage of eggs in the retail market. Sometimes, it may take up to 7 to
10 days for the eggs to reach 7°C from an initial temperature of 25 to 30°C (Anderson et al., 1992). In the United States, most eggs are sold after approx. 19 days of laying with an average sell by date of approx. 30 days (Bell et al., 2001; Patterson et al., 2001). Thus, the eggs undergo continuous temperature fluctuations, from the time they are laid to the time of processing. These temperature fluctuations can result in penetration and growth of *Salmonella* spp. from the shell surface or SE growth within the egg if the egg contents are contaminated.

Mathematical models describe the behavior of microorganisms under the influence of various environmental factors. Predictive models are economical and convenient for evaluating microbial growth compared to traditional laboratory microbiological testing. The USDA-FSIS risk assessment of *Salmonella* spp. in shell eggs and egg products stated that available data for estimating growth parameters and complete growth data of *Salmonella* spp. suitable for predictive models are lacking (FSIS, 2005). The objective of this research was to develop and validate a predictive model of *Salmonella* spp. growth in liquid whole egg under varying temperature conditions.

**MATERIALS AND METHODS**

**Preparation of Liquid Whole egg**

Fresh shell eggs (< 1 day after lay) were collected from the University of Nebraska, Lincoln poultry farm. Selected eggs free from blood spots were washed with warm water to remove external visible contamination. The eggs were sanitized by dipping in ethanol (70%) for 15 min and allowed to dry. Shell eggs were broken in a sterile bio safety
cabinet (Bellco Glass Inc., Vineland, NJ) with an alcohol sanitized knife. Egg albumen and yolk were separated aseptically and collected in sterile containers. Yolk and albumen were blended separately using a Waring blender (Blender; Waring Commercial Laboratory Blender, New Hartford, CT) attached to a rheostat (Superior Electric Co., Bristol, CT) set at 20-35 rpm and 120 v.

**Total Solids and pH adjustment**

Total solids of the yolk and albumen were estimated following AOAC methods (AOAC, 1970). The required quantities of yolk and albumen were mixed to obtain liquid whole egg of 24% total solids. The pH of the liquid whole egg was measured using pH meter (Model Accumet Basic/AB15, Fisher Scientific, Pittsburgh, PA) and adjusted to 7.8 using 1N NaOH or 1N HCl.

**Bacterial Cultures**

Five serovars (Enteritidis phage types 4 and 13, Typhimurium TM-1, Blockley, and Heidelberg) of *Salmonella* were used. The stock culture of each serovar was thawed under refrigeration and three subsequent transfers were made in 10 ml tryptic soy broth (TSB; BD Bacto, Sparks, MD) and incubated for 16-18 h at 35°C. A 2 ml aliquot of each serovar was transferred aseptically to a sterile centrifuge tube and centrifuged at 6,000 x g for 10 min at 4°C (Beckman GS-15R, Fullerton, CA). The supernatant was discarded and the bacterial cell pellet was reconstituted in 10 ml of 0.1% sterile peptone water (PW; BD Difco, Sparks, MD) and serially diluted (1:10) in PW to obtain appropriate concentrations for inoculation.
Sample Inoculation

Appropriate volume of the five serovar cocktail of *Salmonella* spp. was added to liquid whole egg to obtain ca. 2.5-3 log CFU/ml of *Salmonella* spp and blended for 1 min. Portions (5ml) of the inoculated liquid whole egg were transferred to vacuum pouches (6.35 cm x 12.7 cm; Prime Source, Kansas City, MO; 3 mil standard barrier nylon pouch, oxygen transmission rate of 3,000 cm$^3$/m$^2$/24 h at 23°C and 1 atm) and were heat sealed.

Isothermal and Dynamic Profiles

*Salmonella* growth data was collected at isothermal temperatures of 5, 7, 10, 15, 20, 25, 30, 35, 37, 39, 41, 43, 45 and 47°C to cover the entire bio-kinetic growth temperatures and also for the two sinusoidal heating-cooling profiles (5-15°C and 10-40°C). For both isothermal and sinusoidal profiles, *Salmonella* spp. population was determined until stationary phase was reached. Programmable water baths with water circulation capabilities (RTE 740, Thermo Neslab, Portsmouth, NH) were used to follow the temperature profiles. A commercial software package (NesCom Software, Portsmouth, NH) was used to program the temperature profiles in the water baths. The pouches containing inoculated product were immersed in water baths maintained at constant temperatures for isothermal profiles and in programmable water baths for sinusoidal profiles mentioned above. A pouch was removed after 10 min equilibration time and the initial *Salmonella* spp. was determined. Remaining pouches were removed at specific intervals until 2 to 3 points in stationary phase of *Salmonella* were attained at each profile.
**Microbial Enumeration**

The pouches were removed from the water bath at predetermined intervals and massaged for 2 min to uniformly mix the contents of pouch. Surfaces of the pouches were sprayed with alcohol, wiped with paper towel and aseptically opened. Appropriate dilutions of the sample were prepared in PW and either spread or spiral plated on xylose lysine deoxycholate (XLD) agar (Difco) for all profiles. Both XLD and tryptic soy agar (TSA) were used for enumeration of *Salmonella* spp. for temperature profiles above 35°C to evaluate microbial injury. XLD and TSA plates were incubated at 35°C for 24 and 48 h, respectively. Typical colonies were counted and microbial populations expressed as log CFU/ml.

**Modeling**

**Primary Model**

Dynamic model (Baranyi and Roberts, 1994) includes a set of differential equations (Eq. 2.1a and 2.1b) for predicting the growth of microorganisms under time-varying temperature conditions.

\[
\frac{dy}{dt} = \frac{1}{1 + e^{-Q}} \mu_{\text{max}} \left( 1 - e^{y - y_{\text{max}}} \right) \]

(2.1a)

\[
\frac{dQ}{dt} = \mu_{\text{max}} \]

(2.1b)

with initial conditions \( y = y_o \) at \( t = 0 \) and \( Q = \log_e(q_o) \) where \( q \) is a measure of the initial physiological state of cells.
The four-parameter Baranyi model obtained by analytically solving above two equations was used for bacterial growth at isothermal conditions (Baranyi and Roberts, 1994):

\[
y(t) = y_0 + \mu_{\text{max}} F(t) \log_e \left( 1 + \frac{e^\mu_{\text{max}} F(t)}{e^{y_{\text{max}} - y_0}} - 1 \right)
\]

where

\[
F(t) = t + \frac{1}{\nu} \log_e \left( e^{-\nu t} + e^{-h_0} - e^{-\nu t - h_0} \right)
\]

\[y(t)\] is \(\log_e\)CFU/g of cell concentration at time, \(t\)

\[y_0\] is initial cell concentration in \(\log_e\)CFU/ml; \(y_{\text{max}}\) is maximum cell concentration in \(\log_e\)CFU/ml

\(\mu_{\text{max}}\) is maximum specific growth rate in terms of \(\log_e\)CFU/ml in 1/h

\(\nu\) is rate of increase of the limiting substrate, assumed to be equal to \(\mu_{\text{max}}\)

\(h_0\) is equal to \(\mu_{\text{max}} \times \lambda\); \(\lambda\) is lag-phase duration in h.

Analysis of results and fitting of the model to growth data were done in \(\log_e\)CFU/ml units and the results were reported in \(\log_{10}\)CFU/ml.

Growth data (selective medium) obtained from each isothermal profile was plotted against time and the starting values of parameters (\(y_0\), \(h_0\), \(\mu_{\text{max}}\) and \(y_{\text{max}}\)) were estimated. The value of the lower asymptote and upper asymptote were given as starting values for \(y_0\) and \(y_{\text{max}}\), respectively. Slope of exponential phase of bacterial count with respect to time was considered as starting value of the maximum growth rate (\(\mu_{\text{max}}\)). The starting value for \(h_0\) is calculated by multiplying the maximum growth rate (\(\mu_{\text{max}}\)) by lag time (\(\lambda\)). The lag time is the time difference between starting up of profile and exponential growth.
A nonlinear regression procedure, PROC NLIN, in SAS package (SAS, 2006) was used to fit the data to the models with the Marquardt iterative method for estimation of parameters (SAS programs are given in Appendix A.1 and A.2). The given starting values were converged to estimate values the parameters (before fixing) after several iterations using the nonlinear procedure. Baranyi and Roberts (1994) suggested that in situations where the pre-inoculation history of the cells is identical, the value of \( h_0 \) is approximately constant. The \( h_0 \) value was fixed at the average value of \( h_0 \) estimated for all the temperatures and the observed data were again fitted at fixed \( h_0 \) using the model to estimate the other three parameters (after fixing parameters).

In nonlinear regression, \( R^2 \) value should not be used for deriving conclusions like linear regression. Therefore, pseudo-\( R^2 \) (Eq. 2.3) defined by Schabenberger (2005) was used to evaluate the goodness of fit.

\[
Pseudo - R^2 = 1 - \frac{SSR}{TSS} \]

(2.3)

where SSR is sum of squares of residuals and TSS is total sum of squares.

Root Mean Squared Error (RMSE) (Eq. 2.4) was also used to evaluate the model performance.

\[
RMSE = \sqrt{\frac{(O - P)^2}{N - p}} \]

(2.4)

where \( O \) and \( P \) are observed and predicted microbial populations in \( \log_{10} \) CFU/g, \( N \) is the number of observation, and \( p \) is the number of model parameters. The above two
statistics were calculated for each temperature after fitting the observed growth data into the Baranyi model.

**Secondary Model**

The modified Ratkowsky equation was used as secondary model equation in present study to describe the effect of temperature on growth rate, as described by (Zwietering et al., 1991):

\[ \mu_{\text{max}} = a(T - T_{min})^2 \left(1 - \exp(b(T - T_{\text{max}}))\right) \]

\[ T_{min} \text{ - theoretical minimum temperatures beyond which growth of the organism is not possible. Starting value was given 7°C.} \]

\[ T_{\text{max}} \text{ - theoretical maximum temperatures beyond which growth of the organism is not possible. Starting value was given 45°C.} \]

\[ a \text{ and } b \text{ are regression coefficients.} \]

The starting value of parameter “a” was determined by calculating the slope when \( \sqrt{\mu_{\text{max}}} \) values were plotted against \( T \) until the optimum growth rate occurred (Eq. 2.5).

\[ \sqrt{\mu_{\text{max}}} = a(T - T_{\text{min}}) \]

Starting value for \( b \) regression coefficient was taken as 1.

The first term in Equation (2.5) describes the growth rate up to the optimum growth while the second term represents the decrease in growth rate, as the temperature goes beyond the optimum growth temperature. The maximum specific growth rate obtained from isothermal profiles at various temperatures was then fitted into the modified Ratkowsky four parameter equations (Eq.2.5) PROC NLIN procedure was used to estimate the
parameters of secondary model (SAS program is given in appendix A3.). RMSE and pseudo-$R^2$ were used to evaluate the performance of the secondary models.

**Dynamic model**

Computer simulation algorithm was developed for predicting *Salmonella* spp. growth under dynamic temperature conditions. The secondary model (Eq. 2.5) was incorporated into the differential form of the Baranyi model (Eq. 2.1a, 2.1b) and then solved numerically using the fourth-order Runge-Kutta method by using the MATLAB 7.0 package to predict the microbial population at each time step of 5 min (MATLAB program is given in Appendix 4) for 5-15 and 10-40°C sinusoidal profiles. Root Mean Square Error (RMSE) (Eq. 2.4) was used to compare the observed and predicted values (Jeyamkondan et al., 2001).

**RESULTS AND DISCUSSION**

The types and numbers of the foodborne pathogens present in foods determine the human health risk (Rose et al., 1995; Holcomb et al., 1999). Microbial growth in food can be characterized by the parameters lag time, growth rate, and death rate at any specific point of time. These parameters are affected by the intrinsic factors associated with the food product (e.g., pH and water activity), and the extrinsic factors associated with food processing, storage and handling (e.g., processing method, temperature, preservatives, etc.). Mathematical models that combine microbial growth parameters with environmental conditions (Gompertz, 1825; Baranyi et al., 1992) have been important tools for predicting microbial growth in contaminated foods.
Selective media (XLD) was used to determine *Salmonella* spp. populations for all temperatures and of temperatures >35°C, non-selective medium (TSA) was also used to estimate injured cells (Fig. 2.6). *Salmonella* spp. populations differed by < 0.5 log CFU/ml between selective and non-selective media at 37, 39 and 41°C, indicating minimal injury. Difference in growth rate of *Salmonella* spp. in liquid whole egg and egg yolk (Gumudavelli et al., 2007) can be found in Fig. 2.7 at different temperatures. It is evident that *Salmonella* grew faster in egg yolk than liquid whole egg which concluded that same models can not be used for different food matrix. Growth rate of *Salmonella* spp. in both yolk and liquid whole egg differed maximum at temperatures >35°C. At lower temperature 10 and 15°C the difference in growth rate of *Salmonella* spp. in egg yolk and liquid whole egg was lesser than the higher temperatures.

**Primary Model**

Primary models were fitted for *Salmonella* spp. growth data in liquid whole egg obtained at isothermal conditions of 10, 15, 20, 25, 30, 35, 37, 39, 41, and 43°C (Fig 2.2). No growth of *Salmonella* spp. was observed at < 8°C as well as ≤ 45°C in liquid whole egg (Fig. 2.1). Gurtler and Conner (2009) reported no growth of SE in whole shell egg at 7°C for 8 days, which is in agreement with our results. RMSE and pseudo-$R^2$ values calculated for fitting Baryani model for each isothermal profile are shown in (Fig. 2.3).

The $h_0$ values were estimated for growth data of *Salmonella* spp. at all isothermal temperature profiles individually and an average value of $h_0$ (3.51) was fixed and used for fitting the Baryani model for all temperatures profiles to estimate rest of the three
parameters, $y_0$, $y_{max}$, and $\mu_{max}$. Juneja et al. (2007) and Gumudavelli et al. (2007) reported $h_0$ values as 1.75 (Salmonella spp.) and 1.34 (SE) for Salmonella growth in fresh chicken and egg yolk, respectively. The differences in $h_0$ values could be due to differences in the substrate used in the studies. $h_0$ represents the physiological conditions of cells before they were used up for the experiment. This indirectly impacts the lag phase and growth rate of bacterial cells. We got higher $h_0$ value in comparison to Gumudavelli et al. (2007) which clearly demonstrate the difference in growth rate as well as lag phase observation of Salmonella cells in different medium. It can be assumed that Salmonella cells experienced a longer lag phase and slower growth rate in whole egg than egg yolk. Difference in growth rate can be observed from secondary model fitting (Fig. 2.7) as Gumudavelli et al. (2007) used egg yolk to observe the growth parameters of Salmonella spp. cells. Amézquita et al. (2005) and Gumudavelli et al. (2007) used a similar approach for developing growth model of Clostridium perfringens and SE in cooked boneless ham and egg yolk, respectively. Juneja et al. (2007) stated that Baranyi model resulted in better estimated growth rate than the modified Gompertz model or the logistic model in their study of Salmonella growth in chicken for best fit of secondary model.

**Secondary model**

Microbial growth curve includes a lag phase followed by an exponential phase, followed by a decreasing growth rate (down to zero), resulting in a maximum number of organisms which is stationary phase. A growth model with three parameters can describe bacterial growth curve (Zwietering et al., 1990). Ratkowski et al. (1982) described a square root
model given below which was based on the observations that the square root of specific growth rate is linear with temperature at lower temperatures.

\[ \mu_{\text{max}} = [b(T - T_{\text{min}})]^2 \] .................................(2.7)

Later, Ratkowsky et al. (1983) expanded the previous model to describe the growth rate around the optimum and the maximum temperatures. The expanded square root model was

\[ \mu_{\text{max}} = (b(T - T_{\text{min}}) - \{1 - \exp[c(T - T_{\text{max}})]\})^2 \] .................................(2.8)

A disadvantage of the expanded form is that it predicts positive values of growth rate even for temperatures above \( T_{\text{max}} \). So, the model does not hold well for temperatures above \( T_{\text{max}} \). Hence, Zwietering et al. (1991) modified the Ratkowsky model and is expressed as:

\[ \mu_{\text{max}} = [b(T - T_{\text{min}})]^2 \cdot \{1 - \exp[c(T - T_{\text{max}})]\} \] .................................(2.9)

Zwietering et al. (1991) stated that the modified form of Ratkowsky model was most suitable for describing the growth rate and the asymptote as a function of temperature.

The secondary model (Eq. 2.9) fitted to the estimate growth rate of *Salmonella* spp. in liquid whole egg is shown in Fig. 2.4. The theoretical minimum (\( T_{\text{min}} \)) and maximum (\( T_{\text{max}} \)) temperatures for *Salmonella* spp. growth in liquid whole egg were estimated as 6.10 and 45.19°C, respectively although *Salmonella* spp. growth was not observed from experimental data at 7 and 45°C. The \( T_{\text{min}} \) and \( T_{\text{max}} \) are model parameters which are usually 5-10°C below or above the observed minimum and maximum temperatures of
growth, respectively (Ross and Dalgaard, 2004). The RMSE and Pseudo-$R^2$ values were 0.06 h$^{-1}$ and 0.99, respectively for the secondary model fitting. Modified Ratkowsky model was used by Juneja et al. (2007) for secondary modeling of \textit{Salmonella} growth rate in chicken. Gumudavelli et al. (2007) also used same model and stated its robustness for constructing dynamic model.

**Dynamic model**

Validation of the dynamic model is essential to evaluate the construction and robustness of model (Oscar, 2005). Two sinusoidal temperature profiles were selected for validation of \textit{Salmonella} spp. growth model in liquid whole egg. Parameters obtained from primary model and secondary model fit were used as inputs for the dynamic model to predict the growth. Parameters $y_o$ (2.5), $y_{\text{max}}$ (8.7) and $h_\theta$ (3.51) values from primary model, $T_{\text{min}}$ (6.10), $T_{\text{max}}$ (45.19), $b$ (0.0016) and $c$ (0.2014) parameter values from secondary model were used. Baranyi et al. (1995) developed a dynamic model for predicting the growth of \textit{B. thermosphacta} in broth solutions which predicted well for temperature varying conditions. Amezquita et al. (2005) integrated the dynamic model with heat transfer model for predicting the growth of \textit{C. perfringens} in cooked, boneless ham and predicted \textit{C. perfringens} growth curves showed good agreement with validated results. Huang (2003) developed simulation algorithm to dynamically estimate and predict the growth of \textit{C. perfringens} in cooked ground beef and the results of computer simulation matched closely with the experimental data with the absolute errors of $\leq 0.5 \log_{10}$ CFU/g.
Validation

The observed and predicted values for *Salmonella* spp. growth in liquid whole egg under varying temperatures is shown in Fig. 2.5. The RMSE values for the 5-15°C and 10-40°C sinusoidal profiles were 0.28 and 0.23 log CFU/ml, respectively. Gumudavelli et al. (2007) also reported RMSE values ≤ 0.29 log CFU/ml for validation profiles. The developed dynamic model predicted growth of *Salmonella* spp. well in liquid whole egg.

Gurtler and Conner (2009) reported an increase of 0.4 and 4.5 log CFU/ml in SE population at 10°C for 4 days and 23°C for 2 days, respectively in liquid whole egg. These increases in *Salmonella* population are low in comparison to this study. The differences may be due to the difference in total solids and/or pH of liquid whole egg used in the two studies. Kim et al. (1989) concluded that pH of egg increases during egg storage, results in thinning of the albumin which may allow SE to be effective in reaching the yolk for accessing the nutrients required for growth and reproduction. Gurtler and Conner (2009) observed an increase of 1.5 log CFU/ml in *Salmonella* spp. counts at 37°C for 24 h which is lesser than the increase (6.5 log CFU/ml) observed in present study. These differences may be due to different *Salmonella* spp. serotypes used in the studies. Gast and Holt (1995) reported differences in SE growth between (12 strains of SE individually inoculated into separate samples of whole egg at a population of 5 CFU/ml, increased to levels of between 3.9 and 7.5 log CFU/ml within 24 h at 37°C, demonstrating the disparity in growth characteristics between serotypes of *Salmonella*. Latimer et al. (2002) reported no increase in SE populations for 720 h days at 10°C while the stationary phase was achieved with in 480 h at 10°C in our study. This difference can
be due to the use of whole intact shell eggs, while liquid whole egg was used in current study. Mixing the yolk and egg white probably lowered the pH of liquid whole egg close to pH 7.0 and an increase in the nutrient value of the product.

Baryani model fitted well for all the isothermal profiles of *Salmonella* spp. growth in liquid whole egg. The maximum growth rate obtained from each primary model was then fitted as a function of temperature using the modified Ratkowsky model. The RMSE (0.06 h⁻¹) and psuedo-$R^2$ (0.99) values suggest that the secondary model performed well for observed data. Combination of primary and secondary model resulted in a dynamic model whose predictions were also validated for two sinusoidal profiles. The dynamic model also performed satisfactorily and therefore can be used for predicting growth of *Salmonella* spp. under isothermal as well as temperature varying conditions.
REFERENCES


![Figure 2.1. *Salmonella* spp. growth in liquid whole egg at 47°C](image)
Figure 2.2. *Salmonella* spp. growth data on XLD from different isothermal profiles fitted to the Baranyi model (▲ Rep.1, ■ Rep.2, – predicted).
Figure 2.3. RMSE and Pseudo-$R^2$ values at each temperature obtained after growth data fitted to Baranyi model
Figure 2.4. Secondary model describing the growth rate of *Salmonella* spp. in liquid whole egg at different temperatures
Figure 2.5. Validation of the predictive model at different time-varying temperature profiles

\[ \text{Figure 2.6. } \textit{Salmonella} \text{ spp. growth data on TSA and XLD at higher temperatures} \]
Figure 2.7. Comparison of growth rate values of *Salmonella* spp. in different medium
The one-letter amino acid codes used in text.

A     Alanine
C     Cysteine
D     Aspartic acid
E     Glutamic acid
F     Phenylalanine
G     Glycine
H     Histidine
I     Isoleucine
K     Lysine
L     Leucine
M     Methionine
N     Asparagine
P     Proline
Q     Glutamine
R     Arginine
S     Serine
T     Threonine
V     Valine
W     Tryptophan
Y     Tyrosine
INTRODUCTION

Egg and egg products have a variety of uses as an ingredient in foods like mayonnaise, ice cream, noodles, pet food, etc. Egg white is mainly used for its functional properties. In addition to its use for the functional properties, egg whites are being used for preparation of hydrolysates for their anti-hypertensive, anti-cancer and anti-adhesive properties. The enzymatic hydrolysis products of egg white are being used in the health care industry for their angiotensin I- converting enzyme (ACE) inhibitory activity. The hydrolysates of egg yolks, egg white, milk, soy sauce, fish and tuna have been found to reduce the blood pressure in spontaneously hypertensive rats (SHRs) and hypertensive human objects (Miguel et al., 2004). Peptides from egg white and yolk have also been shown to have antibacterial activity against certain microorganisms and their antioxidant potential (Davalos et al., 2004). Therefore, egg peptides may open up profitable new product opportunities for the egg industry. While the egg white hydrolysates can provide various health benefits, their manufacturing processes have not been evaluated for microbiological safety.

Traditional egg white hydrolysate manufacturing processes involve hydrolysis of egg white with pepsin, trypsin, chymotrypsin and similar enzymes to yield peptides of appropriate properties (Miguel et al., 2004). This involves co-incubation of the liquid egg white with a particular enzyme or a sequence of enzymes at their temperature and pH optima for sufficient time to hydrolyze the proteins. While some enzymes have pH optima (pepsin, 2.0) which would prevent the growth of pathogens, other enzymes may
have both temperature and pH optima that would allow the growth and potentially produce heat stable toxin. Spore forming foodborne pathogens *C. perfringens* and *B. cereus* that can survive traditional heating processes are of concern. The raw material (egg white) solutions are often pasteurized to destroy indigenous flora. These heat treatment can heat activate which further results in germination and outgrowth of *C. perfringens* and *B. cereus* which can further outgrow and produce toxins.

The objective of this study is to evaluate the potential germination and outgrowth of *C. perfringens* and *B. cereus* spores during the manufacture of egg white hydrolysates using chymotrypsin and trypsin.
LITERATURE REVIEW

Bacterial Spores

Basic Information

Bacterial spores are highly specialized, differentiated cell types which can survive adverse environmental conditions. Spores are formed by a number of bacteria to survive the unfavorable conditions for growth, but bacterial endospores (spores) are the longest surviving organism, being the toughest among all these cell types (Errington, 2003). Spores of spore forming organisms are ubiquitous and present challenges to the food industry as they can survive several of the food processing unit operations. Spore formation involves a unique process of cell division, engulfing the smaller cell, leading to the sacrifice of original bacterial cell for producing a single spore. Spores have a dormant metabolism (Lewis, 1969) and are highly resistant to adverse conditions, such as starvation, high temperatures, ionizing radiation, chemical solvents, detergents, hydrolytic enzymes, desiccation, pH extremes and antibiotics (Setlow, 2000). Because of their metabolic dormancy, bacterial spores do not require any nutrients or energy source to survive (Keynan, 1972). This dormancy and their resistance are the main reasons of survival for extreme periods of time. Numerous reports have documented the isolation of viable spores from environmental samples such as dried soil in herbarium collections, paleosols, ancient lake sediments, permafrost soils, ice cores and old cans of meat. These samples ranged in age from several decades to thousands of years (Sussman and
Spore Structure

The structure of the spore contributes to its unique properties of spores (Tipper and Gauthier, 1972; Warth, 1978). The spores consist of a core, surrounded by the inner membrane, cortex, outer membrane, coat and in some species, the exosporium. Core, the innermost part of spore contains the spore cytoplasm which includes cytoplasmic proteins, ribosomes and DNA. The water contents of core cytoplasm and vegetative cell cytoplasm is 30-50% and 70-88%, respectively (Potts, 1994; Setlow, 1994; 2000). This dehydrated state plays an important role in spore longevity, dormancy and resistance (Setlow, 2000). The pH of the cytoplasm is less in spore coat when compared to vegetative cell, with a value 6.3-6.5 (Setlow and Setlow, 1980). The core contains large quantities of small acid soluble proteins (SASP; Setlow, 1988). These proteins form a complex with DNA and force DNA into a special compressed condition (Frenkiel-Krispin et al. 2004; Douki et al. 2005) and protecting its DNA from damage (reviewed in Setlow, 1988; 1995). Core cytoplasm contains large amounts of divalent cations (Ca$^{2+}$) which forms a complex with the spore-specific compounds Pyridine-2,6-dicarboxylic acid (dipicolinic acid or DPA; Powell, 1953). During germination, DPA and calcium are excreted from the core and play important roles in several steps of the germination process (Paidhungat et al., 2001; de Vries, 2004). Most of the lipids in inner membrane surrounding the spore coat of dormant spores are immobile, while in germinated spores and vegetative cells, the membrane is fluid and the membrane lipids are highly mobile.
The inner membrane was thought to be the main permeability barrier of spores (Setlow, 1994; 2000), and is an important site for spore germination receptors (Hudson et al., 2001; Paidhungat et al., 2001).

Cortex built around the inner membrane is a thick cell wall composed of modified peptidoglycan (Warth and Strominger, 1972), is electro-negatively charged (Gould and Dring, 1975) and loosely cross-linked (Popham and Setlow, 1993). During germination, lytic enzymes in the dormant spore degrade the cortex peptidoglycan. The spore coat is an intricate protein structure generally consisting of three distinct layers built around the outer membrane (Henriques et al., 2004). The coat protects the cortex peptidoglycan from enzymatic attack (Driks, 1999; 2002; Setlow, 2000). The inner layer of the coat contains a lytic enzyme, which helps in degradation of cortex during germination (Paidhungat et al., 2001; Bagyan and Setlow, 2002; Ragkousi et al., 2003). In many species, the spore coat is surrounded by a membrane-like structure called exosporium and contributes to the spore hydrophobicity and adherence properties (Koshikawa et al., 1989; Faille et al., 2002).

Fig 3.1 Internal structure of a bacterial spore (Foster and Johnstone, 1990)
**Spore Formation**

Sporulation is initiated by phosphorylation of the master transcription regulator, *Spo0A*. Together with $\sigma H$, phosphorylated *Spo0A* triggers the asymmetric sporulation division, septation. Septation produces two distinct cells with very different fates, the smaller pre-spore (also known as fore-spore), which develops into the spore, and the larger mother cell necessary for spore formation but lyses finally.

The changes in gene regulation are coupled to morphogenesis and to each other by inter-compartmental signaling, eventually leading to the development of the resistance properties that characterize bacterial spores. Subsequent to maturation of the spore of the mother cell programmed cell death occurs and the mature spore is released into the environment.

![Sporulation cycle of Bacillus subtilis](Errington, 2003)

*Fig. 3.2 Sporulation cycle of Bacillus subtilis* (Errington, 2003)
Spore Germination

Spore germination involves a series of degradative reactions involving destruction of compact spore structure and ultimately leads to loss of spore dormancy and resistance. The subsequent steps that lead to cell-division and increase in number through cell division is termed as outgrowth, is considered to be a separate process, distinct from germination (Campbell and Leon, 1958). Activation step can enhance germination by several treatments like heat-treatments, time, and certain chemicals (Keynan and Evenchick, 1969; Foster and Johnstone, 1990). Spores comprise of sensors which trigger the germination when exposed to favorable condition. Sensors located in inner membrane, nutrient receptors or germinant receptors (Hudson et al., 2001; Paidhungat et al., 2001). Specific molecules or agents (germinants) recognize a signal of conditions suitable for growth. Some of the germinants are amino acids (especially L-alanine), sugars and ribosides (Hornstra et al. 2005). Igarashi and Setlow (2005) demonstrated interaction between sub-domains from different receptors, supporting the hypothesis of interaction between different receptors and formation of complexes (McCann et al. 1996). Binding of germinant to its receptor triggers transmittance of unidentified signals resulting in fast, irreversible changes which ultimately lead to a fully germinated spore.

Bacillus cereus

*B. cereus* belongs to the *Bacillus cereus* group which also includes *B. weihenstephanensis, B. mycoides, B. pseudomycoides, B. thuringiensis, and B. anthracis*
(Jensen et. al., 2003). *B. cereus* can cause systemic and local infections, and severe ocular infections (Kotiranta et al., 2000; Callegan et al., 2003; Chan et al., 2003). However, *B. cereus* is known for its ability to cause food-poisoning, and is an important spoilage organism in pasteurized dairy products as well. The cells are rod-shaped, straight, typically 0.5-2.5 μm in diameter and 1.2-10 μm in length and are mostly arranged in pairs or chains (Holt et al., 1994). The cells stain Gram-positive, although positive staining is often difficult to obtain in older cultures (Varnam and Evans, 1991). *B. cereus* cells are motile by peritrichous flagella (Varnam and Evans, 1991) and the endospores are centrally or pericentrally positioned. *B. cereus* is a mesophilic, facultatively anaerobic bacterium (Griffiths and Schraft, 2002) and is able to grow at redox-potential below -200 mV (Varnam and Evans, 1991). *B. cereus* has an essential requirement for three L- amino acids: threonine, leucine, valine (Agata et al., 1999) for growth. The temperature range of growth is 4-55°C (optimum 30-40 °C) (Roberts et al., 1996). Psychrotrophic strains are common and growth may occur at temperatures of 4-5 °C. The minimum water activity (a_w) for growth is 0.93 and the pH range is 4.3 - 9.3 (Forsythe, 2000).

*B. cereus* spores are ubiquitous, and have been isolated from a wide variety of environments (Kramer and Gilbert, 1989; Kotiranta et al., 2000; Granum, 2001). *B. cereus* has long been considered to be a soil inhabitant. However, recent genome analysis revealed that *B. cereus* is specialized in protein metabolism indicating a symbiotic or parasitic niche rather than a saprophytic life-style as a benign soil bacterium (Ivanova et al., 2003). *B. cereus* is capable of causing anthrax-like symptoms due to the presence of a plasmid that is highly similar to one of the characteristic plasmids of *B. anthracis*
(Hoffmaster et al., 2004). Several researches have concluded that the major germinant of
*B. cereus* spores is inosine (Yousten, 1975; Hornstra et al., 2007). Glycine and other
neutral L-amino acids and purine ribosides induce germination (Warren and Gould, 1968;
Griffiths and Schraft, 2002; Hornstra et al., 2006). L-alanine is the most effective amino
acid stimulating germination (Yousten, 1975; Griffiths and Schraft, 2002; Hornstra et al.
2007).

**B. cereus in Foods**

The microbial safety of food has been improved significantly over the last few decades.
*B. cereus* spores can enter the food chain through raw materials due to their ubiquity. It is
a major problem in convenience foods and mass catering (Guinebretière et al., 2006). The
high resistance of the spores allows *B. cereus* to survive most drying and cooking
processes and can grow well in cooked food due to lack of a competing microbiota. It is
practically impossible to completely avoid the presence of *B. cereus* in raw milk samples
because *B. cereus* spores are ubiquitous and are present in the farm environment like soil,
cattle feed and cattle. *B. cereus* spores germinate more efficiently in pasteurized milk
than in raw milk (Wilkinson and Davies, 1974; te Giffel et al., 1995). Hence, germination
prior to pasteurization is reduced, but enhanced after pasteurization, thus increasing not
only the chances for survival of the pasteurization resulting in outgrowth and spoilage
during storage. Langeveld et al. (1996) showed that the probability of disease caused by
consumption of pasteurized milk containing high numbers of *B. cereus* is very low. Thus,
the main issue with *B. cereus* in dairy products has been spoilage rather than poisoning.
*B. cereus* causes two distinct types of food poisoning: the emetic type and the diarrheal
The emetic syndrome is associated with fried or cooked rice and pasta. While going through events leading to outbreaks, it was found that these foods were stored at room temperature after preparation which allowed *B. cereus* to grow rapidly and produce toxin in food. The heat stable, circular peptide antibiotic toxin responsible for emetic symptoms is known as cereulide, (Agata et al. 1995) is produced by non-ribosomal peptide synthetase (Horwood et al. 2004). These potassium ionophore properties exhibited by cereulide can be responsible for the disease symptoms caused by *B. cereus* (Mikkola et al., 1999). The *B. cereus* cereulide is heat stable and cannot be destroyed by normal heating of food. The symptoms include nausea, vomiting, malaise and followed by diarrhea (Kramer and Gilbert, 1989). Generally, the symptoms are relatively mild and disappear within 24 h, however there has been one case of liver failure and death caused by cereulide (Mahler et al., 1997). The diarrheal type of food poisoning is caused by enterotoxins, and is mostly associated with foods rich in proteins. *B. cereus* produces several enterotoxins along with other toxins and virulence factors, such as phospholipases and haemolysins (Granum and Lund, 1997; Schoeni and Wong, 2005). The enterotoxins are not heat-stable, and thought to be formed in the digestive tract after ingestion of food containing *B. cereus* spores which later form vegetative cells (Kramer and Gilbert, 1989; Granum, 2001). Symptoms like abdominal pain, profuse watery diarrhea, rectal tenesmus, and occasionally nausea and vomiting can be seen within 8-16 h after ingestion of contaminated foods (Kramer and Gilbert, 1989). In a recent outbreak, three deaths were reported and a new enterotoxin, CytK was identified (Lund et al. 2000). Majority of *B. cereus* foodborne is
under response due to the mild symptoms (Kramer and Gilbert, 1989). Risk assessment have indicated that *B. cereus* is a hazard of major importance in various foodstuffs, especially cooked chilled foods (Carlin et al., 2000; Nauta et al., 2003). The reported foodborne outbreaks and cases attributed to *B. cereus* in North America, Europe and Japan range from 1% to 22% for outbreaks covering 0.7 - 33% of the cases (Griffiths and Schraft, 2002).

*Clostridium perfringens*

The genus *Clostridium* belongs to Bacillaceae family. *Clostridium perfringens* is a Gram-positive, anaerobic, endospore-forming and rod-shaped bacteria (McClane, 2001). *C. perfringens* is an anaerobic organism, but can withstand 5% of oxygen (Futter and Richardson, 1971). *C. perfringens* is ubiquitous in nature including soil, water source and the intestinal tract of humans and animals (McClane, 2001). *C. perfringens* was first described in 1892 by Welch and Nuttall known earlier commonly as *C. welchii* (Hatheway, 1990). *C. perfringens* can grow vigorously between 20°C and 50°C and shows hemolysis when grown on blood agar plate (Hatheway, 1990). Based upon their ability to produce a variety of toxins, *C. perfringens* has been categorized into types A-E. *C. perfringens* cells survive stomach passage and sporulate in the intestinal lumen when large quantities of cells are ingested with food. During lysis of the mother-cell to release the spore, the enterotoxin is released. *C. perfringens* has been associated with many animal diseases including enterotoxemia, myonecrosis, hemorrhagic gastroenteritis and many other enteric diseases. According to a survey of foodborne disease outbreaks in the United States from 1998-2002, *C. perfringens* Type A is the third most common cause of
foodborne infection (Lynch et al. 2006). In addition to foodborne illness, *C. perfringens* type A are also capable of causing non-foodborne illness such as antibiotic associated diarrhea (Sarker et al., 2000). The virulence of *C. perfringens* is attributed to its ability to produce multiple toxins, a short generation time (8 min) and the ability of spores to survive environmental stress.

**Clostridium perfringens** in foods

*C. perfringens* type A is a common cause of human gastrointestinal illness in the United States and Europe (Bean et al. 1996; Todd, 1997). *C. perfringens* type A is also implicated in non-foodborne animal and human diseases (Borreillo et al., 1985; Brett et al., 1992; Jackson et al. 1986; Collie and Mcclane, 1998). It was estimated that approximately 5% to 20% of all cases of antibiotic-associated diarrhea (ADD) and non-foodborne sporadic diarrhea are caused by *C. perfringens* type A. Symptoms associated with *C. perfringens* type A food poisoning are caused by the *C. perfringens* enterotoxin (CPE) (Sarker et al., 1999). The disease develops with the consumption of food contaminated with enterotoxigenic *C. perfringens* (Rood, 1997). Spores of *C. perfringens* can survive cooking process traditionally used to prepare foods and can grow to large populations if held at improper temperatures. When foods containing large numbers of *C. perfringens* are ingested, the majority of the vegetative cells are destroyed by the acidic condition of the stomach. However, a small number of the population will survive and enter into the intestine. The intestine is an environment that favors *C. perfringens* to multiply and then sporulate. CPE is produced and released into the environment when the endospores are fully matured and the mother cell undergoes autolysis (Rood, 1997; Zhao
Symptoms of *C. perfringens* type A food poisoning typically start around 12 h after consumption of contaminated foods, with diarrhea and cramps being the most commonly reported symptoms. In normal adults, symptoms usually persist for 12-24 h and then resolve spontaneously (McClane, 2000, Sarker et al., 2000). However, in immunocompromised individuals such as the elderly, *C. perfringens* type A food poisoning are the fifth leading cause of death from food poisoning in the United States (Brett et al., 1992, Lynch et al., 2006). The disease is diagnosed by the characteristic delayed onset of symptoms as well as detection of toxin in the feces of patients (Rood, 1997).

*C. perfringens* has been isolated from many foods with majority of cases from meat products (CDC, 2006). Raw meat like 66% of fresh swine meat, 26% of fresh cattle meat, 3% of cattle carcasses, 79% of poultry carcasses, 12% of pork organ meat, 39% of ground pork meat, and 81% of processed pork products are contaminated with *C. perfringens* (National Research Council, 1999). Processing of meat increases distribution of this pathogen: 2% of whole muscle meat samples are contaminated, while after emulsification 49% of the samples are infected (Taormina et al., 2003). Other sources of *C. perfringens* are spices; 59% of different spice samples are contaminated (Boer and Boot, 1983). Microorganisms encounter many temperature effects during processing of cooked food. Subsequently, these foods are cooled and kept below 7°C or are held above 60°C to inhibit bacterial growth. Spores of *C. perfringens*, however, survive cooking. Subsequent hot holding at temperatures ≥ 60°C does not reduce spore numbers and germination will occur when the temperature is not sufficiently high (Strong and Ripp,
Foods that are inadequately cooled permit growth of *C. perfringens* to high cell numbers (Juneja et al. 2001; Juneja and Marks, 2002).

**Functional Peptides from Eggs**

Short chain peptides derived from different sources such as egg yolk, egg white, lysozyme, fish, milk etc. have been shown to possess antimicrobial properties. Antimicrobial peptides are small molecular weight segment of proteins which are effective against bacteria, viruses, and fungi. Generally, these peptides are positively charged and have both hydrophobic and hydrophilic side chains, which make them soluble in aqueous environments and also allow them to enter lipid-rich membranes. The embryo present in egg is protected by egg white proteins against microbial invasion. Two major egg white proteins, lysozyme and ovotransferrin, are reported to possess antimicrobial activities (Fitzgerald and Murray, 2006). A 92-amino-acid-long peptide (OTAP-92) isolated from ovotransferrin was reported to exhibit antimicrobial activity through a membrane damage mechanism (Ibrahim et al., 2000). A peptide (IVSDGDGMNAW), isolated from egg lysozyme, showed antimicrobial activity against Gram negative bacteria *E. Coli* K-12 (Mine and Kovacs-Nolan, 2004). An active peptide was obtained from an egg white protein after fermentation with *Saccharomyces cerevisiae* (yeast) (Kassaify et al., 2005). Ingestion of this product has been proved to increase the non-specific activity of neutrophils. Peptides derived from a tryptic digest of egg proteins were shown to trigger the nonspecific immune defense system by stimulating superoxide anions (Mine and Kovacs-Nolan, 2004).
Bioactive peptides derived from different sources play an important role in the body's regulatory system. Antihypertensive peptides derived from various food proteins are major types of this group. Although egg contains more than 40 different proteins, the ACE (Angiotensin Converting Enzyme) inhibitory peptides reported were mostly derived from ovalbumin (López-Fandiño et al., 2007).

Hypertension

According to the World Health Organization (WHO) 17.5 million people deaths have been attributed to cardiovascular diseases in 2005, 31% of all global mortality (Padwal et al., 2009). Hypertension is one of the well-defined risk factors in cardiovascular disease and is defined as a condition in which systolic blood pressure is greater than 120 millimeters of mercury (mm Hg) and/or a diastolic pressure greater than 90 mm Hg. In North America, 79.4 million people have some type of cardiovascular disease; of those 72 million have hypertension (Whelton, 2004).

The renin-angiotensin system is an important pathways for controlling blood pressure. In humans, the main components of the renin-angiotensin system (RAS) are renin, angiotensin-converting enzyme (ACE), and angiotensinogen. Renin is synthesized in the human kidneys, stored in the afferent arterioles, and released in response to hemodynamic, neurogenic, and ionic signals (Hackenthal et al., 1990; Sealey and Laragh, 1990). Renin is an aspartyl protease that has a very high specificity for angiotensinogen (Zhu et al., 2006). Angiotensinogen is a 58 kilodalton (KDa) protein synthesized and released from the liver (Nasjletti and Mason, 1972). Angiotensin-converting enzyme
(ACE) [EC 3.4.15.1], which is also known as kinase II, is a metallo-protease with two zinc active catalytic sites. Renin cleaves angiotensinogen and releases amino terminal decapeptide angiotensin I [DRVYIHPFHL] (Ang I). ACE then cleaves Ang I and releases two carboxy-terminal amino acids (HL). The resulting octapeptide is called angiotensin II (Ang II) (Ng and Vane, 1967). Ang II is a potent vasoconstrictor, whereas Ang I is biologically inactive. Ang II directly stimulates vascular smooth muscle contraction, which increases systematic vascular resistance and elevates blood pressure. It also stimulates secretion of aldosterone from the adrenal cortex. Aldosterone produces kaliuresis and increases sodium and water retention in the body. Additionally, ACE also hydrolyzes and inactivates bradykinin [RPPGFSFPR], a potent vasodilator (Sealey and Laragh, 1990). Therefore, excessive ACE activity leads to high blood pressure or congestive heart failure through an increased rate of vasoconstriction. Thus, inhibition of ACE is the therapeutic target for antihypertensive drug development (Hackenthal et al., 1990). The pharmacological ACE-inhibitors (i.e., captopril and enalpril) are widely used for the treatment of hypertension. However, these ACE inhibitors are associated with adverse effects including dry cough, hyperkalemia, hypotension, renal failure, decrease in white blood cells, and angioedema (Silverberg et al., 2002). Pregnant women or patients suffering from kidney problems are urged to avoid synthetic ACE inhibitors (Ben-Sira and Oliveira, 2007; Pravenec and Petretto, 2008). Therefore, there is an interest in searching for non-pharmacological methods of prevention and management of hypertension. Bioactive peptides derived from food sources are generally regarded as
safer and economical alternative for the prevention and treatment of hypertension (Champagne, 2006).

Fig. 3.4 Rennin-angiotensin system

**Antihypertensive Peptides from different sources**

Several ACE-inhibitory peptides have been reported in different food sources (e.g., food-grains, vegetables, fruits, seeds and legumes, milk and dairy products, seafood, meat, and egg) showing *in vitro* ACE-inhibitory activity as well as *in vivo* antihypertensive effects in animals (spontaneously hypertensive rats [SHRs]) (Opie, 1987; Champagne, 2006; Iwai and Matsue, 2007; Bakris et al., 2008) or even in human subjects (Williams et al. 2004).

Eggs are an excellent source of well-balanced nutrients. Although egg contains more than 40 different proteins, the ACE-inhibitory peptides from eggs were mostly derived from ovalbumin (López-Fandiño et al., 2007). ACE-inhibitory peptides derived from egg proteins are listed in Table 3.1. The peptic hydrolysate of ovalbumin could produce several ACE-inhibitory peptides. Three potent ACE-inhibitory peptides, FFGRCVSP, ERKIKVYL, and FRAHPFL were isolated from ovalbumin with IC$_{50}$ values of 0.4, 1.2,
and 3.2 M respectively (Lopez-Fandino et al. 2007). Ovokinin (FRAHPFL) is one of the most intensively studied vasorelaxing peptides from ovalbumin (Fujita et al., 1995a). Ovokinin lowered the blood pressure in SHRs significantly when administered orally (Fujita et al., 1995b). Another peptide (RAHPFL), lacking the N-terminus Phe (F) residue of ovokinin, was isolated from ovalbumin pepsin hydrolysate (Miguel et al., 2004). Under in vitro conditions, this peptide showed weaker ACE-inhibitory activity (IC50 = 6.2 μM) than that of ovokinin (IC50 = 3.2 μM), but exhibited significant antihypertensive activity in SHRs (Miguel et al., 2005). This hexapeptide (RADHPF) has also been isolated from chymotryptic digestion of ovalbumin; it has been shown to induce nitric oxide-dependent vasorelaxation in an isolated mesenteric artery as well as an antihypertensive effect in SHRs (Matoba et al., 1999). Another promising peptide, YAEERYPIL was identified from pepsin ovalbumin hydrolysate. This peptide showed significant antihypertensive activity in SHRs at a dosage rate of 2 mg/kg (Miguel et al., 2006b; Potter et al., 2009), but was totally degraded after simulated gastrointestinal digestion, and its ACE-inhibitory activity decreased; surprisingly, however, one of its main fragments, YPI, significantly decreased blood pressure in SHRs at a dosage rate of 2 mg/kg (Nakaoka et al., 1987). Pepsin also exhibited the highest proteolytic activity toward ovalbumin, although there was not a direct relationship between the level of intact protein and the resultant ACE inhibition (Fujita et al., 2000).

ACE-inhibitory peptide (RDHP) was obtained by hydrolyzing rice protein with trypsin (Chen et al. 2007). Optimum conditions included pH and temperature of 8.02 and 37ºC. Peptides were also obtained from wheat (IAP) and barley (EVSLNSGY) with
antihypertensive activity (Kajihara et al., 2008). ACE-inhibitory peptides were obtained after treating egg white proteins with pepsin, trypsin and chymotrypsin (Miguel et al., 2004) where pH of egg white solution was adjusted to 7.8 and 8.0 for chymotrypsin and trypsin respectively for hydrolysis. ACE-inhibitory and free-radical scavenging properties were recognized in peptides derived from soybean protein hydrolysates which have been extensively studied (McCue et al., 2005; Farzamirad and Aluko, 2008). ACE inhibitor peptides were also prepared by digesting glycinin with either bovine trypsin or chymotrypsin at an enzyme-substrate ratio of 2% (w/w) at 37 °C/18 h (Gouda et al., 2006). A fermented soybean product, known as douchi, contains a potent ACE inhibitor (Zhang et al., 2006). IAYKP, IAY, and KP peptides exhibiting ACE-inhibitory activity were obtained by adjusting Spinach Rubisco (10mg/mL) pH to 2.0 with 1.0 N HCl and then digestion by pepsin (E/S) 1/100) for 5 h at 37 °C (Yang et al., 2003).

ACE inhibitory peptides could be released by enzymatic hydrolysis (trypsin and chymotrypsin) of water-insoluble proteins (actin, myosin, and collagen), from porcine skeletal muscle, and from myosin after 18 h of digestion at 37°C (Arihara et al., 2001). Peptic hydrolysates of regulatory proteins of porcine, such as tropomyosin and troponin, also exhibited in vitro ACE-inhibitory activity. A novel ACE-inhibitory peptide, PTHILWGD, was isolated by acid extraction from tuna muscle (Kohama et al., 1988). Previous research demonstrated that in vitro incubation of milk proteins with gastrointestinal proteases (pepsin, trypsin, and chymotrypsin) resulted in the release of ACE inhibitory peptides (Yamamoto and Takano, 1999). Milk fermented by Enterococcus faecalis can also produce some ACE-inhibitory peptides (LHLPLP,
LVYPFPGPIPNSLPQNIPP, VLGPVRGPFP, and VRGPFPIIV) (Miguel et al., 2006a).

Two ACE-inhibitory peptides (LQP and MAP) were isolated from enzyme-modified cheese (Tonouchi et al., 2008). The Danish skim milk cheese was added with starter culture for 16 h/37°C followed by treatment with protease N Amano 48 h at 35°C.

Table 3.1 Examples of ACE-inhibitors peptides derived from eggs

<table>
<thead>
<tr>
<th>Protein</th>
<th>Sequence</th>
<th>IC₅₀ (µM)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Ovalbumin</td>
<td>LW</td>
<td>6.8</td>
</tr>
<tr>
<td></td>
<td>FRADHPFL</td>
<td>3.2</td>
</tr>
<tr>
<td></td>
<td>RADHPFL</td>
<td>6.2</td>
</tr>
<tr>
<td></td>
<td>FFGRCVSP</td>
<td>0.4</td>
</tr>
<tr>
<td></td>
<td>FGRCVSP</td>
<td>6.2</td>
</tr>
<tr>
<td></td>
<td>ERKIKVYL</td>
<td>1.2</td>
</tr>
<tr>
<td></td>
<td>YAEERYPIL</td>
<td>4.7</td>
</tr>
<tr>
<td>Ovotransferrin</td>
<td>KVREGT</td>
<td>9.1</td>
</tr>
</tbody>
</table>

Safety of egg white hydrolysates Manufacturing Process

Microorganisms are associated with raw agricultural products such as harvested plants, slaughtered animals and raw materials in food industry. In 1989, FDA considered the
responsibility of food manufacturer for the application of aseptic processing. Among these, the requirement of biological validation of the heat treatment applied to the product has proved to be the most difficult (Dignan et al. 1989). While foodborne pathogens belonging to the genus Bacillus and Clostridium have been isolated from eggs, foodborne illness outbreaks from egg products have not been reported. Majority of the thermal processes employed in the food industry are designed to destroy vegetative forms of foodborne pathogens. In some cases the heat treatments serve to activate spores. Thus it is important to predict the ability of spores to survive a heat treatment and also the effect of environmental conditions on their subsequent growth. Interactions of environmental factors affecting bacterial growth and survival can be safely studied by conducting a microbial challenge studies (Rose, 1987). The aim of MCT is to simulate the fate of relevant organisms during processing. Therefore, the MCT procedure is generally regarded as a means to make sure that a potentially hazardous organism will not survive the process (Notermans and in’t Veld, 1994). There are some important factors to be considered while performing microbial challenge testing (Vestergaard, 2001) which include 1) microorganism selection 2) inoculation level 3) inoculation method 4) length of study 5) product composition and storage 6) microbiological analysis.

The ideal organisms for challenge testing are those that have been previously isolated from similar formulations. Additionally, pathogens from known foodborne outbreaks should be included to ensure the formulation is robust enough to inhibit those organisms as well. Multiple specific strains of the target pathogens should be included in the challenge study (FDA, 2009). It is common to challenge a food formulation with a
"cocktail" or mixture of multiple strains in order to take into account potential strain variation (FDA, 2009). It is also important to incubate and prepare the challenge suspension under standardized conditions and format. Shifts in the incubation temperature used to propagate the challenge organisms and the storage temperature of the product have been shown to change the length of the lag period of organisms used in the challenge study (Curiale, 1991). In certain applications, surrogate microorganisms may be used in challenge studies in place of specific pathogens (FDA, 2009). For example, it usually is not possible to introduce pathogens into a processing facility; therefore, it is desirable to use surrogate microorganisms in those cases. An ideal surrogate is a strain of the target pathogen that retains all other characteristics except its virulence (FDA, 2009).

The inoculum level used in the microbiological challenge study depends on whether the objective of the study is to determine product stability and shelf life or to validate a step in the process designed to reduce microbial numbers. Typically, an inoculum level of between $10^2$ - $10^3$ cells/g of product is used to ascertain the microbiological stability of a formulation (FDA, 2009). Higher inoculum levels may be appropriate for other products. Depending on the product formulation, some of the inoculum may die off initially before adapting to the environment. If too low of an inoculum level is used, the incorrect assumption could be made that the product is stable when it is not. Conversely, if the inoculum level is too high for this purpose, the preservation system or hurdles to growth may be overwhelmed by the inappropriate inoculum size, leading to the incorrect conclusion that the formulation is not stable.
The preparation of the inoculum to be used in microbiological challenge testing is an important component of the overall protocol. Typically, for vegetative cells, 18 - 24 h cultures revived from refrigerated broth cultures or slants or from cultures frozen in glycerol are used (FDA, 2009). The challenge cultures should be grown in media and under conditions suitable for optimal growth of the specific challenge culture. Bacterial spore suspensions may be stored in water under refrigeration or frozen in glycerol. Spore suspensions should be diluted in sterile water and heat-shocked immediately prior to inoculation (FDA, 2009). Quantitative counts on the challenge suspensions may be conducted to aid in calculating the dilutions necessary to achieve the target inoculum in the challenge product. Correct procedures should be used when carrying out challenge tests with certain pathogens. The method of inoculation is another extremely important consideration when conducting a microbiological challenge study. Every effort must be made not to change the critical parameters of the product formulation undergoing challenge.

It is prudent to conduct the microbiological challenge study up to the desired shelf life of product (FDA, 2009). It is also desirable to test the product over and significantly beyond its entire shelf life because sub lethal injury may occur in some products. This can lead to a long lag period, where it may not be possible to culture the inoculum, but over time, a small number of the injured cells can recover and grow in the product.

Several intrinsic factors like pH, water activity and extrinsic factors like temperature can effect significantly on microorganisms growth. It is important to control and note down the conditions under which studies are done. Test samples should be stored in same
conditions as intended for product storage to obtain accurate results. The selection of enumeration media and method depends on the type of pathogens or surrogates used in the study. It is highly recommended to check the background flora present in raw materials used for preparation of the product. Sometimes, this background flora can hinder the growth of organism of our interest and give false result.
REFERENCES


CHAPTER IV

Risk Evaluation of Egg White Hydrolysates Manufacturing Process for Spore Formers: *Bacillus Cereus* and *Clostridium Perfringens*
ABSTRACT

Spore forming foodborne pathogens of the genus *Bacillus* and *Clostridium* are a great concern in food industry due to ability of the spores to survive normal heat treatments used in food manufacturing. The germination and outgrowth of *B. cereus* and *C. perfringens* spores during the egg white hydrolysates (EWH) manufacturing process was evaluated. Egg white solution (2.5%) was inoculated with heat activated *B. cereus* and *C. perfringens* separately to provide a spore population of ca. 2.0 – 3.0 log spores/ml. EWH was prepared by enzymatic hydrolysis of the inoculated egg white solution and maintained at optimum pH of enzymes (trypsin and chymotrypsin) for 24 h at 37°C. *B. cereus* and *C. perfringens* populations were enumerated before and after heat shock, at 0, 4, 8, 12, 18 and 24 h of hydrolysis by plating on corresponding selective medium. SDS-PAGE was performed on EWH obtained during egg white hydrolysis (non-inoculated egg white) with trypsin and chymotrypsin. *B. cereus* population was increased by > 3.5 log CFU/ml during the EWH preparation using chymotrypsin, while minimal increase was observed during trypsin hydrolysis. No increase in *C. perfringens* population was observed irrespective of the enzyme used. Hydrolysis of major egg proteins by chymotrypsin was completed within 4 h of incubation, while minimal hydrolysis of egg white proteins was observed with trypsin subsequent to 24 h incubation. Those enzymes which doesnot have optimal conditions for the growth of pathogens should be used for the manufacturing of egg white hydrolysates.

**Keywords:** *Bacillus, Clostridium, egg hydrolysates, spore*
INTRODUCTION

Eggs are used in preparation of several food products like mixes, bakery foods, noodles, mayonnaise, salad dressings, candies, ice cream, pet foods, etc. (Kovacs-Nolan et al., 2005). Egg white and egg white solids have been used primarily for their functional properties in the food industry. In addition, egg whites are increasingly being used for their health benefits such as antimicrobial, anti-adhesive, immuno-modulating, anti-cancer, angiotensin converting enzyme (ACE) inhibitor, protease inhibitor and anti-hypertensive properties (Kovacs-Nolan, 2005).

Heart disease is a major cause of mortality and remains a major health concern in North America (Lo et al., 2005). Hypertension (blood pressure greater than 140 mm Hg systolic and/or 90 mm Hg diastolic pressure) is a major risk factor for heart disease (Izzo et al., 2003; Kannel et al., 2003). Hypertension and cardiovascular diseases afflicts 28.7, 22.0 and 44.2 % of the population in USA, Canada and Europe, respectively (Kearney et al., 2004). Rennin-angiotensin system is a hormone system which regulates blood pressure and water balance in human body. ACE (dipeptidyl carboxypeptidase, EC 3.4.15.1) is a zinc metallopeptidase that plays a central role in regulating blood pressure through the rennin-angiotensin system (Laragh et al., 1972). ACE converts angiotensin I to angiotensin II, a potent vasoconstrictor and inactivates bradykinin, a potent vasodilator. Thus, inhibition of ACE may lead to a reduction in blood pressure and ACE inhibitory drugs are commonly prescribed for treating hypertension (Grivas and Grivas, 1999).
Food protein-derived angiotensin converting enzyme (ACE) inhibitory peptides are being evaluated for reducing hypertension and some are being produced commercially (Hata et al., 1996). Although synthetic antihypertensive drugs for inhibiting ACE have been successful in lowering hypertension, they exhibit adverse side effects such as cough and angioedema (Beltrami and Zimgale, 2006). Therefore, ACE inhibitory peptides from food proteins are being evaluated as an alternative for prevention and treatment of hypertension (Fitzgerald et al., 2004; Miguel and López-Fandiño, 2005).

Enzymatic hydrolysis products of egg white are being used in the health care industry for their ACE inhibitory activity. The hydrolysates of egg yolks, egg white, milk proteins, soy sauce, fish sauce and tuna have been shown to lower blood pressure in spontaneously hypertensive rats (SHRs) and hypertensive human subjects (Miguel et al., 2004). Peptic and chymotryptic digest of ovalbumin resulted in an octapeptide ovokinin (FRAHPFL) and a hexapeptide (2-7 fragment of ovokinin), respectively (Miguel and Aleixandre, 2006; Fujita et al., 1995) with ACE inhibitory activity. Additional peptides derived from egg with ACE inhibitory activity include ESIINF, YRGGLEPINF, YAEERPIL (Miguel et al., 2007a, Miguel et al., 2007b), and LVREGT (Lee et al., 2006). While these egg white hydrolysates provide health benefits, their manufacturing processes have not been evaluated for microbiological safety.

Traditional EWH manufacturing processes involve sequential hydrolysis of egg white with pepsin, trypsin, chymotrypsin and/or similar proteases to yield peptides with specific properties (Miguel et al., 2004). This involves co-incubation of liquid egg white with appropriate enzyme at its optimum temperature and pH for sufficient length of time to
hydrolyze the egg white proteins. While some enzymes have the pH optima at low pH values (pepsin, pH optima ca. 2.0) that would prevent growth of foodborne pathogens, other enzymes may have both the temperature and pH optima that would allow growth of microorganisms. This may result in growth of foodborne pathogens present in raw materials to hazardous levels and potentially produce toxins. Thus, it is necessary that microbiological safety of these processes is evaluated to assure safety of the final products. The organisms of concern during EWH manufacturing include the spore forming pathogens *C. perfringens* and *B. cereus*, the spores of which can survive traditional heating processes and can grow and produce toxins. *Salmonella* spp. has a high prevalence in eggs and poultry facility than mentioned spore formers but worst it would be worthwhile to evaluate the process for organisms like spore formers which are difficult to kill and avoid due to their heat resistance and ability to survive thermal treatment usually involved in egg white hydrolysates manufacturing process. Spores of *Bacillus* and *Clostridium* spp. have been isolated from eggs and poultry facilities (Siragusa et al., 2004; Siragusa et al., 2006; Ansah et al., 2009). The objective of this study was to evaluate the potential germination and outgrowth of *C. perfringens* and *B. cereus* spores during the manufacture of egg white hydrolysates.

**MATERIALS AND METHODS**

**Microorganisms**

Three different enterotoxin-producing strains of *C. perfringens*, NCTC 8238 (Hobbs serotype 2), NCTC 8239 (Hobbs serotype 3) and NCTC 10240 (Hobbs serotype 13) and seven strains of *Bacillus* spp. producing emetic or diarrheal toxins (*B. cereus* ATCC
14579, FERN 1, FERN 40, 7401, 665; Bacillus spp. FERN 110; B. thuringiensis FERN 7) were used in the study.

**Preparation of spores**

Spores of *C. perfringens* strains were prepared following the procedure outlined by Juneja et al. (1993). Briefly, an aliquot (0.1 ml) from the stock culture was inoculated into 10 ml of freshly prepared fluid thioglycollate medium (FTM; Becton Dickinson and Company, Cockeysville, Md). The spores were heat activated for 20 min at 75°C in circulated water bath (Isotemp 3013H, Fisher Scientific, Fair Lawn, Nj) and then cooled in an ice chilled water followed by the incubation for 18 h at 37°C. A 1.0 ml portion from this culture was transferred to 10 ml of freshly steamed FTM and then incubated for 4 h at 37°C. The fresh culture (1%) was transferred to modified Duncan Strong (DS) medium and incubated aerobically for 24 h at 37°C. The original DS formulation was modified by replacing starch with 0.4% raffinose (Sigma Chemical Co., St. Louis, Mo) and supplemented with 100 mg/L of caffeine (Sigma Chemical Co., St. Louis, Mo) to improve sporulation. Spores of each strain were harvested by centrifugation at 7,012 x g for 20 min at 4°C (CS-15R, Beckman, Palo Alto, Ca) and washed twice with 50 ml of sterile distilled water. The spore crop of each strain was re-suspended in 10 ml sterile deionized water and stored separately at 4°C until use. A spore cocktail containing all three strains of *C. perfringens* spores was prepared immediately before experiments by mixing approximately equivalent numbers of spores from each strain suspension.
Spores of *Bacillus* spp. strains were prepared following the procedure outlined by Beuchat et al. (1997). Briefly, stock cultures were activated in BHI broth (Becton Dickinson and Company, Cockeysville, Md) and incubated at 30°C. Three successive loop transfers at 24 h intervals were made before spreading 0.1 ml aliquots of each strain onto the sporulation medium (Nutrient agar Becton Dickinson and Company, Cockeysville, Md) supplemented with 0.05 g of MnSO$_4$/L). The plates were incubated for 72 h at 30°C and the spores were harvested by depositing 5 ml of sterile distilled water on the surface of each plate and rubbing gently with a sterile bent glass rod. The suspension of spores and vegetative cells was filtered through sterile glass wool and collected in a sterile tube. The washing and filtering procedures were repeated twice. Pooled suspensions were centrifuged at 2,600 x g for 20 min at 5°C (CS-15R, Beckman, Palo Alto, Ca) and the supernatant liquid was discarded. The resulting pellet was suspended in 100 ml of sterile distilled water and centrifuged at 6,000 x g for 10 min at 5°C. This procedure was repeated twice and the final pellet was suspended in sterile de-ionized water and stored at 4°C for use in subsequent experiments. Spore cocktail containing all seven strains of *Bacillus* spp. spores was prepared immediately before experiments by mixing approximately equivalent numbers of spores from each spore suspension.

**Bacterial enumeration**

For enumeration of spore population, samples were heated for 20 min at 75°C or for 5 min at 80°C for *C. perfringens* and *B. cereus*, respectively. Ten-fold serial dilutions of samples were prepared in sterile 0.1% peptone water (PW; BD Difco, Sparks, MD) and
appropriate dilutions were spread or spiral-plated on Tryptose-Sulfite-Cycloserine agar (TSC; Oxoid, Basingstoke, UK) without egg-yolk and Mannitol-Egg Yolk-Polymyxin (MYP) Agar (Becton -Dickinson and Company, Sparks, Md) used for enumeration of C. perfringens and B. cereus, respectively. TSC plates were overlaid with a layer of (8-10 ml) TSC agar and incubated for 24 h at 35°C in an anaerobic chamber (Bactron IV, Sheldon Manufacturing Co., Or) where as MYP plates were incubated aerobically for 24 h at 35°C. Typical C. perfringens and B. cereus colonies were enumerated and counts were expressed as log CFU/ml.

**Preparation of Egg White Solution**

Freshly laid eggs were obtained from the University of Nebraska – Lincoln poultry farm. Eggs were washed with warm water to remove visible dirt and other particulate material from the shells. The washed eggs were subsequently sanitized by dipping in 70% ethanol for 15 minutes and dried in a biosafety cabinet (Belco Glass Inc. Vineland, Nj). The sanitized eggs were broken with an ethanol flamed knife and the egg white was collected in a sterile container. A 2.5% (wt/wt) solution of Egg white was prepared using Milli-Q water. The pH of the egg white solution was adjusted to 8.0 and 7.8 with 1 M HCl for hydrolysis with trypsin and chymotrypsin, respectively. The egg white solutions for each enzyme hydrolysis were equally divided into three portions (ca. 100 ml each) and used for following: (i) *Clostridium* inoculation (ii) *Bacillus* inoculation (iii) pH monitoring and SDS-PAGE (non-inoculated).
Measurement of pH

The pH of egg white solution was measured at 0, 4, 8, 12, 18 and 24 h intervals during hydrolysis using non-inoculated samples by directly immersing the electrode of pH meter (Model Accumet-Basic/AB15, Fisher Scientific, Pittsburgh, Pa) in the sample.

Egg White Inoculation

The heat activated spores of *C. perfringens* (75°C/20 min) and *Bacillus* spp. (80°C/5 min) were added to the egg white solution to obtain a final spore concentration of ca. 2.0 - 3.0 log CFU/ml, respectively.

Enzymatic Hydrolysis

Trypsin (EC 3.4.21.4 type I, 10,900 U/mg) and chymotrypsin (EC 3.4.21.1 type I-S, 44 U/mg from bovine pancreas) were purchased from Sigma Chemical Co. (St. Louis, Mo). Trypsin and chymotrypsin were added to the inoculated egg white to obtain a substrate/enzyme ratio of 1/100 and 5/100 (wt/wt), respectively. The hydrolysis was carried out in water bath set and maintained for 24 h at 37°C.

Sample Collection

Samples were collected at regular intervals (0, 4, 8, 12, 18 and 24 h) from egg white during hydrolysis and analyzed for total and spore populations of *C. perfringens* or *B. cereus* populations. At the end of 24 h incubation period, the enzymes were inactivated by placing the samples in a boiling water bath for 15 min. Samples were obtained at 0, 4,
8, 12, 18 and 24 h from non-inoculated egg white solution during chymotrypsin hydrolysis and heated at 100°C for 15 min for inactivation of enzyme and stored at -20°C. During trypsin hydrolysis, Samples were taken at 0, 4, 8, 12 and 24 h from non-inoculated batch

**Sodium Dodecyl Sulfate-Polyacrylamide Gel Electrophoresis**

Appropriate dilutions of egg white solution and enzymes (trypsin and chymotrypsin) and non-diluted egg white hydrolysates (protein digest) collected at different times during hydrolysis were used for SDS-PAGE experiment. All samples were added to Laemmli sample buffer (Bio-Rad Laboratories, Hercules, Ca) with 5% β-mercaptoethanol (Sigma-Aldrich) in equal proportions (50%; wt/wt). The samples and molecular weight standard were boiled for 5 min followed by cooling to room temperature. Three molecular weight standards (markers) were used: (i) Broad-range protein standard (Biorad Precision Plus Standards) containing pure recombinant proteins of the following molecular weights (MW), 250, 150, 100, 75, 50, 37, 25, 20, 15, and 10 kDa, (ii) SDS-PAGE broad range (Biorad natural standards) protein unstained standards of 200, 116.3, 97.4, 66.2, 45. 31, 21.5, 14.4 and 6.5 kDa and (iii) SDS-PAGE polypeptide (Biorad natural standards) protein unstained standards of 26.6, 17.0, 14.4, 6.5, 3.5 and 1.4 kDa for comparative purposes. Aliquots (10 and 40 µl) of each sample were loaded onto each well of 16.5% and 10-20 % precast gels (Bio-Rad Laboratories, Hercules, Ca), respectively. Electrophoresis was carried out at 200 V, for approximately 30 min and 60 min in 16.5% and 10-20 % precast gels, respectively, using Tris-Tricine-SDS running buffer (Biorad Laboratories Hercules, Ca). Gels were fixed in a solution of 40% (v/v) methanol and 10%
(v/v) acetic acid, stained with 0.025% (w/v) Coomassie Blue R-250 in 10% acetic acid, destained in 10% (v/v) acetic acid and finally washed with deionized water. The molecular weight and band intensities were recorded in Kodak Image Station 440 CF (Kodak, New Haven, Ct).

RESULTS AND DISCUSSION

The SDS-PAGE patterns of hydrolysates obtained at 0, 4, 8, 12, 18 and 24 h of egg white hydrolysis with chymotrypsin and trypsin on 16.5 % and 10-20 % gel are shown in Figs. 1-4, respectively. Similar types of bands were observed for all samples irrespective of the gel concentration. The egg white protein samples produced clear bands near 76, 46 and 14 kDa compared with molecular weight standards (Figs. 1-3). These bands may represent ovotransferrin (76 kDa), ovoalbumin (46 kDa) and lysozyme (14.3 kDa) (Miguel and Aleixandre, 2006; Galyean and Laney, 1980). Protein bands near 25, 15 and less than 15 kDa were observed for chymotrypsin and trypsin (Figs. 1-4). Chymotrypsin and trypsin have a molecular weight of 25 kDa (Burell, 1993) and 24 kDa (Cunningham et al., 1954; Walsh et al., 1970), respectively. Bands lower than the molecular weight of the enzymes (trypsin and chymotrypsin) could be due to impurities or partial degradation of the enzymes.

Hydrolysates obtained from tryptic digest of egg white solution did not show any change in the band patterns at 0, 4, 8, 12 and 24 h of hydrolysis. The inability of trypsin to hydrolyse egg white proteins may be due to the presence of ovomucoid which inhibits the activity of bovine and porcine trypsin (Liu et al., 1971; Zehenley, 1980). Hydrolysates
obtained during hydrolysis of egg white with chymotrypsin at 0 h showed no significant change in bands of egg white proteins at 46 and 14 kDa while 76 kDa band (ovotransferrin) observed in digested egg white sample. This protein may have produced smaller peptides (< 6.5 kDa) which cannot be visualized properly using SDS-PAGE analysis. This particular protein band completely disappeared after 4 h of hydrolysis with chymotrypsin. Yoshino et al. (2004) reported that pepsin digestion of raw egg white proteins of 76.6 kDa mol wt. was rapidly digested within 30 min of hydrolysis. The 14 kDa band (lysozyme) was completely digested (Figs. 1 and 2) at 0 h during chymotrypsin hydrolysis of egg white. All samples were heated for 15 min at 100°C for inactivating enzyme before storing them for SDS-PAGE experiment. It is possible that the 2 to 3 min for samples heating (come up time) may have been enough for the enzyme to digest certain protein as in the case of 14 and 76 kDa protein bands during egg white hydrolysis with chymotrypsin. Proctor and Cunningham (1988) reported that some proteolytic enzymes such as trypsin, chymotrypsin, and papain do not hydrolyze lysozyme, unlike pepsin. However, these enzymes hydrolyze denatured lysozyme. The protein band near 46 kDa (ovoalbumin) is not prominent at 4 h hydrolysate sample compared with time 0 h of hydrolysis and was observed throughout the hydrolysis period. Information about bands < 6.5 kDa could not be obtained due to absence of lower molecular weight markers, also the staining of proteins did not enable lower molecular weight peptides and markers to be clearly visualized. Cheng et al. (2008) reported that some low molecular weight fragments (< 6.5 kDa) cannot be visualized during chicken leg bone hydrolysis with trypsin. Mahmoud (1994) suggested that these partially hydrolyzed proteins were
more difficult to define because they encompass a wide range of products with varying degrees of hydrolysis and Coomassie Blue staining of proteins did not enable lower molecular peptides to be visualized. Majority of the egg white hydrolysis by chymotrypsin was completed within 4 h of hydrolysis. Cheng et al. (2008) and Guerard et al. (2002) also observed faster enzymatic digestion of proteins during hydrolysate preparation initially followed by a slower hydrolysis for chicken leg bone and tuna waste hydrolysates, respectively.

The pH of egg white solutions during trypsin and chymotrypsin hydrolysis are shown in Fig. 5. During chymotrypsin hydrolysis of egg white solution, pH of the solution decreased from 7.8 to 6.5 within the first 4 h indicating hydrolysis. The pH reduction could be due to the peptide residues released during proteolysis (Cheng et al., 2008). The pH drop was minimal subsequent to 4 h, and pH 6.2 was observed at the end of 24 h incubation. Similar decrease in pH for initial 2 h of hydrolysis was observed by Cheng et al. (2008) during hydrolysis of chicken leg bone protein with alcalase and trypsin, resulting in an ultimate pH of 6.7-6.9 (from 8.0 after) 12 h of hydrolysis. In contrast, changes in pH values of trypsin hydrolysis of egg white solution were not observed throughout the incubation period. This may be due to absence of egg white proteins hydrolysates as evident from SDS-PAGE results.

*C. perfringens* spores did not germinate and outgrow in egg white solution during EWH preparation using either of the enzymes (trypsin and chymotrypsin; Figs. 6 and 7). *C. perfringens* populations (before heat shock) decreased during EWH preparation using trypsin and chymotrypsin by 0.84 and 1.05 log CFU/ml, respectively. Also, *C.
perfringens populations (after heat shock) decreased by 0.27 log and 0.13 CFU/ml, respectively for the same. Survivors of C. perfringens were recovered from the samples after enzyme activation step in hydrolysis carried out with chymotrypsin after 24 h. It may be due to the presence of some hydrolysates or breakdown of complex proteins present in egg white by chymotrypsin as observed from SDS-PAGE pattern. The inability of C. perfringens to germinate and outgrow during egg white hydrolysis with trypsin could be explained due to the presence of various antimicrobial factors in the egg white which act as antimicrobial like lysozyme. Egg white proteins were present in their native state due to inhibition of trypsin activity during hydrolysate preparation process. Lysozyme is a 14.6 kDa single peptide that can cause microbial cell lysis by cleavage of β (1–4) glycosidic linkages between N-acetylmuramic acid and N-acetylglucosamine in the peptidoglycan layer of the bacterial cell wall (Proctor and Cunningham, 1988). Lysozyme has been successfully used in the cheese industry as a bio-protectant for more than 20 years to control Clostridium tyrobutyricum that causes the ‘late blowing’ of hard and semi-hard cheeses as a result of butyric fermentation (Carini et al., 1985). Literature on the inhibitory effect of lysozyme against C. perfringens is limited (Osa et al., 1990), although the ability of lysozyme to inhibit several other Clostridium species including C. tyrobutyricum, C. thermosaccharolyticum and C. botulinum has been well documented (Carini et al., 1985; Hughey and Johnson, 1987). Zhang et al. (2006) showed that lysozyme can inhibit C. perfringens growth and destroy the vegetative cells. Iron binding protein (ovotransferrin) present in egg white can be another reason for the ability of C. perfringens to grow during trypsin hydrolysis of egg white. Even though egg white
hydrolysis with chymotrypsin resulted in degradation of ovotransferrin and lysozyme into smaller peptides, *C. perfringens* spore germination and outgrowth was not observed. It can be due to antimicrobial activity of smaller peptides obtained due to the hydrolysis of lysozyme (Mine et al., 2004).

In contrast, *B. cereus* populations (before heat shock) increased in both types of egg white hydrolysates preparation using trypsin or chymotrypsin by 0.27 and 3.72 log CFU/ml, respectively (Figs. 8 and 9). Also, *B. cereus* counts (after heat shock) increased by 0.15 and 1.18 log CFU/ml, respectively for the same. *B. cereus* survived the enzyme inactivation step subsequent to enzymatic hydrolysis carried out with chymotrypsin. Spores of *B. cereus* are resistant to egg-white lysozyme and can grow in presence of lysozyme (Buchanan and Gibbons, 1974). Hughes (1971) demonstrated the resistance of *B. cereus* cell walls to lysozyme at concentrations as high as 100µg/ml. This resistance is specifically due to the deacetylation of N-acetylglucosamine residues of the wall peptidoglycan (Araki et al., 1972; Hayashi et al., 1973) and is responsible for resistance of *B. subtilis* and *B. megaterium* to lysozyme (Hayashi et al., 1973). Greater increase in *B. cereus* population was observed during hydrolysis with chymotrypsin compared to trypsin. The differences in *B. cereus* growth can be attributed to degree of hydrolysis or break down of egg white proteins into small proteins and the resulting pH reductions. Chymotrypsin hydrolysis of egg white resulted in a pH decrease from 7.8 to 6.5 conducive to the growth of *B. cereus* optimum pH for growth is from 6 to 7. However, similar reduction in pH was not observed during trypsin hydrolysis, probably due to the minimal hydrolysis observed with trypsin.
The difference in growth of *C. perfringens* and *B. cereus* could be due to aerobic conditions followed during hydrolysis. *C. perfringens* grows well under anaerobic conditions, while aerobic conditions favor *B. cereus* growth. Ovotransferrin, a biologically active protein of egg white is important in the inhibition of microbes (Board and Hornsey, 1979) as it deprives microorganisms of Fe$^{3+}$, a property that is accentuated by an alkaline nature (pH 9-10) of avian egg whites (Sharp and Whitaker 1927). Tranter and Board (1981) reported that chelation of Fe$^{3+}$ by ovotransferrin in the egg white influenced swelling of germinating *Bacillus* spores and prevented the outgrowth to normal vegetative cells. An increase in both length and breadth of the spores was observed in egg white, as the germinating spore entered the second phase of swelling, resulting in a ‘balloon like’ form. Finally, the germination of the spore continued, although outgrowth was inhibited.

Iron in the ferrous form (Fe$^{2+}$) present in most of the biological fluids (near pH 7) is oxidized to ferric (Fe$^{3+}$) in equilibrium with atmospheric oxygen and later will be hydrolysed to form polynuclear complexes of extremely low solubility (Spiro and Saltman, 1969). Such iron is not available for microorganisms unless they are able to solubilize iron from these polymers by the formation of powerful chelates, known as siderophores (Snow, 1970; Neilands, 1972; Lankford, 1973). In most cases, these chelates are excreted into the medium where they solubilize iron before incorporation into the cell through specific transport systems (Cox et al., 1970; Langman et al., 1972; Kadner and Bassford, 1978). In an iron deficient environment like egg white due to the presence of transferrin, microorganisms have to compete with ovotransferrin for iron, an
element essential for microbial growth. Microbial iron-transport compounds play an important role in reversing the bacteriostatic action of ovotransferrin. Tranter and Board (1981) stated that the three main stages in the conversion of *B. cereus* spore to vegetative cell are germination, swelling and outgrowth. Only the last two stages are affected by hen egg white (Hitchins et al., 1963). Saturation of ovotransferrin with iron relieves the inhibition of bacterial growth in egg white (Garibaldi, 1960; Board, 1964). The high pH of the egg white and its iron-deficient state are responsible for inhibition of *B. cereus* outgrowth during egg white hydrolysate preparation using trypsin.

In conclusion *C. perfringens* did not germinate during egg white hydrolysis with either trypsin or chymotrypsin. *B. cereus* was able to germinate and outgrow during egg white hydrolysate preparation with chymotrypsin, but lower growth was observed during trypsin initiated egg white hydrolysis. Trypsin was unable to hydrolyze egg white proteins while chymotrypsin hydrolysed most of the egg white proteins within 4 h.
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Figure 4.1. Changes in SDS-PAGE pattern of egg white hydrolysates obtained from chymotrypsin hydrolysis on 16.5% gel concentration. First three lanes from left represent Markers (Mol. wt. mentioned to the right of bands in kDa). EW: egg white (1%) solution in water; Chy1 (0.125 %) and Chy2 (1.25 %): chymotrypsin solution in water; PD (0, 4, 8, 12, 18 and 24): egg white protein digest at different time points during 24 h hydrolysis with chymotrypsin.

Figure 4.2. Changes in SDS-PAGE pattern of egg white hydrolysates obtained from chymotrypsin hydrolysis on 10-20% gel concentration. First three lanes from left represent Markers (Mol. wt. mentioned to the right of bands in kDa). EW: egg white (1%) solution in water; Chy1 (0.125 %) and Chy2 (1.25 %): chymotrypsin solution in water; PD (0, 4, 8, 12, 18 and 24): egg white protein digest at different time points during 24 h hydrolysis with chymotrypsin.
Figure 4.3. Changes in SDS-PAGE pattern of egg white hydrolysates obtained from trypsin hydrolysis on 16.5% gel concentration. First three lanes from left represent Markers (Mol. wt. mentioned to the right of bands in kDa). EW: egg white (1%) solution in water; try1 (0.025 %) and tryt2 (0.25 %): trypsin solution in water; PD (0, 4, 8, 12 and 24): egg white protein digest at different time points during 24 h hydrolysis with trypsin.

Figure 4.4. Changes in SDS-PAGE pattern of egg white hydrolysates obtained from trypsin hydrolysis on 10-20% gel concentration. First three lanes from left represent Markers (Mol. wt. mentioned to the right of bands in kDa). try1 (0.025 %) and tryt2 (0.25 %): trypsin solution in water; PD (0, 4, 8, 12 and 24): egg white protein digest at different time points during 24 h hydrolysis with trypsin.
Figure 4.5. Changes in pH values during egg white hydrolysates preparation
Figure 4.6. Survival of *C. perfringens* in egg white hydrolysates preparation using trypsin
Figure 4.7. Survival of *C. perfringens* in egg white hydrolysates preparation using chymotrypsin
Figure 4.8. Survival of *B. cereus* in egg white hydrolysates preparation using trypsin
Figure 4.9. Survival of *B. cereus* in egg white hydrolysates preparation using chymotrypsin
CHAPTER V

SUMMARY AND RECOMMENDATIONS
In the first part of the research, the dynamic model for the growth of *Salmonella* spp. in liquid whole egg was developed and validated. Additional research can be done in direction of developing dynamic growth predicting models for different blended egg products. Also, the validation of present dynamic model can be done for real time profiles followed by various food or egg processors.

In the second part of the research, risk associated with egg white hydrolysates manufacturing process was evaluated for spore formers like *B. cereus* and *C. perfringens*. This study clearly showed that under optimal conditions of enzymes like chymotrypsin followed for egg white hydrolysis can result in favorable conditions for certain pathogens to grow. Further the effect of pH change in liquid whole egg on the growth of *B. cereus* can be studied. Also the peptides obtained during egg white hydrolysis can be analysed further for their ACE-inhibitory activity. Other enzymes can also be analyzed for their ability to hydrolyse egg white protein.

The presence of toxins produced by *B. cereus* can also be studied to evaluate the safety of egg white hydrolysates against toxins and its rate of production. The thermal treatment involved during inactivation of enzymes (100°C/15 min) can kill spores or vegetative cells of spore formers but is ineffective to destroy toxins produced by these organisms.
APPENDIX
A.1. A sample SAS code for fitting the growth data of *Salmonella* spp. in the Baranyi model at 37°C

```sas
data egg37;
input t logy;
CFU = 10**logy;
lny=log(CFU);
datalines;
0 2.322219295
2 2.653212514
4 3.51851394
6 3.579783597
8 4.431363764
10 5.62324929
12 6.812913357
14 8.190331698
16 8.301029996
18 9.041392685
20 8.740362689
22 8.77815125
24 8.819213357
0 2.431363764
2 2.86923172
4 3.139879086
6 3.255272505
8 3.755874856
10 5.77815125
12 6.681241237
14 7.544068044
16 8.322219295
18 8.792391689
20 8.698970004
25.5 8.86332286
;
title "Baranyi fitting";
proc nlin data=egg37 method=marquardt;
parms yo=5.45 ymax=20.2 mumax=1.31 h0=5.26;
model lny=yo+mumax*t+log(exp(-mumax*t)+exp(-h0)-exp(-mumax*t-h0))-log(1+(exp(mumax*t+log(exp(-mumax*t)+exp(-h0)-exp(-mumax*t-h0)))-1)/(exp(ymax-yo)));
output out=baranyimodel30 parms=yo ymax mumax h0 p=predbar r=residbar;
run
```
A.2. Code for fitting the growth data of *Salmonella* spp. in the Baranyi model at 37°C after fixing $h_o$

data egg37;
input t logy;
CFU = 10**logy;
lny=log(CFU);
datalines;
0  2.322219295
2  2.653212514
4  3.51851394
6  3.579783597
8  4.431363764
10 5.62324929
12 6.812913357
14 8.190331698
16 8.301029996
18 9.041392685
20 8.740362689
22 8.77815125
24 8.812913357
0  2.431363764
2  2.86923172
4  3.139879086
6  3.255272505
8  3.755874856
10 5.77815125
12 6.681241237
14 7.544068044
16 8.322219295
18 8.792391689
20 8.698970004
25.5 8.86332286
;
title "Baranyi fitting";
proc nlin data=egg37 method=marquardt;
parms yo=6.34 ymax=20.23 mumax=1.31; h0=3.51;
model lny=yo+mumax*t+log(exp(-mumax*t)+exp(-h0)-exp(-mumax*t-h0))-
log(1+(exp(mumax*t)+log(exp(-mumax*t)+exp(-h0)-exp(-mumax*t-h0))))/exp(ymax-yo));
output out=baranyimodel30 parms=yo ymax mumax h0 p=predbar r=residbar;
run;
A.3. SAS code for fitting the maximum specific growth rates in the modified Ratkowsky equation

data secondary;
input T mu;
sqmu = sqrt(mu);
datalines;
10  0.04
15  0.15
20  0.32
25  0.506
30  0.83
35  1.12
37  1.315
39  1.23
41  1.01
43  0.8
;
title "ratkowsky-Bara";
proc nlin data=secondary method=marquardt;
parms b = 0.0218 Tmin = 10 Tmax = 43 c=1;
model mu=b*((T-Tmin)**2)*(1-exp(c*(T-Tmax)));
output out=fullmodel parms=b c Tmin Tmax p=predfull r=resifull;
run;
A.4. Example of the MATLAB codes for Ratkowsky-Baranyi dynamic model

```matlab
t = 0:15:20*60;
T = (17.5*sin(2*pi.*t/(4*60)))+27.5;
Tempvec = [t' T'];
primod = [2.63, 8.35, 1.7788];
secmod = [2.8988, 47.6981, 0.00164, 0.3706]
out = baranyi_dyn(Tempvec, secmod, primod)
```

```matlab
function out = baranyi_dyn(Tempvec, secmod, primod)

% time - time vector in minutes
% temp - temperature vector in degrees celcius
% Secondary model: mu = a(T-Tmin)^2. (1-exp(b(T-Tmax)))
% a, b, Tmin, and Tmax are parameters of the secondary model
% yo - initial innoculum value in log(CFU/g)
% ymax - maximum microbial population in log (CFU/g)
% h - paremeter in the Baranyi model.

close all
clc;
TIME_min = Tempvec(:, 1);
Temp_C = Tempvec(:, 2);
Tmin_C = secmod(1);
Tmax_C = secmod(2);
a = secmod(3);
b = secmod(4);
y0 = primod(1);
ymax = primod(2);
h0 = primod(3);
clc;

% Define Time Step
dt_min = 15;
dth = dt_min/60;

% Create time vector - user input - it is assumed in uniform time steps

```
csT = spline(t,Temp_C);
TT = ppval(csT,tt);

n = length(TT);
tfinal = tt(n)

% SOLVE DYNAMIC BARANYI'S MODEL USING RUNGE-KUTTA 4th ORDER

% Spline interpolation of temperature to obtain midpoints of each time interval
% Oversampling the time and temperature vectors by a factor of two for
% calculation purposes

csT = spline(tt,TT);
t1 = [t(1):dth/2:tfinal]; % new time vector containing midpoint values
T1 = ppval(csT,t1) % new temperature vector containing midpoint values
nn = length(t1);

% Discretized Secondary Model
% secondary model predicts growth rate as a function of temperature
% determine the growth rate for the oversampled temperature vector.

% initialize growth rate vector
mumax = [];

for i = 1:length(T1)
  if T1(i) > Tmax_C
    mumax(i) = 0;
  elseif T1(i) >= Tmin_C
    mumax(i) = a*(T1(i) - Tmin_C)^2*(1-exp(b*(T1(i) - Tmax_C)));
  elseif T1(i) < Tmin_C
    mumax(i) = 0;
  end
end

% Numerical Solution of dQ/dt using RK4
% Define Initial Condition

% "work to be done", constant for given microorganism & given substrate
% h0 = 1.7788;

alpha = exp(-h0) % initial physiological state of cells
q0 = 1/(exp(h0)-1); % initial bottleneck substance
Q = [];
Q(1) = log(q0);  % initial condition for dQ/dt

% Runge-Kutta algorithm
i = 1;
for j = 2:n

% PREDICTION
k1 = dth*(mumax(i));
k2 = dth*(mumax(i+1));
k3 = dth*(mumax(i+1));
k4 = dth*(mumax(i+2));
i = i+2;

% CORRECTION
Q(j) = Q(j-1) + ((1/6)*(k1 + (2*k2) + (2*k3) + k4));
end

% Numerical Solution of dy/dt using RK4
% Define Initial Condition
y = [ ];
y(1) = log(10^y0);  % initial condition for dy/dt - user input

% Define Maximum Cell Population - constant for given microorganism
% & given substrate.
ymax = log(10^ymax);

% Spline interpolation of Q to obtain midpoints of each time interval
% Oversample or increase the length of Q vector by a factor of two

csQ = spline(tt,Q);
t1 = [t(1):dth/2:tfinal]; % new time vector containing midpoint values
QQ = ppval(csQ,t1);  % new Q vector containing midpoint values

% Runge-Kutta algorithm
i = 1;
for j = 2:n

% PREDICTION
k1 = dth*(mumax(i))*(1/(1+exp(-QQ(i))))*(1-exp(y(j-1)-ymax));
k2 = dth*(mumax(i+1))*(1/(1+exp(-QQ(i+1))))*(1-exp(y(j-1)+(k1/2)))-
ymax));
k3 = dth*(mumax(i+1)*(1/(1+exp(-QQ(i+1))))*(1-exp((y(j-1)+(k2/2))-ymax)));
k4 = dth*(mumax(i+2)*(1/(1+exp(-QQ(i+2))))*(1-exp((y(j-1)+(k3))-ymax)));
i = i+2;

% CORRECTION
y(j) = y(j-1) + ((1/6)*(k1 + (2*k2) + (2*k3) + k4));
end

ypred = y./log(10);
out = [tt' ypred'];

% FIGURE
figure, plotyy(tt,ypred,tt,TT);
grid on
xlabel('Time (h)')
ylabel('log(CFU)/mL')
title ('Time (h), Temp (C), log (CFU)/mL')