ABSTRACT

Title of Thesis: ENHANCEMENT OF THERMAL INACTIVATION OF *CRONOBACTER SAKAZAKII* WITH INCLUSION OF PARABENS

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Master of Science 2016

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Parabens are a family of p-hydroxybenzoic acid esters, which have antimicrobial activity over a broad pH range (4-8). This study was designed to evaluate the enhanced thermal inactivation of *Cronobacter sakazakii* by the inclusion of "parabens" and to ultimately develop mathematical models to describe this effect. A heat-resistant strain, *Cronobacter sakazakii* 607, was heated at three mild heating temperatures in combination with treatments with five parabens in various concentrations. Results showed the presence of parabens significantly enhanced thermal inactivation in a concentration-dependent manner, and the effect increased with increasing alkyl chain length. The concentration of parabens, alkyl side chain length, and heating temperature acted synergistically, causing bacterial inactivation even at low temperatures that were not effective in killing *C. sakazakii*. The survival

data were used to develop primary and secondary mathematical models that accurately describe how this synergistic activity can be applied in the food industry.

ENHANCEMENT OF THERMAL INACTIVATION OF *CRONOBACTER SAKAZAKII* WITH INCLUSION OF PARABENS

By

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Thesis submitted to the Faculty of the Graduate School of the
University of Maryland, College Park, in partial fulfillment
of the requirements for the degree of
Master of Science
2016

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Foreword

My advisor, Dr. Buchanan used to say that, “time management is the most critical skill you should master in your graduate life”. As a graduate student, I devoted a considerable amount of my time in doing research for the past two and a half years. It is quite common to encounter difficulties while pursuing advanced knowledge. When someone is lost in the woods, the guidance of a wise man will lead him to the correct direction with fewer words and gesture. Luckily, Dr. Buchanan is that wise man. It is hard to forget the smile on his face every time I got confused with my project. He always provided comfort and patient guidance. Over the past two years, I have experienced a delay of my visa. That delay killed my bacteria strains. And I broke my automatic submerged coil apparatus. For that mistake, I had to manually rotate the carousel of that machine. Serving (twice) as a teaching assistant for a food microbiology laboratory occupied most of my daytime. However, “only experienced a hellish temper, in order to have the power to create heaven; only the blood flow through the fingers to pop the world farewell” (Rabindranath Tagore). I survived. Other than wisdom, his positive life attitude and energetic spirit moved me so much. Whenever life gives me a scared surprise, I should embrace and process it with positive energy. Complaining is not helpful in many situations whereas finding a solution is the direction that I should move on.

Dedication

Here I dedicate my two and half year research experience and this work to all the people around me for supporting, guiding, helping and encouraging me in all my academic pursuits and dreams. To my loving parents, Rongping & Lin, my trustable and lovely friends Yuqing, Yangyang, Aixia and so on; this is for you all.

Acknowledgements

I would like to take this opportunity to acknowledge some of the lovely people around me. Without them, completion of a Master degree myself would be miserable. First of all, I want to express my sincerest gratitude to Dr. Robert L. Buchanan, my advisor, who provided me the opportunity to pursue my goal of a graduate life and helped me broaden my view of food microbiology and modeling. He is incredibly supportive and guides me towards the right track of my research whenever I get lost. And also, I appreciate Dr. Buchanan for spending time and effects on revising my thesis.

My great thankfulness also goes to the members of my defense committees: Dr. Robert L. Buchanan, Dr. Abani K. Pradhan, Dr. Rohan V. Tikekar, and Dr. Lihan Huang, who are willing to spend their precious time to attend this thesis defense.

To all my sweet labmates and friends: Yangyang Wang, Aixia Xu, Yuqing Ying, Xiaojing Jiang, Ruolin Wang, Min Yan, Abhinav Mishra Miao Guo, Yinzhi Qu and Zhiyuan Lou, your moral support is appreciated.

Last but not least, I would like to dedicate appreciation to my family who supported me all the time in the last two and a half years. Although you are thousands miles away and in a different time zone, your encouragement, support and love kept me overcoming obstacles throughout my graduate study.

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Chapter 1: Introduction

1.1 Background

Cronobacter sakazakii is a gram-negative, rod-shaped bacterium. It has been linked to fatal invasive infections in infants after consuming powdered infant formula (PIF) contaminated with low levels of contamination. While a rare foodborne disease, infected infants suffer from potentially life threatening bacteremia, meningitis and necrotizing colitis, which can result in permanent neurological damage and death. It is controlled through effective pasteurization and good manufacturing practices (GMP) during production of infant formula. The thermal resistance of *C. sakazakii* strains in rehydrated PIF can vary substantially (*30*, *54*, *65*, *92*). Determined using a submerged coil apparatus, the D_{58}-values ranged from 30.5 to 591.9 sec, with *C. sakazakii* 607 being the most heat resistant strain (*30*). Reducing the thermal resistance of *C. sakazakii* would allow PIF to be processed at lower temperatures and may provide a potential means of providing a post-final packaging pasteurization of PIF.

Parabens are a group of p-hydroxybenzoic acids, which possess both antioxidant and antimicrobial activity. They are widely used in foods, pharmaceuticals, and cosmetics, such as food colorings, antacids, toothpastes, mouthwash, hair care products, soaps, body washes, lotions, and eye drops. Past research has established that a shorter heat treatment achieved a desired log-reduction of *Salmonella enteritidis* and *S. oranienburg* in liquid egg albumen when propyl-paraben was added (*49*). In addition, the relative antimicrobial activity of the parabens increases with the

length of alkyl side chain (e.g. methyl, ethyl, propyl, butyl, and heptyl) (*21*, *33*). Parabens have antimicrobial activities over a pH range of 4~8 (*81*). This makes parabens a potentially desirable antimicrobial agent in various food applications.

Thermal treatment is one of the most effective methods to ensure food safety. The effectiveness of theral treatment can be enhanced in antimicrobial agents such as parabens. Therefore, the purpose of this study was to quantitatively characterize the enhanced thermal inactivation of *C. sakazakii* by the inclusion of parabens and to develop mathematical models that describe the effect of parabens on the survival of this microorganism.

1.2 Hypothesis

The hypothesis of this research is that the thermal inactivation of *Cronobacter sakazakii* 607 may be significantly enhanced in the presence of parabens and a synergistic effect may exist between heat treatment and this antimicrobial agent. The rationale of the hypothesis is that a combination of two sub-lethal treatments can be more lethal for *C. sakazakii*.

1.3 Study Approach

Cronobacter sakazakii 607 (heat-resistant) was subjected to isothermal heating under three temperatures (52°C, 55°C, 58°C) in the presence of five parabens (methyl, ethyl, propyl, butyl, and heptyl) at various concentrations. Thermal inactivation was

conducted with a submerged coil apparatus using brain heart infusion (BHI) as the heating menstruum. Surviving cells were surface plated on tryptic soy agar (TSA) and MacConkey agar (MA). After enumeration, survivor curves were plotted and compared using Microsoft Excel®. Primary and secondary models were developed using the Integrated Pathogen Modeling Program (IPMP).

1.4 Potential Impact of Study

High temperature treatment can destroy sensitive nutrients and cause unpleasant sensory defects. Mild heat treatment is desirable for improving the quality of thermally processed foods. If the hypothesis of this study is verified, the results from this research could be applied to thermal processing of nutrient-rich foods, including PIF. A combination of mild heat and inclusion of parabens could effectively kill *C. sakazakii* to meet the safety requirements of food without loss of quality. Additionally, the predictive models can be applied in thermal processing and to predict the efficacy of mild heat treatment in the presence of parabens.

Chapter 2: Literature Review

2.1 The Problem

Over the past several decades, infections with *C. sakazakii* have been associated with contaminated powdered formula products (*40*). According to Bowen and Braden (*11*), among 46 infant cases of *C. sakazakii* infection from 1961 to 2005, including twenty four (24) cases occurred in the U.S. Clinical isolates were obtained from prepared formula (2 cases), opened formula tins (6 cases), and previously unopened formula tins (7 cases) (*11*).

Although *Cronobacter* infections are generally rare, they are frequently lethal for neonates and can be serious among susceptible groups such as people who are immunocompromised and the elderly. The primary symptoms are severe bacteremia and meningitis, with case fatality rates as high as between 40 and 80% (*11*).

Thermal treatment is used as a primary processing means of reducing the risks associated with foodborne pathogens. Thus it has been identified as a practical means of reducing the risk of *C. sakazakii* in infant formulas (*30*). Contrary to common misconceptions among consumers, powdered infant formula (PIF) is not a sterile product, and occasionally contains pathogens capable of causing infections (*92*). The source of *Cronobacter* could be from contaminated raw ingredients or contaminated surfaces in the manufacturing environment. A disadvantage of high temperature treatment prior to dehydration is that it could potentially damage some sensitive

nutrients (*60*). To help control the possible contamination, a combination of mild heat treatment with antimicrobial substances is an area of increased interest in the food industry (*92*).

2.2 The Pathogen

Cronobacter spp. are Gram-negative, rod-shaped, facultative-anaerobic, non-spore-forming bacteria belonging to the family *Enterobacteriaceae*. *Cronobacter sakazakii* was originally classified as *Enterobacter cloacae* but was subsequently designated a separate species (*Enterobacter sakazakii*) on the basis of yellow pigment production, DNA-DNA hybridization, and other phenotypic characteristics (*37*, *38*). *Cronobacter* was proposed as a new genus to include the organisms formerly classified as *Enterobacter sakazakii* in 2007*,* initially comprising eight different organisms including four named species, one unnamed species, and five named subspecies (*39*). Up to 2015, the genus comprises ten species: *Cronobacter sakazakii*, *Cronobacter dublinensis*, *Cronobacter malonaticus*, *Cronobacter muytjensii*, *Cronobacter turicensis*, *Cronobacter universalis, Cronobacter pulveris, Cronobacter zurichensis, Cronobacter helveticus,* and *Cronobacter condiment* (*13*, *58*, *62*)

The microorganism is common among the broad group of coliforms species based on lactose fermentation and resistance to bile salts (*30*). Due to the ubiquitous nature of *C. sakazakii* in inanimate (water, soil, plants) and animate (animals, humans) environments, this microorganism is frequently detected in food production units and a wide spectrum of food, food ingredients, and beverages of animal and vegetable

origins (*41*).

Cronobacter spp. are noted for their ability to survive for extended periods in very dry conditions and have been found in a variety of dry foods, including powdered infant formula, skimmed milk powder, herbal teas, and starches (*20*). A long-term experiment showed that the organism, inoculated at an initial level of 10^6 CFU/ml (when rehydrated) to a PIF product, survived a 2-year storage at room temperature (*31*). Approximately 300 CFU/ml was recovered in the reconstituted product, suggesting a 3.5-log reduction over the 2 years of storage (*31*). Another study found that in PIF, *C. sakazakii* has the highest desiccation tolerance compared to *Listeria monocytogenes*, *Escherichia coli* O157:H7 and *Salmonella enterica*, showing 0.6, 2.3, and 3.6 log reductions at 5, 22, and 35 °C during a 1-year storage period (*65*). Other studies also confirmed that the high tolerance to desiccation provided a competitive advantage for *C. sakazakii* to survive in the dry environment and the accumulation of trehalose in the cells may be linked to the dry resistance (*15*). In general, *C. sakazakii* can grow and survive in a wide range of environmental conditions (Table 2-1).

Table 2-1. Growth and survival conditions of *Cronobacter sakazakii* (*83*)

Growth kinetics	*Optimum*	*Range*
Temperature (°C)	39.4 °C	5.5~45 °C
pH	5~9	Minimum: 3.89
Atmosphere	Aerobic	Aerobic and anaerobic
Water activity (a_w)	0.99	Maximum salt conc. 9.1%
Generation time	20 min	5 h at 10 °C

2.3 Kinetics of Thermal Inactivation

2.3.1 D-value

When bacterial cells or spores are placed in a lethal temperature environment they die exponentially in relation to time. The population of survivors is generally plotted after log-transformation of the bacterial counts. The decrease is typically log-linear over most if not all of a thermal treatment. The D-value is the decimal reduction time, or the time required to destroy 90% of the organisms under a constant temperature. This value is numerically equal to the time required for the survivor curve to traverse one log cycle (*61*). Mathematically, it is equal to the negative reciprocal of the slope of the survivor curve, and is a measure of the death rate of an organism.

2.3.2 z-value

Under the conditions of first order inactivation kinetics, a value that is used to describe the relationship between thermal reduction time (D-values) and heating temperature is the z-value. The z-value refers to the increase in temperature required to cause a one log reduction in the D-values (*61*). It is calculated by developing a thermal death time (TDT) curve that depicts how the log D-value varies with temperature. Mathematically, this value is equal to the negative reciprocal of the slope of the TDT curve. Whereas the D-value reflects the resistance of an organism to a specific temperature, the z-value provides information on the relative resistance of an organism to different temperatures.

2.4 Thermal Resistance

Currently, thermal processing is the most commonly used method for food preservation. It is an efficient and reliable process to reduce the risks associated with foodborne pathogens and inactivate the activity of enzymes, and it is also an economical technology for the food industry (*3*). The effective use of thermal treatments requires accurate information of the heat resistance of the target microorganism. A thermal treatment should be sufficient to inactivate the microorganism of concern while minimizing the loss of nutrients and sensory attributes. However, foodborne pathogens may resist to heat to a different degree. Several factors can influence the heat resistance of a bacterium. These factors may include variations among different species and strains, age of culture, and adaptation history (incubation temperature and cross-protection). The heat resistance of several well-known foodborne bacteria, such as *Listeria monocytogenes*, *Salmonella spp*. and *Escherichia coli* O157:H7, is well referenced in the literature (*27*, *28*).

Strains of *Cronobacter sakazakii* are among the most thermotolerant members of the *Enterobacteriaceae*. The D-values and z-values have been reported by several research groups. Edelson-Mammel and Buchanan (*30*) reported that the D-values at 58 °C for 12 strains of *C. sakazakii* ranged from 30.5 s to 591.9 s, among which *C. sakazakii* 607 was the most heat resistant strain with a z-value of 5.6 °C. The 12 strains were divided into two groups, heat-resistant strains having $D_{58°C} \geq 300$ s and non-heat resistant strains with $D_{58°C} \leq 50$ s (*30*). Breeuwer et al. (*15*) reported the $D_{58°C}$ of *C. sakazakii* 1387-2 was 0.58 h in pH 7 phosphate buffer and 0.5 h in

reconstituted infant formula. Compared to *Salmonella spp*. and *E. coli*, *C. sakazakii* possessed the smallest z-values (*15*). Table 2-2 summaries the D-values and z-values for a variety of *C. sakazakii* strains.

The results of Arroyo et al. (*3*) indicate that the heat resistance of *C. sakazakii* is affected by both intrinsic and extrinsic factors, including bacterial strain, growth conditions and stage (phase and temperature), the characteristics of substrate (eg. pH, a_w), and the recovery conditions. Reducing the pH, or acidification of the substrate decreases its heat resistance, whereas decreasing the water activity shows the opposite effect (*3*). Although the mechanisms are not fully known, it has also been proposed that the interaction of trehalose (a non-reducing disaccharide of glucose) with membrane phospholipids helps stabilize the cell membrane (*23*). Moreover, Arroyo et al. (*3*) demonstrated that increasing the treatment temperature decreases the proportion of sublethally damaged *C. sakazakii* cells within the surviving population. A combination of heating and antimicrobials may enhance the inactivation of the microorganism due to sublethal injury (*3*).

Table 2-2. Summary of D-values and z-values for a variety of *C. sakazakii* strains

Strain	*Temperature (°C)*	*D-value*	*z-value (°C)*	*Medium & Source*
51329	58	30.5 s	N/A	Medium:
NQ2-Environ	58	31.5 s	N/A	rehydrated infant
NQ3-Envrion	58	34.4 s	N/A	formula
LCDC 674	58	36.9 s	N/A	(*30*)
CDC A3	58	37.5 s	N/A	
NQ1-Environ	58	47.9 s	N/A	
EWFAKRC11NNV1493	58	307.8 s	N/A	
29544	58	367.1 s	N/A	
SK 90	58	465.4 s	N/A	
LCDC 648	58	540.9 s	N/A	
4.01C	58	571.9 s	N/A	
607	56	1263.2 s	5.6	
	58	591.9 s		
	60	264.6 s		
	65	35.2 s		
	70	3.9 s		
1387-2	53	20.2 h	3.1	Medium:
	54	7.1 h		phosphate buffer
	56	2.4 h		*medium is
	58	0.48 h		reconstituted
1387-2*	58	0.50 h	N/A	infant formula
16	53	8.3 h	3.6	(*15*)
	54	6.4 h		
	56	1.1 h		
	58	0.40 h		
1360	58	0.34 h	N/A	
145	58	0.27 h	N/A	
NCTC 11467	54	14.9 min	5.6	Medium: TSB
	56	2.7 min		(*57*)
	58	1.3 min		
	60	0.9 min		
	62	0.4 min		
NCTC 11467	54	16.4 min	5.8	Medium: Infant
	56	5.1 min		formula milk
	58	2.6 min		(*57*)
	60	1.1 min		
	62	0.3 min		
Cocktail culture (51329	52	7.36 min	4.22	Medium:
and 4 food isolates) heat	54	2.07 min		rehydrated infant
stressed	56	0.76 min		milk formula
	58	0.27 min		(Shaker et al.,
				2008)

2.5 The Parabens

2.5.1 Introduction

Paraben is defined as a series of parahydroxybenzoates or esters of parahydroxybenzoic acid. Its basic structure consists of a benzene ring, a hydroxyl group in the para (#4) position, and an ester group with a changeable R (Figure 2-1.).

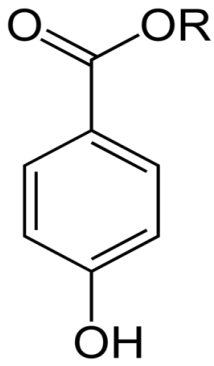


Fig. 2-1. Chemical structure of parabens

As the alkyl substituents change from methyl to butyl or even heptyl, methyl-paraben, ethyl-paraben, propyl-paraben, butyl-paraben and heptyl-paraben are formed. Main physicochemical characteristics of parabens are shown in Table 2-3.

Table 2-3. Physicochemical properties of parabens used in this study (*2*, *94*)

Characteristic	***Methyl***	***Ethyl***	***Propyl***	***Butyl***	***Heptyl***
Chemical formula	$C_8H_8O_3$	$C_9H_{10}O_3$	$C_{10}H_{12}O_3$	$C_{11}H_{14}O_3$	$C_{14}H_{20}O_3$
Molecular weight (g/mol)	152.16	166.18	180.21	194.23	236.31
pKa	8.17	8.22	8.35	8.37	N/A
Solubility (25°C, g/100ml)	2.00	0.86	0.30	0.15	N/A
Melting point (°C)	131	116-118	96-98	68-69	N/A
Boiling point (°C)	270-280	297-298	N/A	N/A	N/A
Octane/water partition coefficient (log value)	1.96	2.47	3.04	3.57	N/A

Although parabens can be derived from natural sources such as a *Microbulbifer* bacterium, blueberries, and root hairs of the New Zealand yam (*4*, *85*, *86*), the commercial para-benzoates are produced synthetically. Parabens were firstly synthesized in 1924 as alternative preservatives for salicylic and benzoic acids (*73*).

They quickly gained acceptance not only in food processing but also in the pharmaceutical and cosmetic industries (*73*). The most commonly used parabens are the methyl, ethyl, and propyl along with their sodium salts. What determined the wide application of parabens as preservatives was their powerful bactericidal and fungicidal properties along with their resistance to the high temperatures used in food processing, and their compatibility with other preservatives such as sorbates and benzoates (*73*). For instance, propyl-paraben is used as a preservative in FDA-approved food colorings, antacids, adult and child toothpastes, teeth whitener and mouthwash, cosmetics and hair care products, soaps, body washes, lotions, over-the-counter oral medications, and eye drops (*49*). According to Davidson (*24*), propyl-paraben has been applied to a variety of foods, including bakery products, beverages, cakes, crusts, pastries, toppings, fillings, fish, flavorings, fruit products, jams, jellies and preserves, malt extracts olives, pickles, salad dressings, sorbitol, and syrups.

2.5.2 Antimicrobial profile

Parabens are an effective antimicrobial agent for both bacteria and fungi. The minimum inhibitory concentration for methyl-paraben is 1000-4000 μg/ml for a range of pathogenic foodborne bacteria (*25*). Methyl-paraben inhibits the growth of four psychrotrophic foodborne pathogens, including *Listeria monocytogenes*, *Yersinia enterocolitica*, *Aeromonas hydrophila* and nonproteolytic *Clostridium botulinum* (*81*).

Propyl-paraben is more effective than methyl-paraben, with up to 1000 ppm of the former and 1000-4000 ppm of the latter needed for bacterial inhibition (*25*). In general, Gram-positive bacteria are more susceptible than Gram-negatives to the parabens. In a reduced-broth medium, 100 ppm propyl-paraben delayed the germination and toxin production by *Clostridium botulinum* type A, and 200 ppm inhibited its growth for 120 hours at 37 °C (*89*). Equivalent inhibition by methyl-paraben requires levels of 1200 ppm (*89*). While few data have been published on the antimicrobial activity of heptyl-paraben, it appears to be quite effective at 10-100 ppm levels for inhibiting both Gram-positive and Gram-negative bacteria (*61*). Heptylparaben is reported to be effective against the malo-lactic bacteria as well (*61*).

Parabens are more effective against yeast and mold. Propyl-paraben appears to be the most effective with 100 ppm or less capable of inhibiting some yeasts and molds, whereas 50-200 and 500-1000 ppm are required for heptyl- and methyl-parabens, respectively (*61*). Parabens can suppress a broad range of foodborne fungi, including *Aspergillus*, *Byssochlamys*, *Candida*, *Fusarium*, *Penicillium*, *Rhizopus*, *Saccharomyces*, and *Zygosaccharomyces*. An *in vitro* study concluded that propyl-paraben completely inhibited fumonisin production by both *Fusarium verticillioides* and *Fusarium proliferatum* at > 180.2 ppm, regardless of the temperature or a_w level (*36*). Parabens act on the germinative and vegetative phases of fungal growth (*93*).

Parabens have a notable advantage over sorbate and other relatively pH-dependent antimicrobials due to its chemical structure. Organic acids are typically more active

against microorganisms when the acids are in their undissociated form (*91*). A large proportion of paraben is in the undissociated form at pH values of most foods because its pKa is 8.5, whereas the antimicrobial activity of sorbic acid (pKa 4.74) diminishes as the pH rises towards neutrality (*81*). Thus, parabens are effective over a wide range of pH, ranging from 4.0 to 8.0 (*1*). As the chain length of the ester group of paraben increases, the antimicrobial activity increases, while the water solubility decreases (*33*).

In addition, combinations of parabens have been proven to have a synergistic effect on bacteria (*21*). This is one means for overcoming the limited solubility of higher alkyl side chain parabens in water. Furthermore, studies have been conducted to investigate the synergistic activity with combination of parabens and other antimicrobials (*43*, *92*).

2.5.3 Regulation

Methyl-paraben (21 CFR 184.1490) and propyl-paraben (21 CFR 184.1670) have been granted generally recognized as safe status (GRAS) by the FDA for direct addition to food at levels not to exceed good manufacturing practices. A maximum level of 0.1 percent can be added to food. FDA has also approved methyl-, propyl- and butyl-paraben (<20 ppm) as synthetic flavoring substances and adjuvants (21 CFR 172.515) for addition to beverages. Heptyl-paraben is permitted by the FDA for direct addition to fermented malt beverages in amounts not to exceed 12 ppm and in non-carbonated soft drinks and fruit in various foods. Consumption of these

compounds in the United States is estimated to be between 222 and 466 mg per person per day (*93*). The Joint FAO/WHO Expert Committee on Food Additives (JECFA) and the Flavor and Flavor and Extract Manufacturer's Association (FEMA) have also approved the use of methyl- and propyl-parabens in food. Use of parabens in food is permitted in Japan and European Union as well.

2.5.4 Toxicity

The general opinion is that the parabens do not affect the health of the consumers. Acute, subchronic, and chronic studies in rodents indicated that parabens are practically non-toxic (*33*, *77*). Also, genotoxicity tests of parabens in a variety of *in vitro* and *in vivo* studies primarily gave negative results (*56*, *71*, *72*, *76*, *78*, *82*). Additionally, the paraben structure does not indicate carcinogenic potential, and experimental studies support these observations (*55*, *64*, *67*, *75*).

However, recent reports have indicated that exposure to parabens may modulate or disrupt the endocrine system and thus may have harmful consequences on human health (*48*). The detection of parabens in a small number of breast tumor tissue samples and adverse reproductive effects of parabens in animals have provoked controversy over the continued use of these substances (*94*).

Based on the FDA report, the average person is estimated to consume as much as 0.6 mg/day (0.01 mg/kg/day) and 0.78 mg/day (0.013 mg/kg/day) of methyl- and propyl-parabens, respectively for an individual weighing 60 kg (*94*). These numbers are

much lower than the lethal dose of acute, short-term, chronic toxicity and carcinogenicity (*33*, *77*). Thus, the restricted dose of parabens use in foods is not anticipated to cause any toxic or carcinogenic effect.

2.5.5 Mechanism

Although the mechanism for parabens is not fully understood, it has been proposed that they act in a manner similar to other weak organic acids such as acetic, lactic, benzoic, and sorbic acids where acidification disrupts the proton motive force that drives ATP formation (*61*). Briefly, hydrogen ions (protons) and hydroxyl ions are separated by the cytoplasmic membrane, with the former, outside the cell, giving rise to acidic pH and the latter, inside the cell, giving rise to pH near neutrality. The membrane gradient thus creates electrochemical potential that the cell employs in the active transport of some compounds such as amino acids. Weak lipophilic acids act as protonophores. After diffusing across the membrane in its undissociated form, the molecule dissociates in the neutral intracellular pH, thereby lowering intracellular pH. This requires that the cell must devote more and more its energy to transport the H^+ ions out of the cell, leaving less energy available for anabolic and homeostatic activities.

Soni et al. (*93*) suggested that the mechanism of propyl-paraben in fungi might be linked to mitochondrial failure that is dependent on induction of membrane permeability transition accompanied by the mitochondrial depolarization and depletion of cellular ATP through uncoupling of oxidative phosphorylation.

2.6 Synergism

A synergistic effect is an effect arising between two or more agents, factors, or substances that produce an effect greater than the sum of their individual effects. It has been observed in multiple studies that investigated the techniques to produce a safer powdered infant formula. The number of *Cronobacter spp*. numbers in contaminated reconstituted infant formula was reduced much more rapidly with increased temperatures and concentrations of caprylic acid (*60*). In another experiment, after inoculation of *C. sakazakii* into powdered infant formula, the sum of near-infared-radiant heating and UV inactivation applied individually was lower than that obtained by the simultaneous application of both technologies (*50*). Moreover, a synergistic action of thymoquinone and mild heat was demonstrated in a recent study, i.e. the antimicrobial activity of thymoquinone was enhanced with increase in temperature (*92*). However, little information is available in the scientific literature concerning the impact of parabens on the thermal inactivation. The only report found was a 2011 study that reported propyl-paraben sensitized *Salmonella* in liquid egg white during thermal pasteurization (*49*).

2.7 Modeling

2.7.1 Predictive microbiology

Predictive microbiology is a rapidly developing and promising area of food microbiology, which has attracted significant attention worldwide in recent years. The emergence of the concept was driven by Australian scientists Esty and Meyer in 1922

as a means to evaluate thermal processes for *Clostridium botulinum* type A spores (*34*). A simplified working definition of predictive microbiology is the development and use of mathematics (and statistics) to describe the behavior of microorganisms in a laboratory media and food as a function of environmental conditions (*45*).

Predictive microbiology has been greatly expanded to include modeling of growth, survival, inactivation, competition, growth/no-growth boundaries, and risk assessment of microorganism. The application covers prediction of the change in microbial population in a product during food processing, estimation of food shelf life during storage, and estimation of the risk in a particular food to public heath under various conditions (*69*).

2.7.2 Primary model

The primary models refer to mathematical expressions describing microbial inactivation, survival or growth using cell population density against time under a set of particular environmental and cultural conditions (*97*). For primary growth models, there are a number of different options available. They are the Baranyi and Roberts model (*6*, *7*), Gompertz model (*47*), and Buchanan et al. three-phase linear model (*19*). Examples of other growth models include logistic model (*63*), new logistic model (*42*), Baranyi lag model (*9*), McKellar model (*79*, *80*), and Huang model (*52*, *53*).

For thermal inactivation the classic inactivation/survival model is the first order kinetics, which is also named as log-linear model (*5*, *34*, *35*) that describes linear reduction of bacterial counts under a constant temperature (*29*). Other commonly used non-linear models are modified Gompertz model (*18*), Weibull model (*84*), and Buchanan three-phase linear models (*19*). Among these three, the Weibull model has the main advantage of its simplicity and capability of modeling linear survival curves as well as those containing shoulder and/or tail regions (*22*, *84*). It has been effectively applied in modeling survival curves of both spore-forming bacteria and non-spore-forming bacteria.

The inactivation model of Geeraerd et al. model (*44*) is capable of describing independently a smooth initiation (shoulder phase) and/or saturation (tail phase) during a mild heat treatment and offers a possible interpretation from a mechanistic point of view about microbial survival and the tail phenomenon (*69*). However, it could not deal with steep temperature profile. Additional survival/inactivation models were listed in a review article authored by Li et al. (*69*).

2.7.3 Secondary model

The secondary models describe how the growth/survival/inactivation curves change as a result of modifying one or more of the intrinsic or extrinsic growth parameters (eg. pH, temperature, water activity etc.). Most of them have little or no microbiological basis, which makes interpretation of some model parameters difficult and sometimes their performance is not stable (*70*).

Available secondary models can be divided into two groups according to their modeling approaches. The first group, including the Arrhenius models (*68*), the Ratkowsky-type models (square-root models) (*10*, *88*), and probability models (*87*), was established to provide high quality of fit based on the biological interpretability of the parameter values (*70*). The second group models include polynomial models (response surface models) (*46*) and artificial neural networks (*51*). These models are developed with black box modeling approaches (*70*).

2.7.4 Tertiary model

The tertiary models combine one or more primary and secondary models to generate a system to predict the behavior of specific microorganisms under different conditions, which are usually designed and packaged as predictive microbiology software and conveniently used by non-modelers (*8*). Examples of tertiary models are ComBase predictor, and the USDA Pathogen Modeling Program.

Chapter 3: Project Objectives and Methods

3.1 Project objectives

The overall goal of this research was to determine if thermal inactivation of *Cronobacter sakazakii* could be enhanced through the inclusion of parabens and if a synergistic relationship existed between heat treatment and antimicrobial parabens. Studies of thermal treatment with inclusion of esters of parahydroxybenzoic acid on foodborne pathogens were limited, and almost no information was available on the impact of the intrinsic and extrinsic factors that affect the growth and survival of bacteria in the presence of parabens. Mathematical models were not available to describe the inactivation rate of heat-resistant *C. sakzakii* in the presence of parabens. To address this knowledge gap, the study was conducted with two specific objectives, both with the goal of gaining a deeper knowledge of the effectiveness of parabens on thermal inactivation and the potential for development of predictive models that can be applied in the food industry:

- The first objective was to investigate the effects of heating temperature, parabens alkyl side chain length, and parabens concentration on the thermal inactivation of heat-resistant *Cronobacter sakazakii* 607 in a model system.
- The second objective was to develop initial primary and secondary mathematical models that effectually describe different process factors and predict the survival population of this microorganism during thermal processing.

3.2 Materials and Methods

3.2.1 *Cronobacter sakazakii* isolates

Cronobacter sakazakii 607, one of twelve strains obtained from the Food and Drug Administration was used throughout the study (Table 4-1). All stock cultures were stored in brain heart infusion broth (BHI) with 30% glycerol at -70°C. Working cultures were subcultured weekly on brain heart infusion agar (BHIA) plates and stored at 4°C.

3.2.2 Parabens

The parabens used in the study were reagent-grade methyl-paraben (MP), ethyl-paraben (EP), propyl-paraben (PP), butyl-paraben (BP) and heptyl-paraben (HP), which were obtained from Fisher Scientific (catalog No. M245-100, Fair Lawn, NJ), Spectrum Chemical MFG Corp. (catalog No. NC9694474, New Brunswick, NJ), Pfaltz & Bauer (catalog No. 50-749-0028, Waterbury, CT), MP Biomedicals LLC (catalog No. ICN222907, Solon, OH), and Pfaltz & Bauer (catalog No. 50-749-4189), respectively. The chemicals were stored at room temperature.

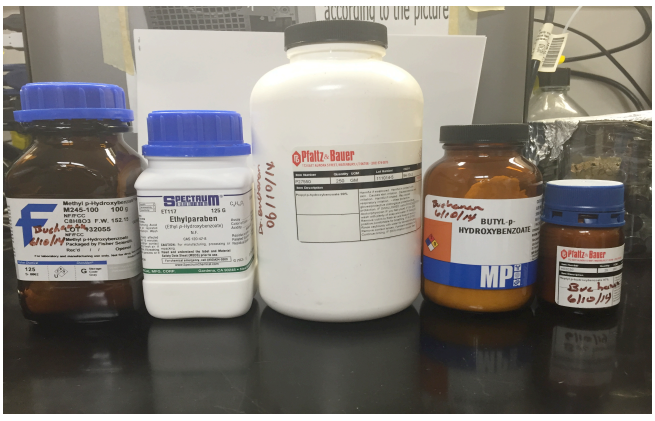


Figure 3-1. Parabens used in this study

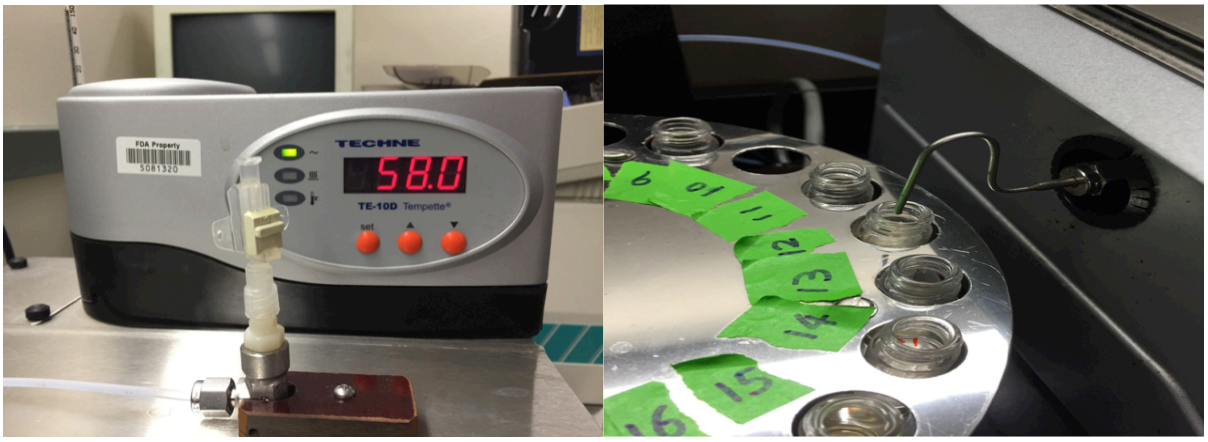
3.2.3 Sample preparation and inoculation

Approximately 24 h before an experiment, 10 ml of BHI was inoculated with *C. sakazakii* 607 strain. The culture was incubated at 37°C for 24 h and then concentrated by centrifugation (3000 rpm, 20 min, 25°C). Before starting a thermal trial, 80 µl of a specific solubilized paraben with the desired concentration was added to a 19-ml BHI blank and then agitated thoroughly. A 1-ml portion of the concentrated culture was transferred to 19 ml of BHI broth and the mixture was vortexed thoroughly and served as zero-time sample. The initial level of *C. sakazakii* 607 in the zero-time samples was approximately 10^8~10^9 CFU/ml.

3.2.4 Determination of thermal inactivation kinetics

Thermal trials were conducted in a submerged coil apparatus using a protocol modified from Edelson-Mammel and Buchanan (*30*). Prior to the start of a heating trial, 9 sterile vials, each with 3.6 ml of sterile 0.1% peptone water were placed on the carousel in a counter-clockwise order. The instrument was pre-equilibrated to 52, 55, or 58°C and preprogrammed to deliver 400 µl aliquots at designated sampling times, i.e. every 90 seconds, through a computer connected to the apparatus. Then, 10 ml of the zero-time sample was injected into the heating coil apparatus using a sterile syringe, and the heat trial initiated immediately. At 90, 180, 270, 360, 450, 540, 630, 720, and 810 seconds, heated samples were dispensed into the vials (served as 10^{-1} dilution), and the vials were immediately capped and put into an ice bucket to halt any further thermal inactivation. The samples were then diluted to 10^{-3} and 10^{-5} using dilution blanks, which contained 9.9 ml sterile 0.1% peptone water.

a. b.



c. d.

Fig. 3-2**. Photographs of the submerged coil apparatus:** (a) complete submerged coil apparatus; (b) screen shot of computer program controlling the apparatus; (c) digital temperature display and port for injecting liquid to be heated; (d) exit port where heated samples were dispensed from the submerged coil.

3.2.5 Plating and enumeration

All the dilutions (10^{-1}, 10^{-3}, 10^{-5}) were surface plated onto tryptic soy agar (TSA) and MacConkey agar (MA) using a spiral plater. This dual plating procedure allowed the degree of injury to be estimated. The TSA plates support the growth of injured and non-injured cells, while the MA plates only allows the growth of non-injured cells (*30*). After plates were dried for 10 minutes at room temperature, they were inverted and incubated at 37 °C for 18-24 h. The plates were enumerated with an automated colony counter (IUL Flash & Grow, Neutec Group Inc., Farmingdale, NY). After enumeration, the counted plates were kept at room temperature for one day to allow the *C. sakazakii* colonies to produce its characteristic yellow pigment. This serve as a means to ensure that only *C. sakazakii* was present and counted. For each paraben at each heating temperature, at least one concentration was repeated independently three times.

3.2.6 Inactivation data analysis

The collected data sets were imported into Excel spreadsheets and transformed to $\log_{10}$ of colony-forming units (CFU). The inactivation curves were plotted for both TSA & MA plates. Comparison of inactivation rates was obtained based on alkyl side chain length, concentration, and heating temperature. Curve trends were recorded.

3.2.7 Modeling

The survivor curves were fitted to the survival/inactivation models using the USDA Integrated Pathogen Modeling Program (IPMP). This software was download from USDA website (http://www.ars.usda.gov/Main/docs.htm?docid=23355). After initial evaluation of various inactivation models, the Weibull model was selected as the primary model due to its flexibility, the small number of model parameters (3), and the goodness of fit. Each set of the data was fitted three times in IPMP to the Weibull model ($Y= Y_0 – K* t^{\alpha}$). The values of the three parameters (Y_0, K, α) were automatically computed by the program (detailed procedures are in Chapter 5). Consideration of secondary models development will be discussed in Chapter 6.

Chapter 4: Collection and Analysis of Inactivation Data

4.1 Preliminary Tests

Preliminary studies confirmed that *Cronobacter sakazakii* 607 was the most heat resistant among all twelve strains tested, and ethanol could be used to solubilize the parabens without confounding the assessment of thermal inactivation kinetics.

4.1.1 Heat resistance evaluation

Twelve strains of *C. sakazakii* were tested at 58°C using the submerged coil apparatus to select the most heat resistant strain. These 12 strains are the same as the strains used by Edelson-Mammel and Buchanan (*30*). As in the 2004 study, *C. sakazakii* 607 was the most heat resistant strain (Table 4-1).

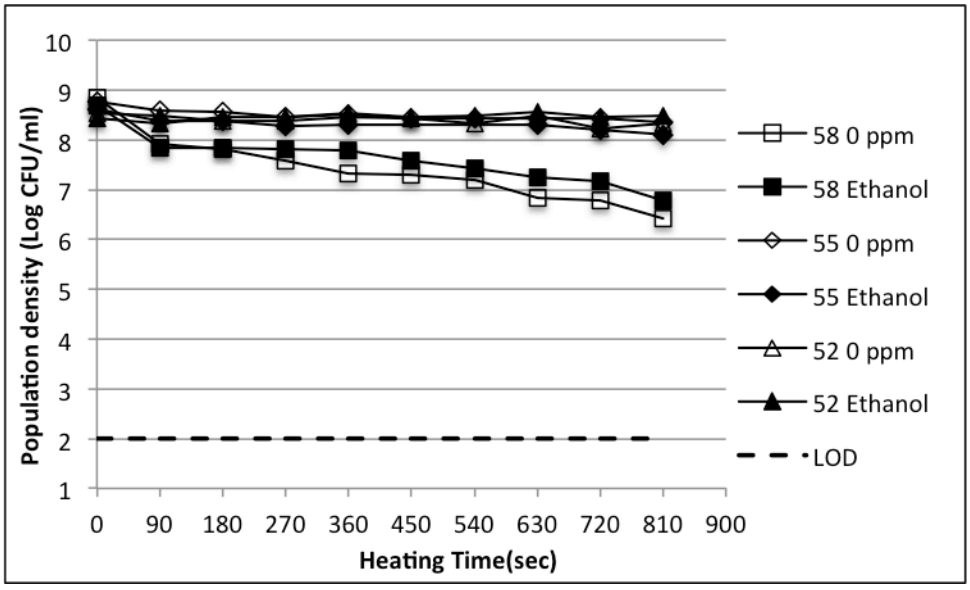
Table 4-1. Preliminary comparison of the $D_{58°C}$ of twelve *Cronobacter sakazakii* strains

Strain	*Source*	*D-value* (sec)*
607	Clinical isolate, F. Khambaty, FDA	455
ATCC 29544	ATCC	196
ATCC51329	ATCC	74
LCDC 674	J. M. Farber, Heath Canada	15.6
CDC A3	J. M. Farber, Heath Canada	26
SK 90	J. M. Farber, Heath Canada	125
LCDC 648	J. M. Farber, Heath Canada	137
EWFAKRC11NNV1493	J. M. Farber, Heath Canada	17
NQ1-Environ	Environmental-food manufacturing, M. Kotewicz, FDA	41.3
NQ2-Environ	Environmental-food manufacturing, M. Kotewicz, FDA	67.5
NQ3-Envrion	Environmental-food manufacturing, M. Kotewicz, FDA	17
4.01C	Dried infant formula, S. Edelson-Mammel, FDA	389

*D-values were collected only once since the purpose was to confirm the most heat resistant strain.

4.1.2 Ethanol effect assessment

For parabens with long alkyl side chains, the compounds are only slightly soluble in water. Accordingly, a small amount (80 µl) of ethanol was added to the stock solution to facilitate dispersing the compounds in BHI. A secondary preliminary study was conducted to ensure that the addition of the ethanol did not change the thermal resistance of *C. sakazakii* 607 in the absence of parabens (Fig. 4-2). The addition of ethanol had no significant effect on the thermal inactivation of *C. sakazakii* at any of the three temperatures used in the inactivation trials.



*Limit of detection is 2.0-log CFU/ml.

Figure. 4-1. Comparison of inactivation curves with 0 ppm paraben and 80 µl 70% ethanol

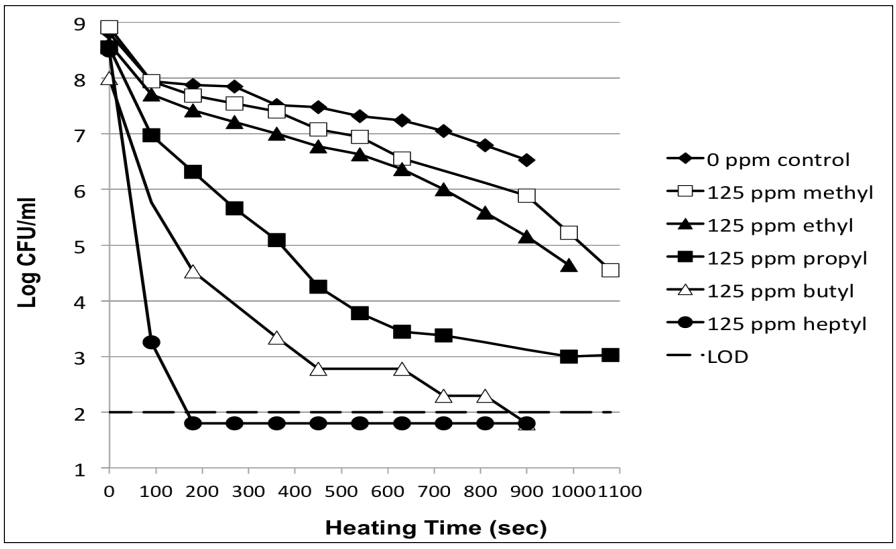
4.2 Results

Three factors (alkyl side chain length, concentration, and heating temperature) were evaluated individually.

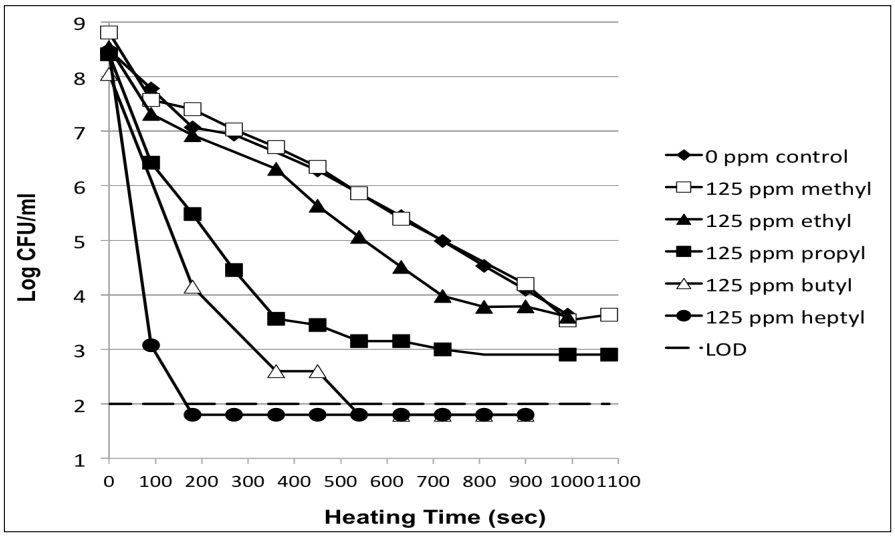
4.2.1 Alkyl side chain length

At each heating temperatures, greater log reductions were observed as the alkyl side chain length of parabens increased. Figure 4-2a depicted the thermal inactivation curves of *C. sakazakii* 607 as affected by addition of 125 ppm of the five parabens at 58°C. When plated on TSA, *C. sakazakii* were reduced by 2.7, 3.0, 5.3, 6.0, and >6.0 log (CFU/ml) after 810 seconds, when exposed to methyl, ethyl, propyl, butyl, and heptylparabens, respectively. More inactivation was observed when plating on MA plates (Fig. 4-2b), providing a means for estimating the degree of injury.

a



b



LOD=lower limit of detection = 2.0 log (CFU/ml). Samples where no viable cells were recovered were assigned a value of log CFU/ml =1.8.

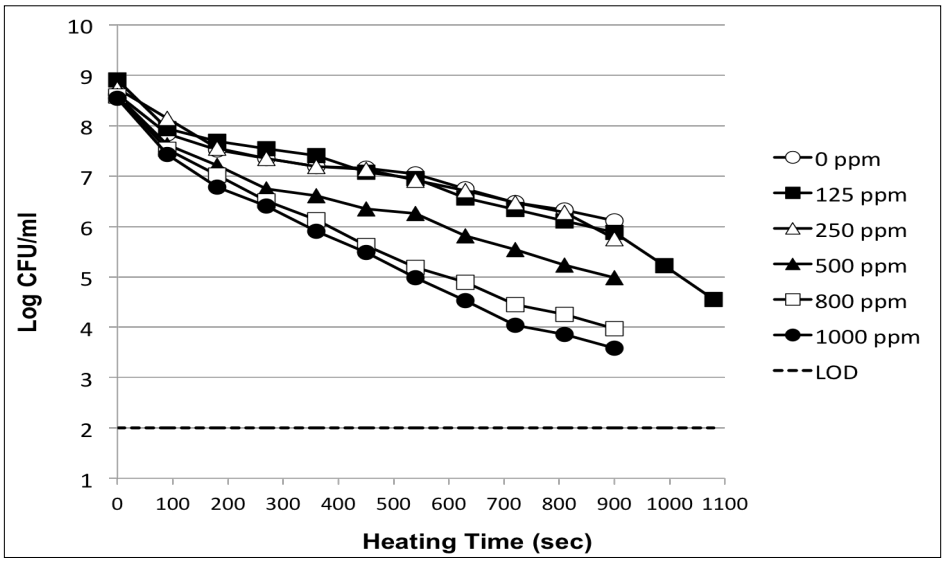
Figure. 4-2. Thermal inactivation of *C. sakazakii* 607 at 58°C with 125 ppm parabens plated onto TSA (a) and MA (b).

4.2.2 Concentration

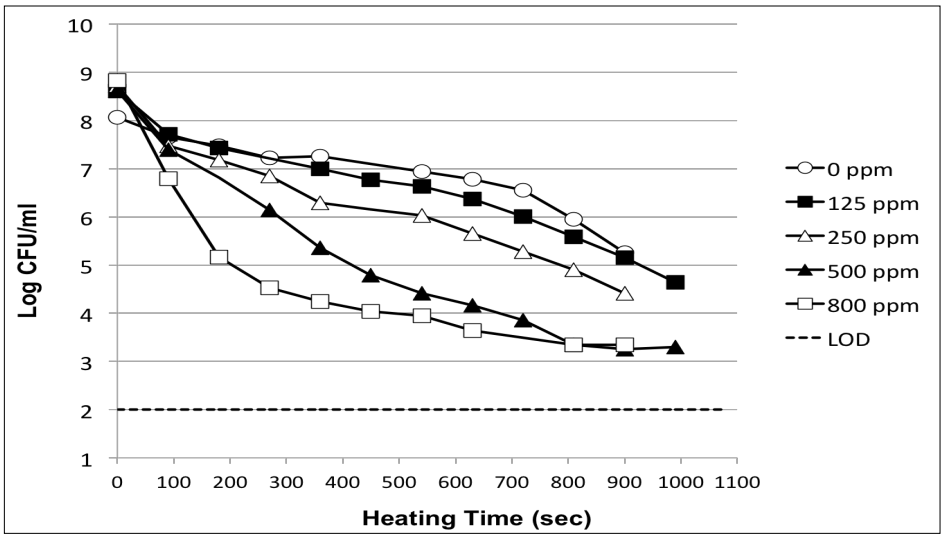
At 58°C, the presence of parabens enhanced the inactivation of *C. sakazakii* in a dose-dependent manner, and the inactivation was strongly dependent on the length of alkyl side chain (Fig. 4-3). The effect was more significant with longer alkyl side chains. For parabens with longer side chain, rapid inactivation was observed during the initial state of heat treatment, although inactivation rate decreased as the heating was prolonged. Methyl-paraben caused 2.5 log reduction at the125 ppm and 250 ppm after 810 seconds; whereas 3.5, 4.5, 5.0 log reduction was achieved at 500 ppm, 800 ppm, and 1000 ppm after 810 seconds, respectively (Fig. 4-3a). Ethyl-paraben produced a 3.0, 3.8, 5.2, and 5.5 log reduction at 125 ppm, 250 ppm, 500 ppm, and

1000 ppm after 810 seconds, respectively (Fig. 4-3b). For propyl-paraben, 3.0, 4.5, 5.0, and 5.5 log reduction were obtained at 0 ppm, 62.5 ppm, 93.75 ppm, 125 ppm, 250 ppm after 810 seconds, respectively (Fig. 4-3c). In contrast, butyl-paraben attained a 3.7 and 5.0 log-reduction at concentrations of 31.25 ppm and 62.5 ppm after 810 seconds, respectively (Figure 4-3d). When concentrations increased even higher, the reductions exceeded the lower detection limit (2.0 log (CFU/ml)), especially for heptyl-paraben (Figure 4-3e).

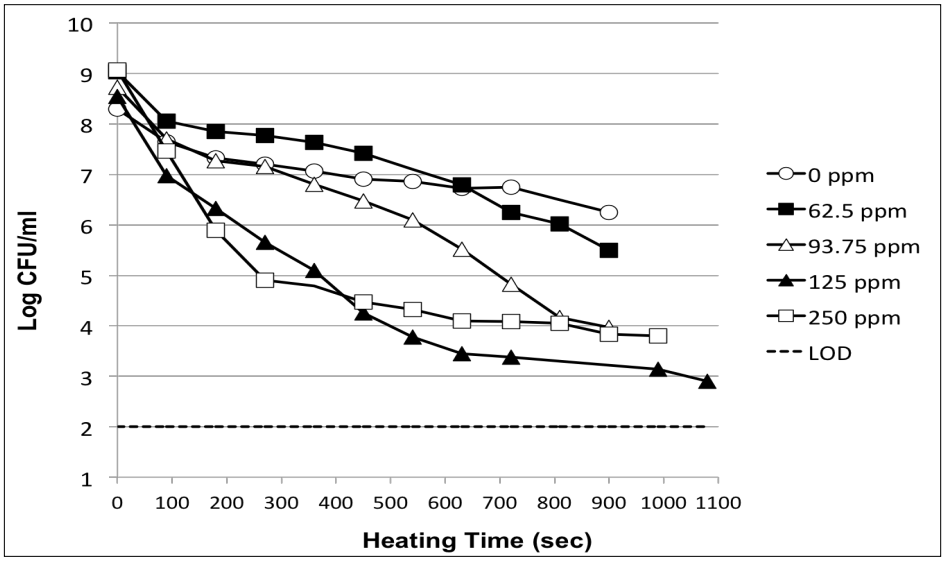
a



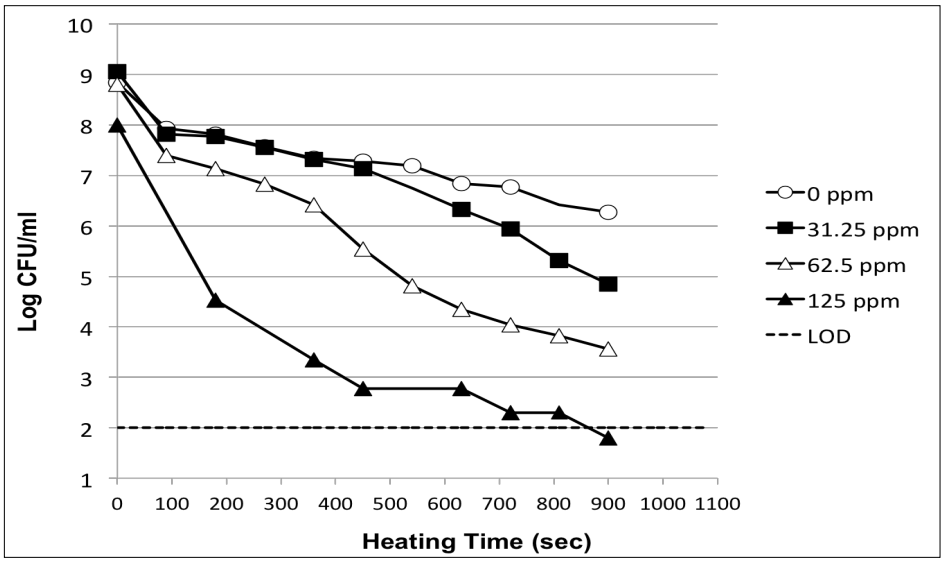
b



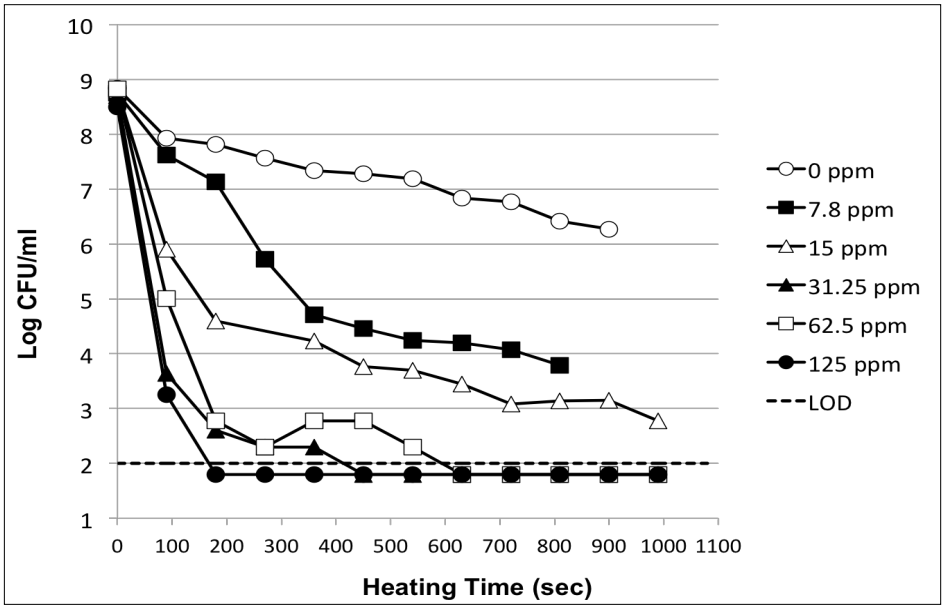
c


Log CFU/ml
Heating Time (sec)
0 ppm
62.5 ppm
93.75 ppm
125 ppm
250 ppm
LOD

d


Log CFU/ml
Heating Time (sec)
0 ppm
31.25 ppm
62.5 ppm
125 ppm
LOD

e


Log CFU/ml
Heating Time (sec)
0 ppm
7.8 ppm
15 ppm
31.25 ppm
62.5 ppm
125 ppm
LOD

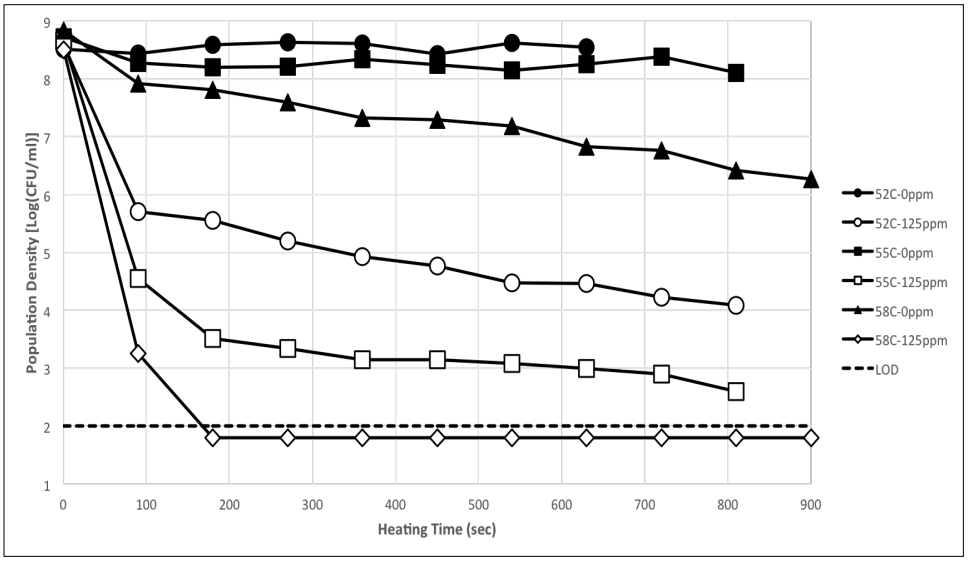
LOD=lower limit of detection = 2.0 log (CFU/ml). Samples where no viable cells were recovered were assigned a value of log CFU/ml =1.8.

Figure. 4-3. Thermal inactivation of *C. sakazakii* 607 at 58°C and plated onto TSA (a) methyl-paraben; (b) ethyl-paraben; (c) propyl-paraben; (d) butyl-paraben; (e) heptyl-paraben.

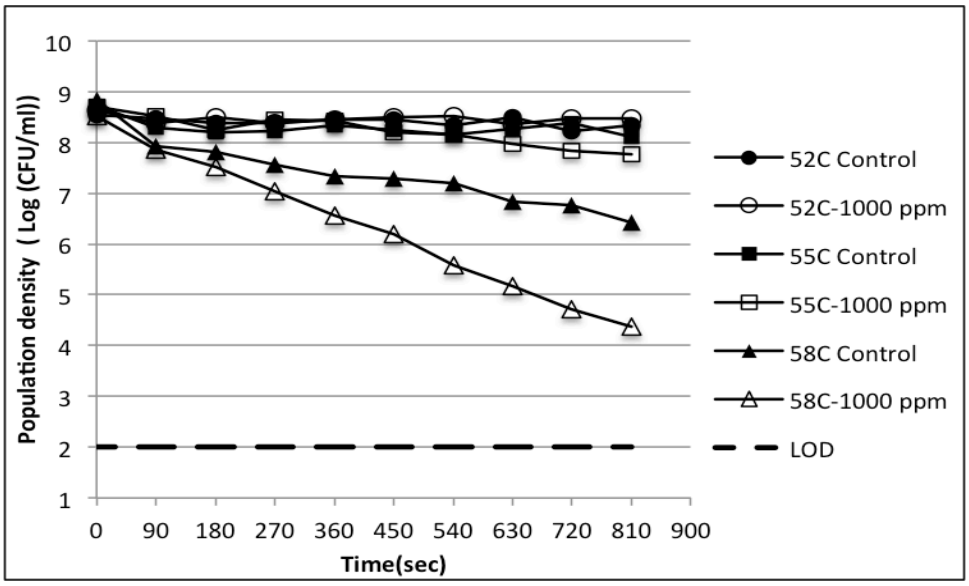
4.2.3 Temperature

Temperatures of 52°C and 55°C were evaluated to determine the effect of parabens at temperatures that normally have little lethal effect on *C. sakazakii* 607. Figure 4-4a clearly demonstrated the relationship between temperature and inactivation in the presence of 0 and 125 ppm heptyl-paraben. With 125 ppm heptyl-paraben, the log-reduction in *C. sakazakii* 607 was 5.0, 6.0, and >7.5 at 52°C, 55°C, and 58°C after 810 seconds, respectively.

a.

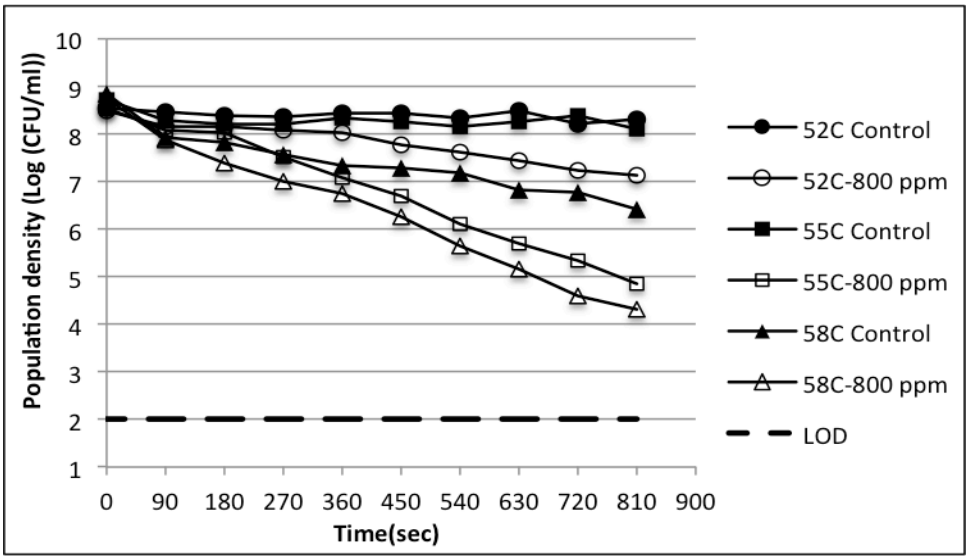


b.

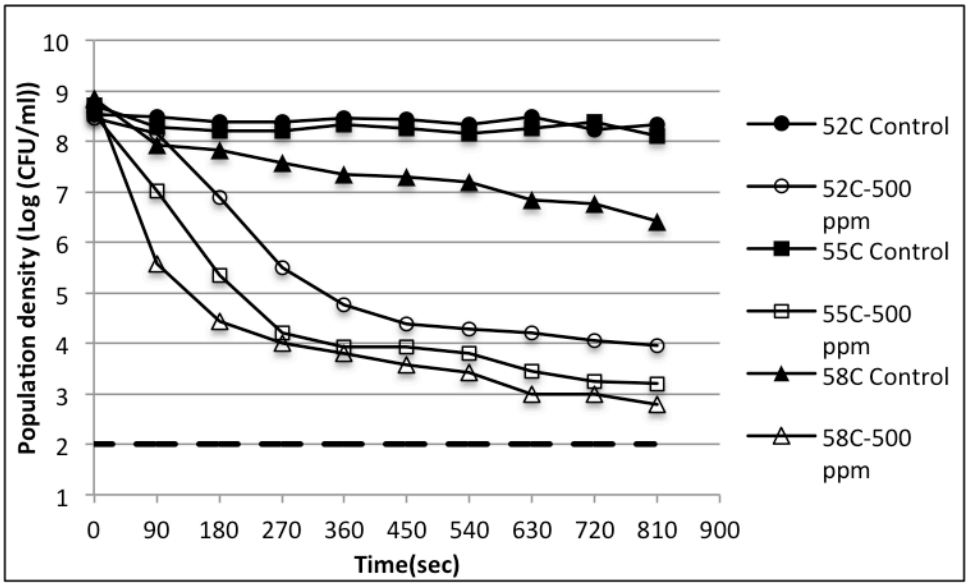

Population density (Log (CFU/ml))
Time(sec)
52C Control
52C-1000 ppm
55C Control
55C-1000 ppm
58C Control
58C-1000 ppm
LOD

.

c.


Population density (Log (CFU/ml))
Time(sec)
52C Control
52C-800 ppm
55C Control
55C-800 ppm
58C Control
58C-800 ppm
LOD

d.

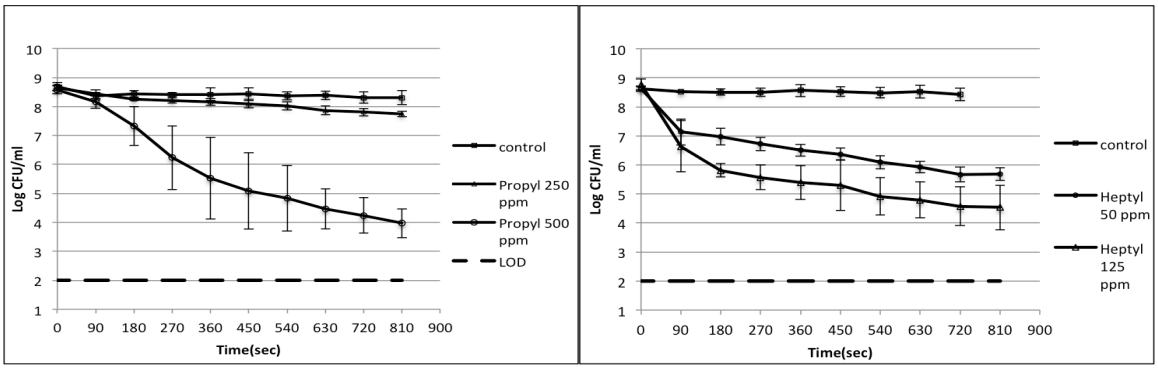

Population density (Log (CFU/ml))
Time(sec)
52C Control
52C-500 ppm
55C Control
55C-500 ppm
58C Control
58C-500 ppm

LOD=lower limit of detection = 2.0 log (CFU/ml). Samples where no viable cells were recovered were assigned a value of log CFU/ml =1.8.

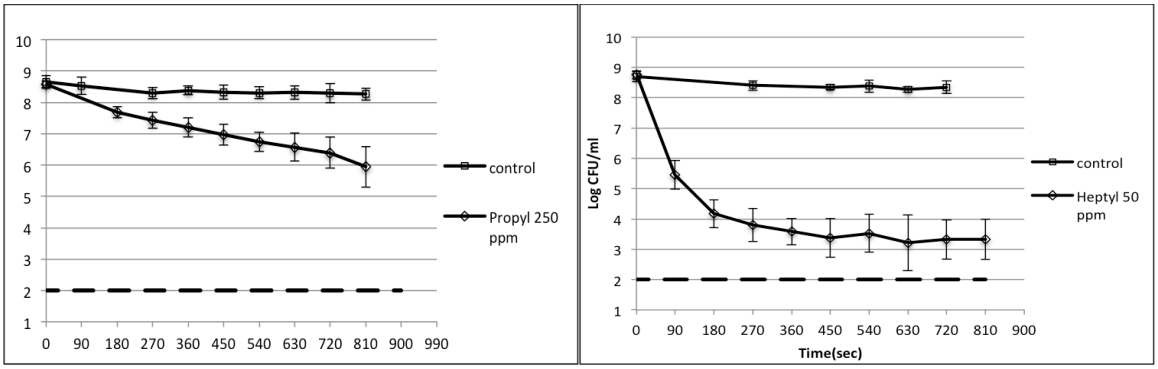
Figure 4-4. Thermal inactivation of *C. sakazakii* 607 at different temperatures in BHI (a) with 0 and 125 ppm heptyl-paraben; (b) with 0 and 1000 ppm methyl-paraben; (c) with 0 and 800 ppm ethyl-paraben; (d) with 0 and 250 ppm propyl-paraben.

4.2.4 Repeatability

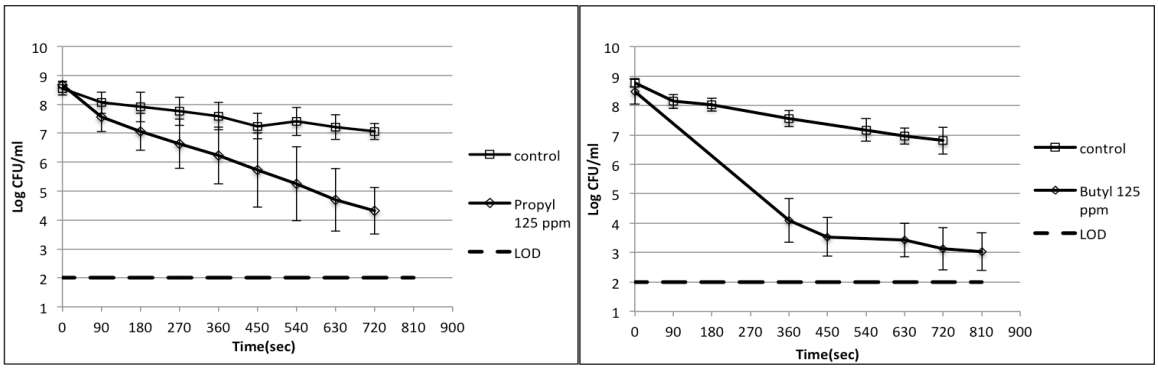
Ideally at least three independent replicates were required to explicate the repeatability of their experiment. While more inactivation information for different concentrations would have been desirable, the triplicate experiment was only conducted independently for at least one concentration for each paraben at one designated heating temperature. Although not all trials were replicated three times, the repeatability was quite satisfactory (Fig. 4-5).



a. b.



c. d.



e. f.

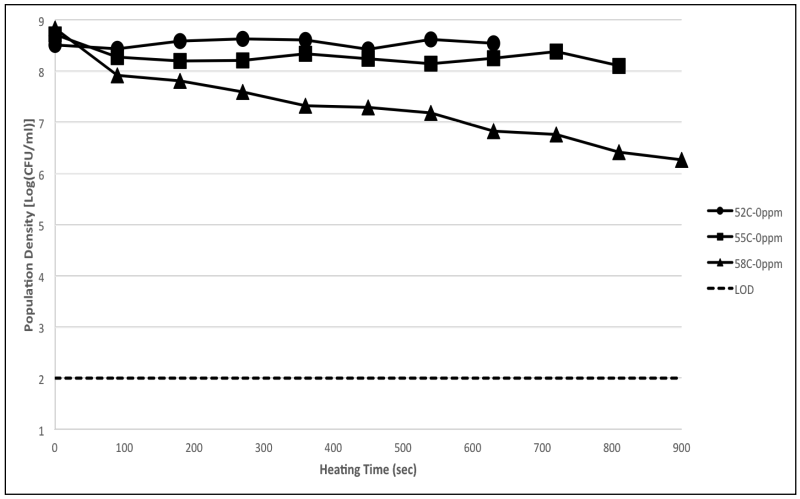
LOD=lower limit of detection = 2.0 log (CFU/ml). Samples where no viable cells were recovered were assigned a value of log CFU/ml =1.8.

Figure 4-5. Inactivation curves by mean of triple independent trials with error bars. (a) propyl-paraben at 52°C; (b) heptyl-paraben at 52°C; (c) propyl-paraben at 55°C; (d) heptyl-paraben at 55°C; (e) propyl-paraben at 58°C; (f) butyl-paraben at 58°C.

4.3 Synergistic relationship

4.3.1 Heat treatment with 0 ppm paraben

In the absence of parabens, the controls (0 ppm) showed a 2.0 log (CFU/ml) decrease during heating at 58°C for 810 seconds. As previously noted, when the temperature was decreased to 55°C, and 52°C, only a 0.5 and 0.2 log-reduction was observed (Fig. 4-6) respectively, indicating essentially no inactivation over the course of the heating duration at these temperatures.

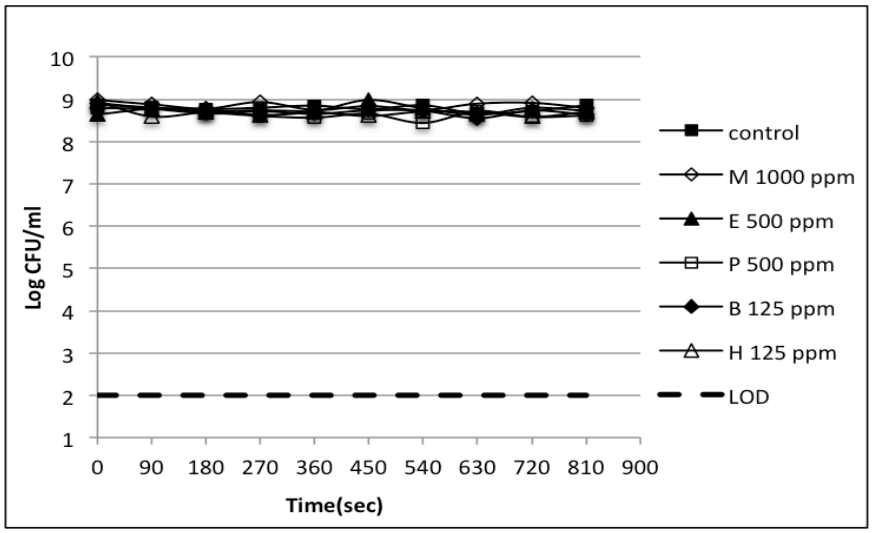


*Limit of detection is 2.0-log CFU/ml.

Figure 4-6. Thermal inactivation of *C. sakazakii* 607 at 52, 55, 58 °C with 0 ppm parabens plated onto TSA.

4.3.2 High concentration of parabens without heat treatment

At room temperature (22°C), even treated with high concentration of each of the five parabens, little or no inactivation was observed (Fig. 4-7).



LOD=lower limit of detection = 2.0 log (CFU/ml).

Figure. 4-7. Inactivation curves of *C. sakazakii* 607 at room temperature with high concentration of methyl-, ethyl-, propyl-, butyl-, and heptyl-paraben plated onto TSA.

4.4 Discussion

In this experiment, a submerged coil apparatus was used to simulate the thermal processing that may occur in food manufacturing. Likewise, instead of a food system, BHI was used as a model system to evaluate the effects of the three factors, paraben concentration, paraben identity, and processing temperatures. The thermal treatments temperatures were purposefully selected to be just above the maximum temperature of growth of *C. sakazakii*. Such temperatures would be most likely to demonstrate any enhancement of inactivation due to parabens supplementation, since higher treatment temperatures would be expected to rapidly inactivate the microorganism.

Figures 4-2, 4-3, 4-4, 4-5,4-6, and 4-7 are representative survivor curves observed throughout the study. The original data for each individual trials are provided in appendix. There was a clear interaction between the thermal treatment and the levels of the different parabens needed to enhance inactivation. At lower heating temperatures (52°C or 55°C), higher concentrations of each paraben were required to achieve the same level of inactivation observed with heating at 58°C. For instance, 15 ppm of heptyl-paraben at 58°C resulted in a 6-log inactivation of *C. sakazakii* 607, which showed a >3-log enhancement of inactivation compared to the 2.5-log inactivation observed in the control samples (Fig. 4-3e). At 52°C, addition of 125 ppm of heptyl-paraben achieved a 4.5-log reduction, while no reduction was observed in the control samples (Fig. 4-4a). Similarly, methyl-paraben, at its maximum concentration (1000 ppm) at 52°C, was not effective in enhancing the bacterial inactivation, whereas at this concentration methyl-paraben caused >2-log additional

kills at 58°C when compared with the control samples (Fig. 4-4b). For short side chain parabens, such as methyl-paraben, ethyl-paraben, and propyl-paraben, they were not effective in killing *C. sakazakii* at the lower heating temperatures (Fig. 4-4b-d). Overall, the enhancement of thermal inactivation increased with the increasing number of carbon atoms in the alkyl side chains, concentration of the parabens, and heating temperature.

The curves generated based on bacterial counts from MA were similar to those from TSA, with a range of 0.2-2.5 log injury observed (Fig. 4-2). As alkyl side chain length or the concentration of parabens was increased, the bacterial injury decreased since more initial population was killed immediately, instead of injured.

In addition to evaluating the impact of each factor, the total inactivation activity was assessed to compare the effects of combined treatment and individual treatment. Without parabens, heating at 58 °C, 55 °C, and 52 °C caused 2.0, 0.5, and 0.2 log reduction in *C. sakazakii*. However, at room temperature, little or no inactivation was observed even with a high concentration of any of five parabens. Only a combination of mild heating and paraben treatment was found to present a sufficient destruction of the bacterial population. The combined effect of mild heating and inclusion of parabens was greater than either treatment alone. Therefore, a synergistic relationship existed between heat treatment and parabens. Furthermore, under a certain heating temperature, the bacterial population decreased linearly with low concentration and

short side chain of parabens, whereas the population decreased exponentially as paraben concentration and side chain length increased (Fig. 4-3).

The results of this part were not subjected to statistical analysis, since the means and standard deviations were not acquired for every combination of the variables. The survival data were collected to develop initial predictive mathematical models of the three effects (see Chapters 5 and 6).

It is unclear at this stage that how paraben works synergistically with mild heat. A potential inactivation hypothesis for most of weak organic acids is the cytoplasmic acidification, that is, undissociated weak acid enters cytosol, dissociates in it, decreases the intracellular pH, and causes reduction of energy to restore homeostasis (*16*, *96*). However, inhibition of growth by weak acid preservatives has been proposed to be due to a number of other actions including membrane disruption (*12*, *95*), inhibition of essential metabolic reactions (*66*), and the accumulation of toxic anions (*32*). For instance, sorbic acid and acetic acid have similar pK_a values (4.76), but sorbic acid is a far more potent preservative (*96*). Sorbic acid is also a more potent uncoupler of the membrane potential than acetic acid, which may explain the effectiveness (*96*). The precise mode of action for parabens is still under investigation. A study hypothesized that propyl-paraben firstly induces the permeabilization of bacterial membranes, causing the release of intracellular molecules and the de-energization of membranes (*14*). Thus, a possible explanation for the synergism is that both parabens and heat caused disruption or leakage of cell

membrane and a combination of two sub-lethal membrane damage resulted in a rapid bacteria death.

Chapter 5: Analysis and Development of Primary Model

5.1 Introduction

Primary models describe the changes in microbial numbers with time under a single set of environmental conditions. In this case, the survival population of *C. sakazakii* 607 during mild heat treatment with and without parabens was evaluated over a fixed course of time period. Based on the review of the literatures, several survival models could be potentially useful. The models include log-linear (*34*), Gompertz (*47*), two- and three-phase linear (*17*, *19*), and Weibull models (*74*, *84*). The major requirement for a primary model that can adequately describe the data set is flexibility in the types of shapes that can be described. To find the best primary model, a user-friendly software was employed during the curve fitting process.

5.2 Methods

5.2.1 Software

The Integrated Pathogen Modeling Program (IPMP) software developed by Dr. Lihan Huang at US Department of Agriculture provides a user-friendly means of considering a number of the mathematical models that have been used to describe microbial behavior in foods. The software includes a set of primary models for mathematically describing inactivation/survival. This software was used to consider several different candidate models. Due to the need of flexibility, the three-parameter Weibull model was chosen as the primary survival model for describing the

inactivation of *C. sakazakii* in the current study. The IPMP includes two formulae for Weibull model in IPMP: a standard version (*84*)

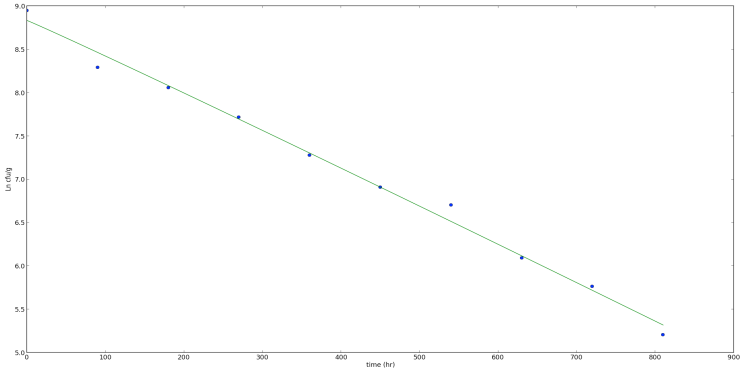
$$Y_t = Y_0 - K * t^{\alpha}$$

and a modified one (*74*)

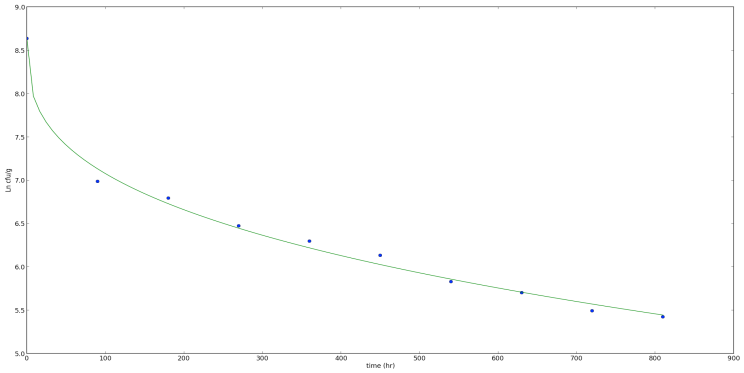
$$Y_t = Y_0 - (t/D)^{\alpha}.$$

The former one was used in fitting the current inactivation data. Y_0 and Y_t are initial population density (log (CFU/ml) and the population density at time t. The other terms are t = time; K is parameter that partially determines the steepness of curves; and α is a shape parameter that can be > 1, = 1, or < 1 (Fig. 5-1).

α=1



α<1



α>1

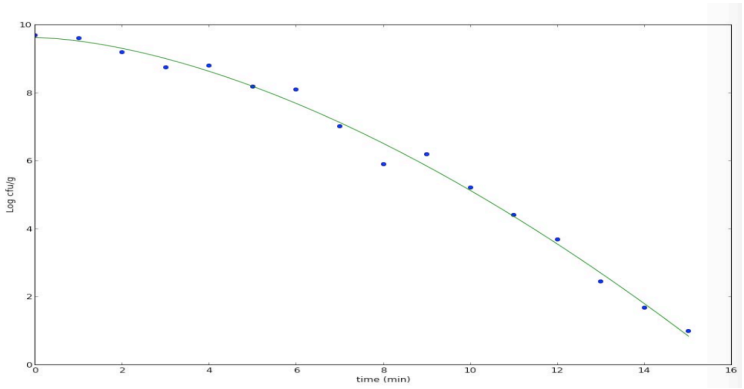
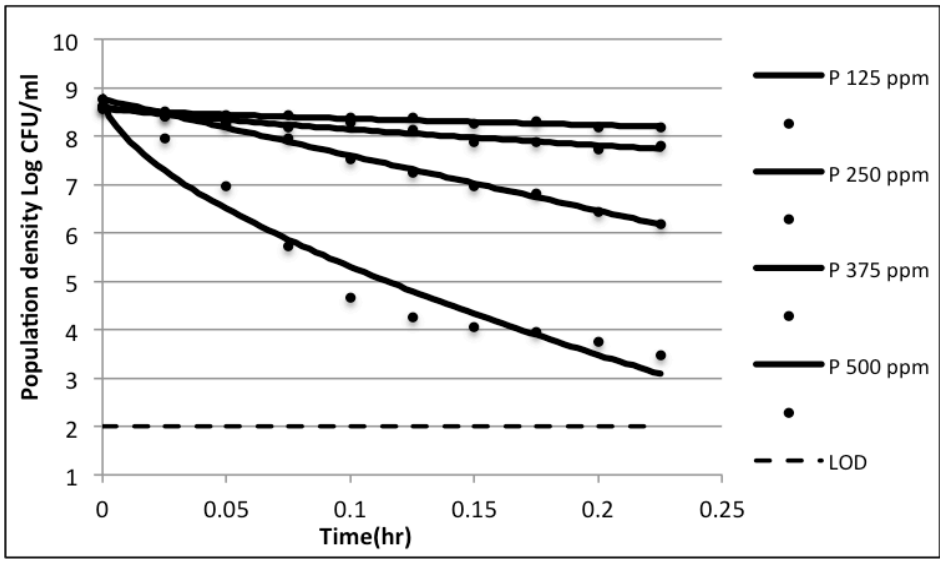


Figure. 5-1. Shape of curves when α is > 1, = 1, or < 1.

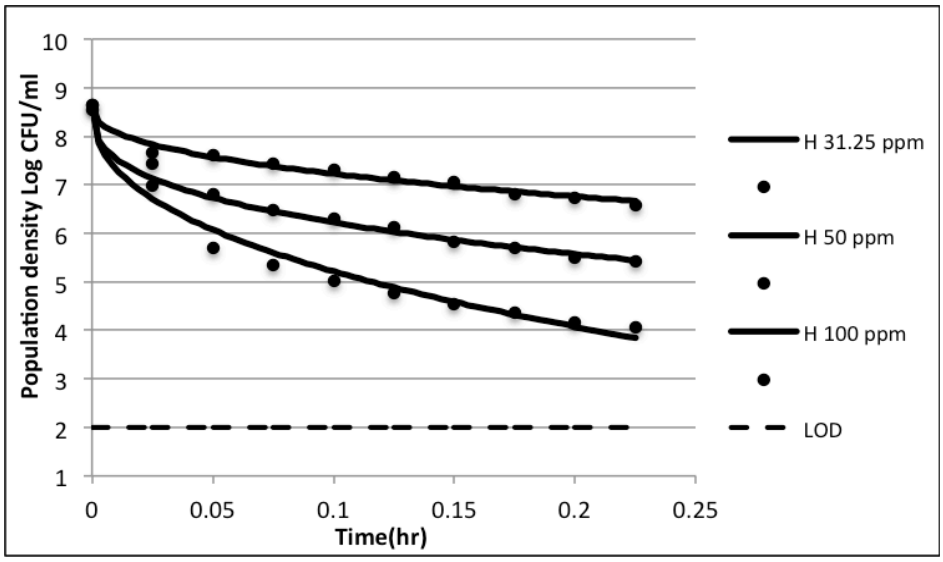
5.2.2 Curve fitting

Each set of data was fitted to the model three times to obtain a statistical mean of each parameter. Because of large amount of data, only a few representative figures are presented to depict the goodness of the fit with the Weibull model (Fig. 5-2a, b, c, d). In almost all cases, the Weibull model fitted the data sets well.

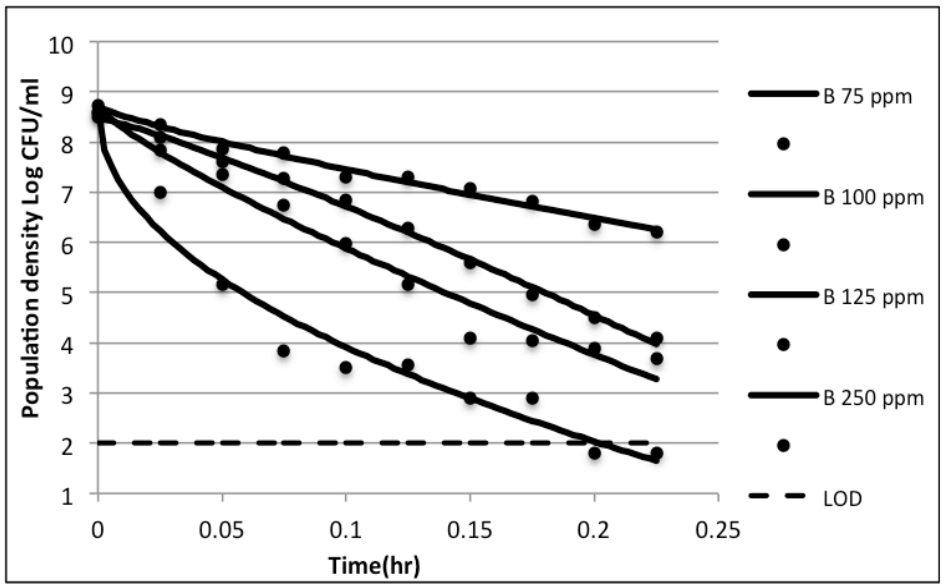
a.



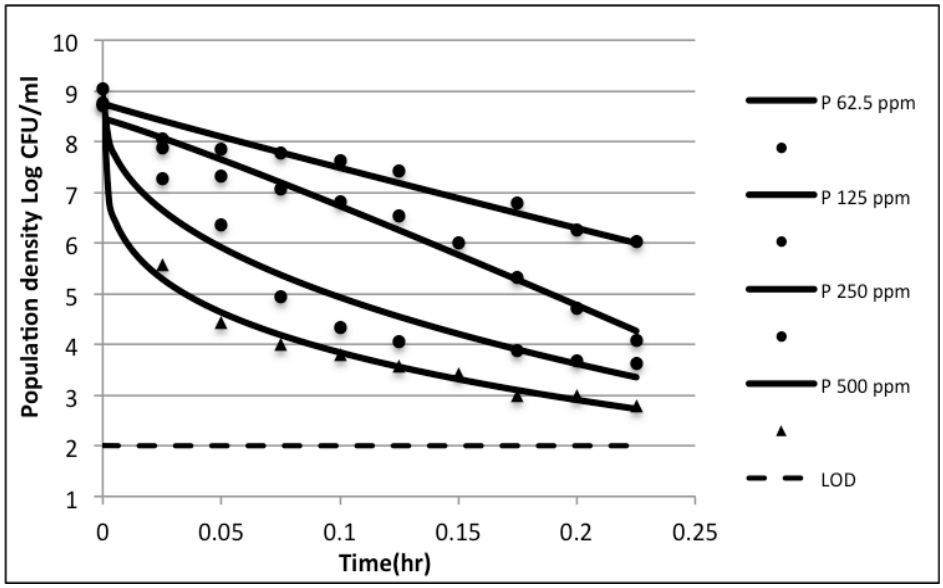
b.


Population density Log CFU/ml
Time(hr)
H 31.25 ppm
H 50 ppm
H 100 ppm
LOD

c.


Population density Log CFU/ml
Time(hr)
B 75 ppm
B 100 ppm
B 125 ppm
B 250 ppm
LOD

d.


Population density Log CFU/ml
Time(hr)
P 62.5 ppm
P 125 ppm
P 250 ppm
P 500 ppm
LOD

LOD=lower limit of detection = 2.0 log (CFU/ml). Samples where no viable cells were recovered were assigned a value of log CFU/ml =1.8.

Figure. 5-2. Comparison of curves generated by Weibull and experimental data points for (a) propyl-parabens at 52 °C; (b) heptyl-parabens at 52 °C; (c) butyl-parabens at 55 °C; (d) propyl-parabens at 58 °C

5.3 Results

The complete list of Weibull model parameters generated for the three heat treatments are presented in Table 5-1, Table 5-2 and Table 5-3.

Table 5-1. Summary of Weibull parameters for survival curves heated at 52 °C in the presence of different concentrations of parabens.

Paraben	***Conc. (ppm)***	***Parameter***	***Rep[a]***	***N[b]***	***Mean***	***L95CI[d]***	***U95CI[d]***
Control	0	Y_0	1	3	8.53	8.35	8.72
			2	3	8.76	8.60	8.91
			3	3	8.78	8.57	8.98
			Mean[c]		8.69	8.51	8.87
		K	1	3	0.444	-0.328	1.22
			2	3	0.313	-0.0881	0.715
			3	3	0.782	0.392	1.17
			Mean		0.513	-0.00831	1.03
		α	1	3	0.515	-0.620	1.65
			2	3	0.200	-0.407	0.807
			3	3	0.159	-0.0487	0.367
			Mean		0.291	-0.359	0.941
Methyl	1000	Y_0	1	3	8.56	8.39	8.73
			2	3	8.56	8.46	8.66
			3	3	8.62	8.47	8.77
			Mean		8.58	8.44	8.72
		K	1	3	0.424	-0.141	0.989
			2	3	0.255	0.108	0.402
			3	3	0.166	-0.119	0.450
			Mean		0.282	-0.0506	0.614

		α	1	3	0.480	-0.425	1.39
			2	3	0.0258	-0.1.66	0.217
			3	3	0.00102	-0.675	0.677
			Mean		0.169	-0.422	0.760
Ethyl	800	Y_0	1	3	8.41	8.20	8.62
			2	3	8.74	8.63	8.85
			3	3	8.38	8.22	8.55
			Mean		8.51	8.35	8.67
		K	1	3	12.4	-9.22	34.0
			2	3	0.644	0.397	0.891
			3	3	8.38	2.50	14.3
			Mean		7.13	-2.11	16.4
		α	1	3	1.68	0.514	2.85
			2	3	0.247	0.0616	0.433
			3	3	1.26	0.780	1.74
			Mean		1.06	0.452	1.67
Propyl	125	Y_0	1	3	8.57	8.46	8.69
		K	1	3	1.07	0.242	1.89
		α	1	3	0.699	0.163	1.23
Propyl	250	Y_0	1	3	8.54	8.37	8.71
			2	3	8.61	8.40	8.83
			3	3	8.71	8.54	8.88
			Mean		8.62	8.44	8.81
		K	1	3	2.35	1.27	3.42
			2	3	2.71	0.889	4.53
			3	3	2.18	1.14	3.23
			Mean		2.41	1.10	3.73
		α	1	3	0.657	0.341	0.973
			2	3	0.756	0.288	1.23
			3	3	0.647	0.318	0.976
			Mean		0.687	0.316	1.06
Propyl	375	Y_0	1	3	8.71	8.46	8.97
			2	3	8.77	8.61	8.92
			Mean		8.74	8.53	8.95
		K	1	3	20.6	12.2	29.0
			2	3	11.2	8.62	13.8
			Mean		15.9	10.4	21.4
		α	1	3	1.23	0.946	1.50
			2	3	0.980	0.820	1.14
			Mean		1.10	0.883	1.32
Propyl	500	Y_0	1	3	8.74	7.38	10.1
			2	3	8.81	7.66	9.96

			3	3	8.74	8.51	8.98
			Mean		8.76	7.85	9.67
		K	1	3	12.3	5.72	18.9
			2	3	14.1	7.87	20.3
			3	3	25.8	18.9	32.8
			Mean		17.4	10.8	24.0
		α	1	3	0.568	0.209	0.927
			2	3	0.603	0.304	0.903
			3	3	1.19	1.01	1.38
			Mean		0.787	0.506	1.07
Butyl	75	Y_0	1	3	8.70	8.57	8.83
			2	3	8.93	8.68	9.18
			Mean		8.81	8.62	9.01
		K	1	3	4.62	3.07	6.16
			2	3	1.27	0.730	1.82
			Mean		2.95	1.90	3.99
		α	1	3	0.865	0.631	1.10
			2	3	0.333	0.0790	0.587
			Mean		0.599	0.355	0.843
Butyl	125	Y_0	1	3	8.83	8.59	9.08
			2	3	8.44	8.20	8.68
			Mean		8.64	8.40	8.88
		K	1	3	16.2	11.6	20.8
			2	3	7.29	3.56	11.0
			Mean		11.8	7.57	15.9
		α	1	3	1.03	0.828	1.22
			2	3	0.959	0.603	1.32
			Mean		0.992	0.716	1.27
Butyl	200	Y_0	1	3	8.77	7.52	10.0
			2	3	8.61	7.06	10.2
			Mean		8.69	7.29	10.1
		K	1	3	11.1	6.45	15.7
			2	3	14.6	6.87	22.2
			Mean		12.8	6.66	19.0
		α	1	3	0.468	0.205	0.732
			2	3	0.572	0.218	0.926
			Mean		0.520	0.211	0.829
Butyl	250	Y_0	1	3	8.04	6.73	9.34
			2	3	8.66	7.96	9.36
			3	3	8.63	7.26	10.0
			Mean		8.44	7.32	9.56
		K	1	3	8.40	5.87	10.9

			2	3	9.47	7.84	11.1
			3	3	11.4	6.88	16.0
			Mean		9.76	6.86	12.7
		α	1	3	0.158	0.0330	0.284
			2	3	0.265	0.179	0.350
			3	3	0.426	0.184	0.669
			Mean		0.283	0.132	0.434
Heptyl	31.25	Y_0	1	3	8.84	8.73	8.95
			2	3	8.61	8.38	8.83
			Mean		8.72	8.56	8.89
		K	1	3	4.18	3.81	4.55
			2	3	3.62	2.90	4.35
			Mean		3.90	3.35	4.45
		α	1	3	0.436	0.381	0.490
			2	3	0.418	0.297	0.539
			Mean		0.427	0.339	0.515
Heptyl	50	Y_0	1	3	8.71	8.62	8.80
			2	3	8.62	8.42	8.82
			3	3	8.50	7.96	9.04
			Mean		8.61	8.33	8.89
		K	1	3	5.87	5.52	6.21
			2	3	5.31	4.76	5.86
			3	3	4.19	2.84	5.53
			Mean		5.12	4.37	5.87
		α	1	3	0.464	0.427	0.501
			2	3	0.345	0.287	0.402
			3	3	0.299	0.131	0.467
			Mean		0.369	0.281	0.457
Heptyl	100	Y_0	1	3	8.62	7.82	9.42
		K	1	3	8.90	6.28	11.5
		α	1	3	0.417	0.240	0.595
Heptyl	125	Y_0	1	3	8.58	8.34	8.83
			2	3	8.97	8.76	9.18
			3	3	8.78	7.98	9.59
			Mean		8.78	8.36	9.20
		K	1	3	6.18	5.65	6.71
			2	3	8.27	7.69	8.84
			3	3	5.36	3.38	7.34
			Mean		6.60	5.57	7.63
		α	1	3	0.225	0.185	0.265
			2	3	0.339	0.301	0.378
			3	3	0.293	0.102	0.485

			Mean	0.286	0.196	0.376

[a]Number of replicate (maximum is 3)
[b]Number of model fitting times for each replicate
[c]Grand mean of each replicate's mean
[d]When the value of L95CI and U95CI is less than 0, the value is not meaningful

Table 5-2. Summary of Weibull parameters for survival curves heated at 55 °C in the presence of different concentrations of parabens.

Paraben	***Conc. (ppm)***	***Parameter***	***Rep[a]***	***N[b]***	***Mean***	***L95CI[d]***	***U95CI[d]***
Control	0	Y_0	1	3	8.81	8.66	8.97
			2	3	8.77	8.69	8.85
			3	3	8.43	8.18	8.68
			Mean[c]		8.67	8.51	8.83
		K	1	3	0.782	0.209	1.36
			2	3	0.652	0.429	0.875
			3	3	0.455	-0.122	1.03
			Mean		0.629	0.172	1.09
		α	1	3	0.468	0.00474	0.931
			2	3	0.354	0.161	0.547
			3	3	0.260	-0.366	0.886
			Mean		0.361	-0.0666	0.788
Methyl	1000	Y_0	1	3	8.61	8.39	8.82
			2	3	8.72	8.60	8.84
			3	3	8.53	8.38	8.68
			Mean		8.62	8.46	8.78
		K	1	3	5.39	-1.97	12.8
			2	3	3.77	3.03	4.51
			3	3	0.614	0.237	0.991
			Mean		3.26	0.434	6.08
		α	1	3	1.24	0.311	2.18
			2	3	0.644	0.509	0.778
			3	3	0.308	-0.0179	0.634
			Mean		0.732	0.267	1.20
Ethyl	500	Y_0	1	3	8.73	8.52	8.93
			2	3	8.56	8.37	8.75
			Mean		8.64	8.45	8.84
		K	1	3	3.17	1.82	4.52

			2	3	4.55	2.03	7.07
			Mean		3.86	1.92	5.79
		α	1	3	0.674	0.380	0.968
			2	3	0.905	0.519	1.29
			Mean		0.790	0.449	1.13
Ethyl	800	Y_0	1	3	8.59	8.19	9.00
			2	3	8.73	8.59	8.87
			3	3	8.52	8.33	8.72
			Mean		8.61	8.37	8.86
		K	1	3	9.21	4.79	13.6
			2	3	5.69	4.19	7.19
			3	3	21.0	15.6	26.4
			Mean		12.0	8.21	15.7
		α	1	3	0.839	0.504	1.18
			2	3	0.826	0.642	1.01
			3	3	1.16	0.988	1.34
			Mean		0.943	0.711	1.18
Propyl	125	Y_0	1	3	8.56	8.36	8.75
			2	3	8.73	8.46	9.00
			Mean		8.64	8.41	8.87
		K	1	3	3.16	0.896	5.42
			2	3	1.75	0.933	2.56
			Mean		2.45	0.915	3.99
		α	1	3	0.866	0.366	1.37
			2	3	0.386	0.114	0.659
			Mean		0.626	0.240	1.01
Propyl	250	Y_0	1	3	8.66	8.33	8.99
			2	3	8.44	8.03	8.86
			3	3	8.53	8.37	8.68
			Mean		8.54	8.24	8.84
		K	1	3	8.92	5.46	12.4
			2	3	11.3	7.36	15.3
			3	3	4.78	3.87	5.70
			Mean		8.34	5.56	11.1
		α	1	3	0.826	0.555	1.10
			2	3	0.869	0.608	1.13
			3	3	0.629	0.498	0.760
			Mean		0.775	0.554	0.996
Propyl	375	Y_0	1	3	8.65	8.38	8.92
			2	3	8.34	8.14	8.54

			Mean		8.49	8.26	8.73
		K	1	3	12.7	8.62	16.7
			2	3	15.3	12.0	18.6
			Mean		14.0	10.3	17.7
		α	1	3	0.946	0.724	1.17
			2	3	0.979	0.827	1.13
			Mean		0.962	0.776	1.15
Propyl	500	Y_0	1	3	8.54	7.38	9.71
			2	3	8.64	7.53	9.75
			Mean		8.59	7.45	9.73
		K	1	3	10.0	6.88	13.1
			2	3	10.2	6.88	13.5
			Mean		10.1	6.88	13.3
		α	1	3	0.334	0.162	0.506
			2	3	0.382	0.193	0.571
			Mean		0.358	0.178	0.539
Butyl	75	Y_0	1	3	8.71	8.45E	8.98
			2	3	8.78	8.50	9.06
			Mean		8.75	8.47	9.02
		K	1	3	8.47	5.64	11.3
			2	3	5.24	2.32	8.17
			Mean		6.86	3.98	9.73
		α	1	3	0.828	0.595	1.06
			2	3	0.824	0.435	1.21
			Mean		0.826	0.515	1.14
Butyl	100	Y_0	1	3	8.48	8.27	8.70
			2	3	8.86	8.25	9.46
			3	3	8.62	7.70	9.55
			Mean		8.65	8.07	9.24
		K	1	3	25.2	19.3	31.2
			2	3	22.9	12.1	33.8
			3	3	15.4	4.11	26.7
			Mean		21.2	11.8	30.6
		α	1	3	1.16	0.994	1.32
			2	3	1.01	0.680	1.34
			3	3	0.815	0.325	1.31
			Mean		0.993	0.666	1.32
Butyl	125	Y_0	1	3	8.71	7.45	9.97
			2	3	8.74	7.94	9.54
			Mean		8.73	7.69	9.76

		K	1	3	12.5	7.69	17.2
			2	3	18.1	10.2	25.9
			Mean		15.3	8.95	21.6
		α	1	3	0.478	0.234	0.722
			2	3	0.801	0.497	1.10
			Mean		0.639	0.366	0.913
Butyl	250	Y_0	1	3	8.86	7.82	9.89
			2	3	8.70	7.59	9.82
			Mean		8.78	7.70	9.85
		K	1	3	11.0	8.44	13.6
			2	3	14.4	10.1	18.6
			Mean		12.7	9.29	16.1
		α	1	3	0.305	0.180	0.429
			2	3	0.477	0.290	0.665
			Mean		0.391	0.235	0.547
Heptyl	15.5	Y_0	1	3	8.71	8.42	8.99
			2	3	8.41	8.18	8.64
			Mean		8.56	8.30	8.82
		K	1	3	13.1	9.56	16.5
			2	3	19.9	16.8	23.0
			Mean		16.5	13.2	19.8
		α	1	3	0.873	0.687	1.06
			2	3	0.933	0.825	1.04
			Mean		0.903	0.756	1.05
Heptyl	31.25	Y_0	1	3	9.02	8.32	9.72
		K	1	3	11.7	9.23	14.2
		α	1	3	0.454	0.321	0.586
Heptyl	50	Y_0	1	3	8.85	8.20	9.50
			2	3	8.61	7.80	9.42
			3	3	8.85	8.03	9.67
			Mean		8.77	8.01	9.53
		K	1	3	6.05	4.85	7.24
			2	3	8.15	6.53	9.78
			3	3	8.49	6.69	10.3
			Mean		7.56	6.02	9.11
		α	1	3	0.128	0.0494	0.206
			2	3	0.181	0.0945	0.267
			3	3	0.233	0.133	0.334
			Mean		0.181	0.0921	0.269
Heptyl	100	Y_0	1	3	8.61	8.06	9.16

			2	3	8.38	7.25	9.51
			Mean		8.49	7.65	9.33
		K	1	3	9.22	8.13	10.3
			2	3	7.99	5.99	9.99
			Mean		8.61	7.06	10.2
		α	1	3	0.174	0.123	0.225
			2	3	0.102	0.00712	0.196
			Mean		0.138	0.0653	0.210
Heptyl	125	Y_0	1	3	8.66	8.18	9.13
		K	1	3	7.34	6.45	8.22
		α	1	3	0.137	0.0883	0.185

[a]Number of replicate (maximum is 3)
[b]Number of model fitting times for each replicate
[c]Grand mean of each replicate's mean
[d]When the value of L95CI and U95CI is less than 0, the value is not meaningful

Table 5-3. Summary of Weibull parameters for survival curves at 58 °C in the presence of different concentrations of parabens.

Paraben	***Conc. (ppm)***	***Parameter***	***Rep[a]***	***N[b]***	***Mean***	***L95CI[d]***	***U95CI[d]***
Control	0	Y_0	1	3	8.57	8.29	8.86
			2	3	8.71	8.59	8.83
			3	3	8.29	8.04	8.54
			Mean[c]		8.52	8.31	8.74
		K	1	3	5.07	3.91	6.23
			2	3	3.63	3.14	4.13
			3	3	6.48	5.29	7.67
			Mean		5.06	4.11	6.01
		α	1	3	0.564	0.397	0.730
			2	3	0.514	0.425	0.604
			3	3	0.617	0.482	0.752
			Mean		0.565	0.435	0.695
Methyl	125	Y_0	1	3	8.56	8.01	9.10
		K	1	3	12.2	6.44	17.9
		α	1	3	1.01	0.592	1.42
Methyl	250	Y_0	1	3	8.68	8.29	9.08
			2	3	8.55	8.43	8.67
			Mean		8.62	8.36	8.87

		K	1	3	6.33	4.24	8.43
			2	3	6.49	5.56	7.43
			Mean		6.41	4.90	7.93
		α	1	3	0.634	0.401	0.867
			2	3	0.725	0.625	0.825
			Mean		0.679	0.513	0.846
Methyl	500	Y_0	1	3	8.57	8.30	8.84
			2	3	8.53	8.20	8.85
			3	3	8.67	8.45	8.89
			Mean		8.59	8.32	8.86
		K	1	3	7.63	6.27	9.00
			2	3	6.54	5.13	7.94
			3	3	7.89	6.58	9.19
			Mean		7.35	5.99	8.71
		α	1	3	0.581	0.460	0.701
			2	3	0.528	0.387	0.670
			3	3	0.634	0.521	0.748
			Mean		0.581	0.456	0.706
Methyl	650	Y_0	1	3	8.49	8.19	8.80
		K	1	3	13.0	7.51	18.5
		α	1	3	1.01	0.716	1.30
Methyl	800	Y_0	1	3	8.59	8.44	8.74
			2	3	8.65	8.37	8.94
			Mean		8.62	8.40	8.84
		K	1	3	11.7	10.8	12.6
			2	3	23.0	14.8	31.2
			Mean		17.3	12.8	21.9
		α	1	3	0.661	0.605	0.717
			2	3	1.18	0.932	1.42
			Mean		0.919	0.768	1.07
Methyl	1000	Y_0	1	3	8.51	8.31	8.71
			2	3	8.49	8.34	8.64
			Mean		8.50	8.32	8.68
		K	1	3	13.5	12.0	14.9
			2	3	17.1	14.8	19.4
			Mean		15.3	13.4	17.1
		α	1	3	0.703	0.627	0.778
			2	3	0.946	0.854	1.04
			Mean		0.825	0.740	0.909
Ethyl	125	Y_0	1	3	8.31	7.84	8.78

			2	3	8.73	8.48	8.98
			Mean		8.52	8.16	8.88
		K	1	3	12.3	6.38	18.3
			2	3	5.21	3.96	6.46
			Mean		8.77	5.17	12.4
		α	1	3	0.997	0.613	1.38
			2	3	0.568	0.406	0.730
			Mean		0.783	0.510	1.06
Ethyl	250	Y_0	1	3	8.63	8.19	9.08
			2	3	8.46	7.99	8.93
			3	3	8.75	8.26	9.24
			Mean		8.62	8.15	9.08
		K	1	3	9.44	7.22	11.7
			2	3	15.2	8.96	21.4
			3	3	10.7	4.85	16.6
			Mean		11.8	7.01	16.5
		α	1	3	0.629	0.452	0.806
			2	3	0.900	0.614	1.19
			3	3	0.805	0.454	1.16
			Mean		0.778	0.507	1.05
Ethyl	500	Y_0	1	3	8.71	8.23	9.18
			2	3	8.79	8.38	9.21
			3	3	8.56	7.97	9.15
			Mean		8.69	8.19	9.18
		K	1	3	11.8	10.0	13.6
			2	3	15.7	11.9	19.6
			3	3	19.3	11.5	27.2
			Mean		15.6	11.1	20.2
		α	1	3	0.557	0.442	0.671
			2	3	0.785	0.613	0.958
			3	3	0.906	0.622	1.19
			Mean		0.749	0.559	0.940
Ethyl	800	Y_0	1	3	8.89	8.04	9.74
			2	3	8.64	8.32	8.96
			Mean		8.77	8.18	9.35
		K	1	3	9.07	7.04	11.1
			2	3	16.9	12.6	21.2
			Mean		13.0	9.82	16.2
		α	1	3	0.318	0.194	0.441
			2	3	0.911	0.734	1.09

			Mean		0.615	0.464	0.765
Propyl	62.5	Y_0	1	3	8.75	8.22	9.28
		K	1	3	11.5	4.62	18.3
		α	1	3	0.956	0.503	1.41
Propyl	93.75	Y_0	1	3	8.41	7.90	8.92
			2	3	8.44	7.96	8.93
			Mean		8.42	7.93	8.92
		K	1	3	19.5	10.6	28.4
			2	3	16.8	7.41	26.1
			Mean		18.1	8.99	27.3
		α	1	3	1.07	0.725	1.41
			2	3	1.15	0.726	1.57
			Mean		1.11	0.725	1.49
Propyl	125	Y_0	1	3	8.65	7.84	9.46
			2	3	8.58	8.24	8.91
			3	3	8.45	7.96	8.95
			Mean		8.56	8.01	9.11
		K	1	3	11.5	8.69	14.3
			2	3	18.3	12.5	24.1
			3	3	21.4	9.98	32.9
			Mean		17.1	10.4	23.8
		α	1	3	0.498	0.331	0.665
			2	3	0.994	0.774	1.21
			3	3	1.09	0.726	1.46
			Mean		0.862	0.610	1.11
Propyl	250	Y_0	1	3	9.16	8.19	10.1
			2	3	8.88	7.79	9.98
			Mean		9.02	7.99	10.1
		K	1	3	8.74	6.68	10.8
			2	3	10.3	7.19	13.3
			Mean		9.50	6.94	12.1
		α	1	3	0.336	0.190	0.482
			2	3	0.414	0.221	0.606
			Mean		0.375	0.206	0.544
Propyl	500	Y_0	1	3	8.84	8.47	9.22
			2	3	8.83	7.50	10.2
			Mean		8.83	7.98	9.69
		K	1	3	8.85	8.00	9.70
			2	3	10.1	6.93	13.2
			Mean		9.46	7.47	11.4

		α	1	3	0.248	0.201	0.295
			2	3	0.334	0.154	0.513
			Mean		0.291	0.178	0.404
Butyl	31.25	Y_0	1	3	8.65	8.08	9.22
			2	3	8.74	8.45	9.03
			Mean		8.69	8.26	9.13
		K	1	3	15.2	5.81	24.7
			2	3	15.1	12.1	18.1
			Mean		15.2	8.96	21.4
		α	1	3	1.04	0.582	1.51
			2	3	0.864	0.718	1.01
			Mean		0.954	0.650	1.26
Butyl	62.5	Y_0	1	3	8.72	8.14	9.31
			2	3	8.69	8.38	9.00
			Mean		8.71	8.26	9.15
		K	1	3	15.1	11.0	19.2
			2	3	16.6	13.8	19.3
			Mean		15.8	12.4	19.3
		α	1	3	0.747	0.543	0.952
			2	3	0.774	0.656	0.892
			Mean		0.761	0.599	0.922
Butyl	75	Y_0	1	3	8.90	7.95	9.85
		K	1	3	13.5	7.95	19.0
		α	1	3	0.627	0.348	0.907
Butyl	100	Y_0	1	3	8.94	7.87	10.0
		K	1	3	9.09	6.20	12.0
		α	1	3	0.340	0.164	0.516
Butyl	125	Y_0	1	3	8.01	7.34	8.68
			2	3	8.68	7.80	9.56
			3	3	8.90	8.12	9.68
			Mean		8.53	7.75	9.30
		K	1	3	9.21	6.96	11.5
			2	3	8.46	6.29	10.6
			3	3	10.4	8.11	12.6
			Mean		9.35	7.12	11.6
		α	1	3	0.305	0.175	0.435
			2	3	0.296	0.162	0.431
			3	3	0.394	0.257	0.531
			Mean		0.332	0.198	0.465
Heptyl	5	Y_0	1	3	18.9	18.1	19.6

		K	1	3	28.7	20.2	37.1
		α	1	3	0.853	0.646	1.06
Heptyl	7.8	Y_0	1	3	8.92	7.81	10.0
		K	1	3	12.2	7.25	17.2
		α	1	3	0.539	0.270	0.808
Heptyl	15.5	Y_0	1	3	8.82	8.36	9.29
			2	3	8.79	7.88	9.69
			Mean		8.80	8.12	9.49
		K	1	3	8.43	7.55	9.31
			2	3	10.0	7.30	12.7
			Mean		9.22	7.42	11.0
		α	1	3	0.259	0.202	0.316
			2	3	0.371	0.212	0.530
			Mean		0.315	0.207	0.423
Heptyl	31.25	Y_0	1	3	8.71	8.17	9.25
			2	3	8.88	8.17	9.58
			3	3	8.65	8.21	9.09
			Mean		8.74	8.19	9.30
		K	1	3	8.65	7.66	9.64
			2	3	8.59	7.17	10.0
			3	3	8.52	7.45	9.60
			Mean		8.59	7.42	9.75
		α	1	3	0.127	0.0820	0.172
			2	3	0.156	0.0845	0.228
			3	3	0.255	0.190	0.320
			Mean		0.179	0.119	0.240
Heptyl	62.5	Y_0	1	3	8.85	7.61	10.1
			2	3	8.64	7.74	9.55
			Mean		8.75	7.67	9.82
		K	1	3	9.75	7.18	12.3
			2	3	9.23	7.43	11.0
			Mean		9.49	7.30	11.7
		α	1	3	0.199	0.0813	0.318
			2	3	0.175	0.0913	0.258
			Mean		0.187	0.0863	0.288
Heptyl	125	Y_0	1	3	8.50	7.66	9.35
		K	1	3	7.74	6.31	9.18
		α	1	3	0.0749	0.00805	0.142

[a]Number of replicate (maximum is 3)
[b]Number of model fitting times for each replicate

[c]Grand mean of each replicate's mean
[d]When the value of L95CI and U95CI is less than 0, the value is not meaningful

A tips for Table 5-1, 5-2, and 5-3 is to not pay much attention to the values of lower 95% confidence internal and upper 95% confidence interval, particularly lower 95% confidence interval because it is not meaningful when the value is less than 0.

5.4 Discussion

In selecting a primary model, three factors were considered: flexibility in terms of the types of survivor curves that could be fitted, the goodness of the fits achieved, and minimizing the number of parameters in the model. Of the models evaluated, the Weibull model best met these criteria. In this model, the critical parameter was α, which determines the shape of curve. Most of the inactivation data fitted as linear or exponential decrease shape, as shown in Figure 5-2a-d. Very few of survivor curves display a shoulder.

When fitting, data points were distributed evenly above or below the curves. For some concentrations of heptylparaben, during the later phase of inactivation, population density was below the detection limit (2.0 log (CFU/ml)), where 1.8 log was assigned. Therefore, a steady tail was created to serve in modeling whereas the tail did not exist in reality. In this case, the data points were fitted better in a two/three-phase linear model or linear model with tail. However, these special cases could not represent the rest of data, which was the basis for eliminating linear model as a survival model option.

Curve fitting was limited to the plate counts recovered from TSA plates since the data included both heat-injured and non-injured cells, which would be normally used to

calculate thermal processing times and temperatures. In the future date, the MA data (non-injured cells) could be fitted to more thoroughly explore the impact of injury in the enhancement of thermal inactivation by parabens.

Chapter 6: Development of Secondary Model

6.1 Introduction

Secondary models are designed to mathematically describe the changes in the parameters derived from primary model as function of one or more environmental conditions such as temperature, pH, or water activity. In this study, uncovering the relationship between Y_0, K, α and one of the condition factors (alkyl side chain length, concentration and temperature) was the purpose of developing a secondary model.

6.2 Methods

The preliminary graphing for K and α with increase of concentration did not present a constant pattern. Thus, an approach was taken where the time to achieve 2.5-log reduction ($t_{2.5R}$). The $t_{2.5R}$ was calculated by re-arranging the Weibull equation and solving for time:

$$t = 10\text{^}\{\log_{10}^{[(Y0 - Y)/K]/\alpha}\}.$$

Using two of Y values, Y=7.5 log CFU/ml and Y=5.0 log CFU/ml in the formula, two t values corresponding to each Y value were calculated with each set of known Y_0, K and α values. The $t_{2.5R}$ values were then computed for each specific condition (alkyl side chain length, concentration, temperature). To simplify the secondary model, a $\log_{10}$ transformation was applied to the $t_{2.5R}$. A linear regression was conducted between log ($t_{2.5R}$) and concentration of each paraben.

6.3 Results

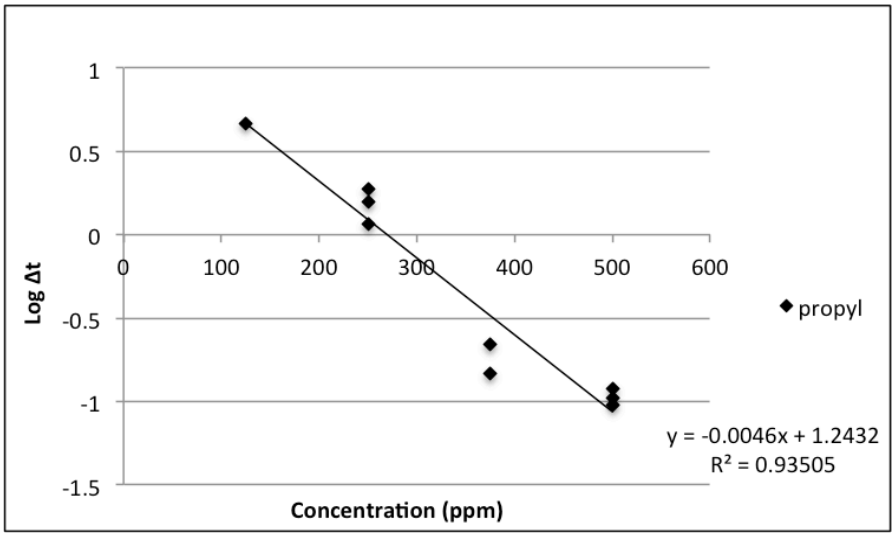
The effectiveness of this approach to secondary modeling as a function of temperature and concentration are summarized in Table 6-1 and Figure 6-1.

Table 6-1. Time to achieve 2.5 log reduction ($t_{2.5R}$) by heating *C. sakszakii* in BHI at 52°C, 55°C, 58°C as affected by parabens concentration and identity

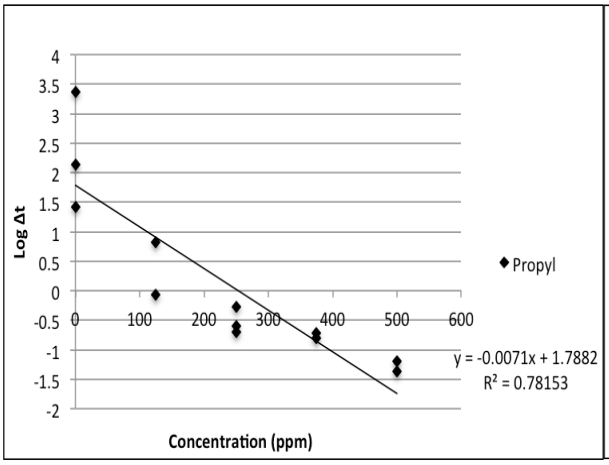
Paraben	***Conc. (ppm)***	***Temperature: 52°C***			***Temperature: 55°C***			***Temperature: 58°C***		
		Log_{10} ($t_{2.5R}$)			***Log_{10} ($t_{2.5R}$)***			***Log_{10} ($t_{2.5R}$)***		
		R1[a]	***R2***	***R3***	***R1***	***R2***	***R3***	***R1***	***R2***	***R3***
Control	0	1.71	5.40	4.29	1.42	2.13	3.37	-0.32	-0.03	-0.52
Methyl	125	-	-	-	-	-	-	-0.69	-	-
	250	-	-	-	-	-	-	-0.45	-0.45	-
	500	-	-	-	-	-	-	-0.63	-0.55	-0.60
	650	-	-	-	-	-	-	-0.71	-	-
	800	-	-	-	-	-	-	-0.85	-0.88	-
	1000	1.89	44.44	-	-0.35	-0.09	2.46	-0.91	-0.86	-
Ethyl	125	-	-	-	-	-	-	-0.69	-0.32	-
	250	-	-	-	-	-	-	-0.73	-0.83	-0.69
	500	-	-	-	0.01	-0.25	-	-0.97	-0.91	-0.94
	800	-0.60	3.08	-0.50	-0.61	-0.35	-0.85	-1.18	-0.87	-
Propyl	62.5	-	-	-	-	-	-	-0.67	-	-
	93.75	-	-	-	-	-	-	-0.86	-0.77	-
	100	-	-	-	-	-	-	-	-	-
	125	0.67	-	-	-0.06	0.83	-	-1.05	-0.87	-0.88
	250	0.20	0.06	0.27	-0.59	-0.71	-0.28	-0.99	-1.06	-
	375	-0.83	-0.66	-	-0.72	-0.80	-	-	-	-
	500	-0.98	-1.02	-0.92	-1.36	-1.19	-	-1.47	-1.28	-
Butyl	31.25	-	-	-	-	-	-	-0.77	-0.84	-
	62.5	-	-	-	-	-	-	-0.92	-0.96	-
	75	-0.25	1.45	-	-0.56	-0.31	-0.92	-0.95	-	-
	100	-	-	-	-0.92	-0.96	-0.89	-1.09	-	-
	125	-0.80	-0.47	-	-1.14	-0.98	-	-1.59	-1.23	-1.11
	200	-1.04	-1.12	-	-	-	-	-	-	-
	250	-2.80	-1.57	-1.20	-1.51	-1.28	-	-	-	-
Heptyl	5	-	-	-	-	-	-	-1.05	-	-
	7.8	-	-	-	-	-	-	-0.99	-	-
	15.5	-	-	-	-0.77	-0.94	-	-1.33	-1.16	-
	31.25	-0.12	-0.03	-	-1.08	-	-	-2.89	-2.21	-1.45

	50	-0.47	-0.50	-0.27	-1.54	-1.96	-1.48	-	-	-
	62.5	-	-	-	-	-	-	-2.03	-2.31	-
	100	-0.96	-	-	-2.34	-3.69	-	-	-	-
	125	-1.06	-0.96	-0.53	-2.21	-	-	-4.60	-	-

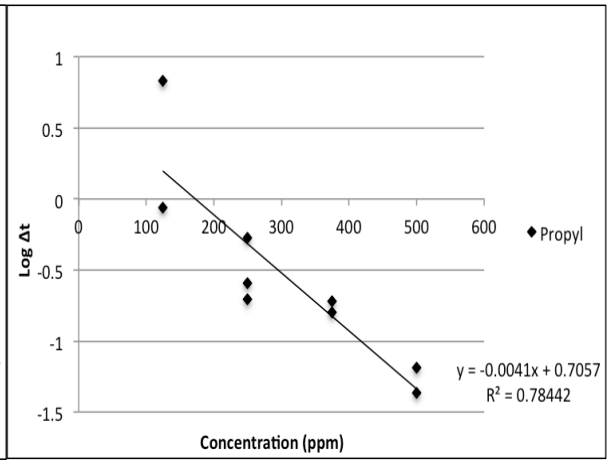
[a]: Number of replicate (maximum is three)



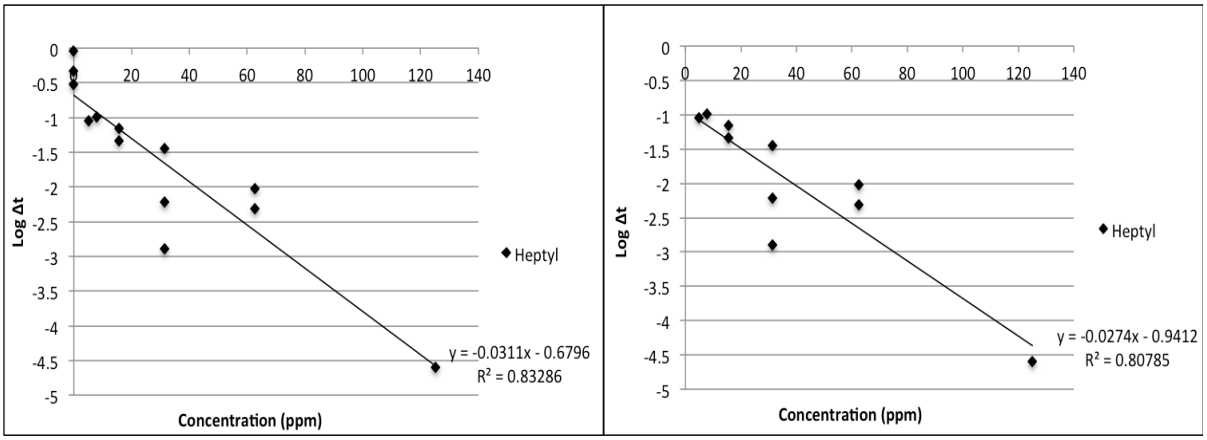
a.



b.



c.



d. e.

Figure 6-1. Linear regression of (a) propyl-paraben without control (0 ppm) at 52°C; (b) propyl-paraben with control (0ppm) at 55°C; (c) propyl-paraben without control (0 ppm) at 55°C; (d) heptyl-paraben with control (0ppm) at 58°C; (e) heptyl-paraben without control (0 ppm) at 58°C

Table 6-2. Summary of parameters from linear regression models of the log transformed $t_{2.5R}$ values at 52°C, 55°C, 58°C.

	***Temperature: 52 °C*[a]**			***Temperature: 55°C***			***Temperature: 58°C***				
Paraben	**Propyl**	**Butyl**	**Heptyl**	**Propyl**	**Butyl**	**Heptyl**	**Methyl[b]**	**Ethyl[b]**	**Propyl**	**Butyl**	**Heptyl**
W/control											
Slope	N/A	N/A	N/A	-0.0071	-0.0147	-0.0381	-0.0006	-0.0009	-0.0019	-0.0076	-0.0274
Intercept	N/A	N/A	N/A	1.7882	1.3376	0.9482	-0.3449	-0.3992	-0.5134	-0.3970	-0.9412
R^2	N/A	N/A	N/A	0.7815	0.6273	0.6470	0.6808	0.6940	0.7374	0.8130	0.8079
W/O control											
Slope	-0.0046	-0.0091	-0.0077	-0.0041	-0.0091	-0.0186	-0.0005	-0.0007	-0.0014	-0.0054	-0.0274
Intercept	1.2432	0.5255	0.0477	0.7057	-0.1853	-0.6656	-0.4139	-0.5243	-0.7021	-0.6067	-0.9412
R^2	0.9351	0.6297	0.7281	0.7844	0.6583	0.6644	0.7071	0.6169	0.8632	0.7149	0.8079

[a]Due to lack of control data (no inactivation observed during sampling time at 52°C), linear regression was conducted without control.

[b]Due to lack of data points, linear regression was not applied for methyl- and ethyl-paraben at 52°C and 55°C.

6.4 Discussion

Based on initial plotting, a direct relationship between any of three parameters from Weibull model was not readily apparent, and may reflect the fact that the shape of the survivor curve is dependent on the interaction of both K and α and any of factors influencing inactivation activity. This prompted the use of an "outcome" based metric for modeling the effects of thermal treatments, parabens inclusion, and their interaction. A 2.5-log reduction was selected as a means of including most of the types of reductions that were observed during the experimental trials.

The initial plotting of $t_{2.5R}$ values versus parabens concentration at 52°C, 55°C, 58°C, generated exponential shape curves. As a mean of simplifying the models several mathematical transformation were evaluated. A log transformation of $t_{2.5R}$ allowed the relationship between $t_{2.5R}$ and parabens concentration to be modeled with a linear model. While generally good, the R^2 values showed deviation from linearity. However, overall this appears to provide a reasonable approach to modeling this complex date set.

The methyl-paraben and ethyl-paraben did not have enough data points to generate a meaningful linear regression since even with a maximum concentration evaluated, little or no inactivation were observed, particularly at the lower thermal treatment temperatures. Therefore, at lower heating temperature, no attempt was made to develop secondary model for methyl-paraben and ethyl-paraben.

Chapter 7: Summary, Conclusions and Recommendations for Future Research

7.1 General discussion of study

Cronobacter sakazakii is one of the microorganisms that possess heat resistance and survival ability under dry condition and therefore, it was used in this study to examine the enhancement of thermal inactivation activity. However, other bacteria strains might be involved in contamination of powdered infant formula such as *Enterobacter cloacae, Klebsiella ozaenae*, *Citrobacter freundii, Salmonella* Typhi *and Shigella dysenteriae* (*26*, *59*).

The mode of action for parabens against microorganisms is not fully understood. It is proposed that in addition to the acidification in cytosol, disruption of cell membrane is probably the main reason. Further research can be invested to discover the real mechanism behind it.

While pasteurization is widely applied in industry, combination of parabens and mild heat treatment is particularly advantageous because it can lead to effective reductions in food-borne pathogens at low intensities, and maximize the likelihood that food quality will be maintained. Although this study was conducted in a liquid model system, which is different from a food system, this combined intervention was proved to effectively inactivate *Salmonella enteritidis* and *Salmonella oranienburg* in liquid

egg albumen (*49*). It is feasible to implement the application in the industry. Additionally, the primary and secondary mathematical models can be used as a tool to predict the effectiveness of thermal inactivation with inclusion of parabens under various conditions.

7.2 Future research

Additional research is needed to more conclusively determine other factors that affect the antimicrobial effectiveness of parabens. For example, the branch position of R-group in paraben (propyl- and isopropyl-paraben) may or may not have an effect on the bactericidal property. Also, the position of hydroxyl group (ortho/2-methyl-paraben, meta/3-methyl-paraben, para/4-methyl-paraben), and number of hydroxyl group (2, 3-dihydroxyl methyl-paraben, 3,4,5-trihydroxyl methyl-paraben) may impact on inactivation rates. Moreover, number and position of double bond/triple bond in the alkyl side chain may play a role in killing microorganisms.

Since the parabens' solubility is low in water and it is related to its chemical structure, partition coefficients of parabens in food need to be investigated. Most food is an emulsion system, which contains lipid phase and water phase at the same time. If parabens can be remained in water phase in a food system, then the antimicrobial effect can be reached to the maximum level, as microbes prefer to live with water. Meanwhile, the concentration of parabens can be lowered down to ensure the safety of human health. A food system contains other intrinsic factors, such as water activity

and pH. More thermal inactivation studies need to be conducted to look into the antimicrobial effects of parabens and intrinsic conditions.

For modeling, other models can be selected to fit the data since IPMP is only one of multiple user-friendly modeling software. For example, OriginPro is a good data analysis and graphing software. Additional validation tests to assess the accuracy of predictive models are needed as well.

7.3 Conclusions

- Thermal inactivation of heat resistant *C. sakazakii* 607 is significantly enhanced by the inclusion of "parabens" in a concentration-dependent manner. The degree of inactivation achieved at a certain concentration increases with the increasing of alkyl side chain length of the parabens. The three parameters act synergistically on thermal inactivation of *C. sakazakii* 607.
- Weibull model is the primary model that describes the change of population density of heat resistant *C. sakazakii* 607 under various conditions (concentration, alkyl side chain length, temperature).
- Linear model is the secondary model proposed to elucidate the relationship between time to achieve 2.5-log reduction and alkyl side chain length.

This entire work yield a conclusion of how thermal inactivation was enhanced synergistically as a result of inclusion of antimicrobial compound or combinations of

multiple process treatments. It also provides insights into how inactivation models can be used to systematically describe enhanced inactivation kinetics.

Table 8-1. List of Log_{10} population density for methyl-paraben and ethyl-paraben at 52 °C

Time (sec)	Methyl-paraben Concentration (ppm)						Ethyl-paraben Concentration (ppm)					
	0			1000			0			800		
Rep[a]	R1	R2	R3	R1	R2	R3	R1	R2	R3	R1	R2	R3
0	8.43	8.78	8.66	8.56	8.58	8.62	8.43	8.78	8.66	8.74	8.57	8.48
90	-	-	-	8.34	8.43	8.37	-	-	-	8.43	8.33	8.15
180	8.47	-	-	8.32	8.40	8.49	8.47	-	-	8.43	8.13	8.17
270	8.40	8.31	8.71	-	8.53	8.39	8.40	8.31	8.71	8.39	8.19	8.08
360	8.41	8.53	8.60	-	8.38	8.44	8.41	8.53	8.60	8.42	8.21	8.03
450	-	8.43	8.74	8.27	8.52	8.49	-	8.43	8.74	8.41	8.20	7.76
540	8.26	8.54	8.60	8.34	8.40	8.52	8.26	8.54	8.60	8.38	7.90	7.61
630	8.31	8.56	8.73	-	-	8.35	8.31	8.56	8.73	8.26	7.73	7.43
720	8.44	8.50	8.63	8.33	8.31	8.48	8.44	8.50	8.63	8.31	7.52	7.23
810	8.27	8.47	8.67	8.39	8.38	8.46	8.27	8.47	8.67	8.25	7.42	7.13

[a]. replicate number (maximum is 3)

Table 8-2. List of Log_{10} population density for propyl-paraben at 52 °C

Time (sec)	Propyl-paraben Concentration (ppm)											
	0			125	250			375		500		
Rep[a]	R1	R2	R3	R1	R1	R2	R3	R1	R2	R1	R2	R3
0	8.54	8.76	8.78	8.60	8.57	8.61	8.72	8.85	8.76	8.45	8.56	8.74
90	8.47	8.38	8.22	8.40	8.25	8.51	8.52	8.38	8.41	8.14	7.94	8.37
180	8.39	8.57	8.36	8.44	8.21	8.23	8.31	8.14	8.28	6.90	6.97	8.09
270	8.37	8.50	8.38	8.42	8.14	8.17	8.31	7.76	7.95	5.49	5.71	7.50
360	8.45	8.63	8.19	8.38	8.04	8.27	8.15	7.40	7.51	4.76	4.66	7.14
450	8.44	8.64	8.20	8.39	7.94	8.14	8.21	7.15	7.25	4.38	4.25	6.59
540	8.33	8.52	8.24	8.26	8.01	7.88	8.17	6.87	6.96	4.29	4.06	6.13
630	8.48	8.46	8.21	8.29	7.70	7.89	8.02	6.40	6.81	4.20	3.94	5.26
720	8.22	8.52	8.18	8.18	7.72	7.74	7.95	5.85	6.44	4.05	3.75	4.92
810	8.32	8.53	8.06	8.18	7.64	7.80	7.78	5.24	6.19	3.96	3.48	4.47

[a]. replicate number (maximum is 3)

Table 8-3. List of Log_{10} population density for butyl-paraben at 52 °C

Time (sec)	Butyl-paraben Concentration (ppm)											
	0			75		125		200		250		
Rep[a]	R1	R2	R3	R1	R2	R1	R2	R1	R2	R1	R2	R3
0	8.54	8.55	8.61	8.70	8.94	8.95	8.50	8.60	8.34	8.01	8.63	8.48
90	8.47	8.44	-	8.54	8.49	8.29	8.03	7.54	7.44	4.35	5.59	7.01
180	8.39	8.45	8.26	8.35	8.47	8.06	8.10	6.52	6.80	2.30	4.03	5.97
270	8.37	8.45	8.59	8.11	8.40	7.72	8.02	4.91	5.18	1.80[b]	3.76	4.11
360	8.45	8.39	8.63	8.06	8.35	7.28	7.60	4.34	4.18	1.80[b]	3.08	3.66
450	8.44	8.40	8.70	7.98	8.48	6.91	7.40	4.18	3.38	1.80[b]	3.30	3.45
540	8.33	8.39	8.62	7.81	-	6.70	7.25	4.09	3.08	1.80[b]	3.00	3.48
630	8.48	8.48	8.61	7.77	8.05	6.09	7.02	3.99	3.08	1.80[b]	2.78	3.41
720	8.22	8.44	8.47	7.53	-	5.76	6.91	3.85	3.15	180[b]	2.60	3.08
810	8.32	8.41	8.55	7.38	8.24	5.20	6.72	3.64	3.20	1.80[b]	2.30	3.00

[a]. replicate number (maximum is 3)
[b]. lower limit of detection = 2.0 log (CFU/ml). Samples where no viable cells were recovered were assigned a value of log CFU/ml =1.8.

Table 8-4. List of Log_{10} population density for hetyl-paraben at 52 °C

Time (sec)	Heptyl-paraben Concentration (ppm)											
	0			31.25		50			100	125		
Rep[a]	R1	R2	R3	R1	R2	R1	R2	R3	R1	R1	R2	R3
0	8.54	8.70	8.61	8.85	8.63	8.71	8.64	8.53	8.53	8.59	8.96	8.76
90	8.47	8.53	8.49	7.93	7.66	7.63	6.99	6.78	7.42	5.71	6.77	7.45
180	8.39	8.59	8.52	7.68	7.60	7.31	6.79	6.83	5.71	5.56	5.87	6.01
270	8.37	8.66	8.49	7.57	7.43	6.92	6.47	6.77	5.36	5.20	5.48	6.03
360	8.45	8.83	8.46	7.34	7.30	6.71	6.30	6.52	5.03	4.93	5.19	6.04
450	8.44	8.71	8.40	7.18	7.17	6.48	6.13	6.49	4.77	4.77	4.83	6.31
540	8.33	8.67	8.42	7.01	7.05	6.21	5.83	6.24	4.54	4.48	4.61	5.67
630	8.48	8.77	8.33	6.89	6.80	6.06	5.70	6.00	4.36	4.47	4.40	5.52
720	8.22	8.63	8.34	6.76	6.73	5.95	5.49	5.56	4.16	4.23	4.15	5.34
810	8.32	-	8.49	6.62	6.59	5.80	5.42	5.80	4.07	4.09	4.09	5.42

[a]. replicate number (maximum is 3)

Table 9-1. List of Log_{10} population density for methyl -araben and ethyl-paraben at 55 °C

Time (sec)	Methyl-paraben Concentration (ppm)						Ethyl-paraben Concentration (ppm)					
	0			1000			0			800		
Rep[a]	R1	R2	R3	R1	R2	R3	R1	R2	R3	R1	R2	R3
0	8.43	8.78	8.66	8.56	8.58	8.62	8.43	8.78	8.66	8.74	8.57	8.48
90	-	-	-	8.34	8.43	8.37	-	-	-	8.43	8.33	8.15
180	8.47	-	-	8.32	8.40	8.49	8.47	-	-	8.43	8.13	8.17
270	8.40	8.31	8.71	-	8.53	8.39	8.40	8.31	8.71	8.39	8.19	8.08
360	8.41	8.53	8.60	-	8.38	8.44	8.41	8.53	8.60	8.42	8.21	8.03
450	-	8.43	8.74	8.27	8.52	8.49	-	8.43	8.74	8.41	8.20	7.76
540	8.26	8.54	8.60	8.34	8.40	8.52	8.26	8.54	8.60	8.38	7.90	7.61
630	8.31	8.56	8.73	-	-	8.35	8.31	8.56	8.73	8.26	7.73	7.43
720	8.44	8.50	8.63	8.33	8.31	8.48	8.44	8.50	8.63	8.31	7.52	7.23
810	8.27	8.47	8.67	8.39	8.38	8.46	8.27	8.47	8.67	8.25	7.42	7.13

[a]. replicate number (maximum is 3)

Table 9-2. List of Log_{10} population density for propyl-paraben at 55 °C

Time (sec)	Propyl-paraben Concentration (ppm)											
	0			125		250			375		500	
Rep[a]	R1	R2	R3	R1	R2	R1	R2	R3	R1	R2	R1	R2
0	8.79	8.40	8.75	8.58	8.74	8.71	8.52	8.51	8.77	8.35	8.48	8.53
90	8.79	8.26	8.42	8.40	8.18	8.25	-	8.09	8.09	7.97	6.48	7.00
180	8.58	8.11	-	8.21	8.26	7.75	7.48	7.82	7.85	7.52	4.45	5.35
270	8.51	8.18	8.21	8.32	8.15	7.49	7.14	7.66	7.49	6.98	3.76	4.21
360	8.52	8.24	8.39	8.12	7.91	7.41	6.85	7.33	7.25	6.66	3.58	3.91
450	8.50	8.07	8.41	8.03	8.07	7.13	6.58	7.18	6.98	6.47	3.34	3.92
540	8.48	8.11	8.32	7.90	7.80	6.81	6.41	7.01	6.72	6.05	3.45	3.81
630	8.44	8.07	8.43	7.90	7.98	6.74	6.06	6.91	6.24	5.58	3.42	3.45
720	8.54	7.95	8.37	7.88	7.74	6.37	5.92	6.90	5.84	5.18	2.90	3.26
810	8.41	8.05	8.33	7.57	7.67	5.82	5.37	6.65	5.45	4.71	2.30	3.20

[a]. replicate number (maximum is 3)

Table 9-3. List of Log_{10} population density for butyl-paraben at 55 °C

Time (sec)	Butyl-paraben Concentration (ppm)											
	0			75		100			125		250	
Rep[a]	R1	R2	R3	R1	R2	R1	R2	R3	R1	R2	R1	R2
0	8.72	8.76	8.42	8.74	8.86	8.51	8.73	8.73	8.53	8.60	8.82	8.58
90	8.28	8.60	-	8.34	8.41	8.08	8.29	7.53	7.33	7.85	5.90	7.00
180	8.20	8.56	-	7.86	8.21	7.60	7.96	-	6.16	7.36	3.87	5.16
270	8.21	8.45	-	7.79	8.23	7.27	7.29	-	4.57	6.74	3.38	3.83
360	8.34	8.50	-	7.31	7.98	6.84	6.79	6.89	4.06	5.98	3.20	3.51
450	8.25	8.43	-	7.31	8.08	6.29	6.05	5.83	3.65	5.16	3.26	3.56
540	8.15	8.43	-	7.08	7.64	5.60	5.14	5.03	3.45	4.11	2.90	2.90
630	8.26	8.44	8.19	6.81	7.50	4.95	4.50	4.60	3.30	4.04	2.78	2.90
720	8.39	8.44	-	6.37	7.41	4.50	4.27	4.47	3.00	3.89	1.80[b]	1.80[b]
810	8.11	8.35	8.26	6.20	7.16	4.09	4.19	4.24	3.26	3.70	1.80[b]	1.80[b]

[a]. replicate number (maximum is 3)
[b]. lower limit of detection = 2.0 log (CFU/ml). Samples where no viable cells were recovered were assigned a value of log CFU/ml =1.8.

Table 9-4. List of Log_{10} population density for heptyl-paraben at 55 °C

Time (sec)	Heptyl-paraben Concentration (ppm)											
	0			15.5		31.25	50			100		125
Rep[a]	R1	R2	R3	R1	R2	R1	R1	R2	R3	R1	R2	R1
0	8.72	8.85	8.51	8.79	8.41	8.94	8.84	8.60	8.82	8.60	8.37	8.65
90	8.28	-	8.26	8.03	-	7.08	5.54	4.94	5.87	3.99	3.73	4.55
180	8.20	-	8.43	7.80	7.18	6.35	4.48	3.64	4.39	3.09	1.80[b]	3.51
270	8.21	8.52	8.46	7.25	6.73	5.26	4.30	3.20	3.89	2.78	1.80[b]	3.34
360	8.34	-	8.36	6.95	5.94	4.58	4.01	3.15	3.58	2.30	1.80[b]	3.15
450	8.25	8.43	8.36	6.60	5.52	4.21	4.06	2.78	3.30	1.80[b]	1.80[b]	3.15
540	8.15	8.53	8.46	6.35	5.10	3.73	4.14	2.90	3.53	1.80[b]	1.80[b]	3.08
630	8.26	8.36	8.18	6.00	4.61	3.72	4.14	2.30	3.20	1.80[b]	1.80[b]	3.00
720	8.39	8.52	8.11	5.53	3.90	3.51	4.03	2.78	3.15	1.80[b]	1.80[b]	2.90
810	8.11	8.30	-	4.97	3.45	3.41	4.06	2.78	3.15	1.80[b]	1.80[b]	2.60

[a]. replicate number (maximum is 3)
[b]. lower limit of detection = 2.0 log (CFU/ml). Samples where no viable cells were recovered were assigned a value of log CFU/ml =1.8.

Table 10-1. List of Log_{10} population density for methyl-paraben at 58 °C

Time (sec)	Methyl-paraben Concentration (ppm)													
	0			125	250		500			650	800		1000	
Rep[a]	R1	R2	R3	R1	R1	R2	R1	R2	R3	R1	R1	R2	R1	R2
0	8.62	8.72	8.28	8.91	8.73	8.55	8.60	8.52	8.64	8.61	8.59	8.78	8.55	8.53
90	7.84	8.09	7.68	7.94	-	8.13	7.62	7.68	8.06	8.05	7.53	8.21	7.43	7.86
180	7.52	8.00	7.26	7.69	7.55	7.78	7.22	7.15	7.45	7.72	-	7.81	6.78	7.51
270	7.35	7.78	6.93	7.54	7.35	7.55	6.75	6.77	7.06	7.52	6.51	7.61	6.41	7.05
360	7.19	7.60	6.73	7.40	7.19	7.25	6.61	6.47	6.80	7.29	6.13	7.26	5.91	6.56
450	7.16	7.41	6.60	7.08	7.14	7.15	6.35	6.39	6.60	7.16	5.62	6.83	5.48	6.20
540	7.05	7.33	6.10	6.95	6.92	6.94	6.26	6.17	6.19	6.49	5.19	6.20	4.99	5.59
630	6.75	7.26	-	6.56	6.72	6.79	5.81	6.17	6.15	6.20	4.90	5.55	4.52	5.17
720	6.47	7.16	6.05	-	6.48	6.50	5.54	5.78	5.92	6.00	4.45	5.16	4.04	4.72
810	6.33	6.98	5.74	-	6.28	6.30	5.23	5.36	5.56	5.52	4.26	4.74	3.86	4.37

[a]. replicate number (maximum is 3)

Table 10-2. List of Log_{10} population density for ethyl-paraben at 58 °C

Time (sec)	Ethyl-paraben Concentration (ppm)												
	0			125		250			500			800	
Rep[a]	R1	R2	R3	R1	R2	R1	R2	R3	R1	R2	R3	R1	R2
0	8.06	8.63	8.61	8.62	8.75	8.72	8.66	8.88	8.62	8.79	8.61	8.82	8.76
90	7.66	-	7.91	7.71	8.07	7.47	7.60	8.00	7,40	7.83	7.58	6.79	7.88
180	7.47	7.81	7.87	7.42	7.71	7.18	7.27	7.61	-	7.28	7.33	5.17	7.40
270	7.23	7.65	7.54	-	7.48	6.85	6.94	7.38	6.14	6.93	6.99	4.52	7.01
360	7.25	-	7.44	7.00	-	6.29	6.63	7.29	5.36	6.41	6.42	4.24	6.76
450	-	7.25	7.30	6.77	7.24	-	6.40	7.00	4.79	5.66	5.60	4.04	6.27
540	6.94	7.19	7.23	6.63	7.03	6.03	5.93	6.48	4.41	5.03	4.83	3.94	5.66
630	6.78	7.02	7.06	6.37	6.94	5.66	5.27	6.13	4.16	4.56	4.31	3.64	5.17
720	6.55	6.98	7.03	6.00	6.58	5.28	4.76	5.59	3.86	4.32	3.90	-	4.60
810	5.95	6.99	6.72	5.59	6.37	4.91	4.38	-	3.34	4.15	3.89	3.34	4.31

[a]. replicate number (maximum is 3)

Table 10-3. List of Log_{10} population density for propyl-paraben at 58 °C

Time (sec)	Propyl-paraben Concentration (ppm)												
	0			62.5	93.75		125			250		500	
Rep[a]	R1	R2	R3	R1	R1	R2	R1	R2	R3	R1	R2	R1	R2
0	8.29	8.70	8.66	9.03	8.73	8.63	8.55	8.76	8.72	9.06	8.76	8.83	8.71
90	7.64	8.34	8.19	8.05	7.70	-	6.97	7.87	7.88	7.47	7.27	5.57	6.97
180	7.33	8.32	8.09	7.85	7.27	7.62	6.32	7.50	7.32	5.89	6.35	4.44	4.95
270	7.21	8.11	7.95	7.76	7.16	7.43	5.66	7.17	7.07	4.90	4.94	4.00	3.94
360	7.06	7.96	7.75	7.63	6.80	7.08	5.09	6.80	6.80	-	4.34	3.79	3.70
450	6.91	7.08	7.75	7.41	6.48	7.11	4.25	6.39	6.53	4.47	4.06	3.56	3.48
540	6.86	7.77	7.57	-	6.10	6.64	3.78	5.96	6.01	4.33	-	3.42	-
630	6.72	7.43	7.49	6.79	5.52	6.39	3.45	5.31	5.31	4.10	3.88	3.00	3.20
720	6.74	7.21	7.24	6.25	4.82	5.87	3.38	4.86	4.72	4.09	3.67	3.00	3.00
810	-	6.90	7.05	6.02	4.17	5.42	-	4.29	4.07	4.05	3.63	2.78	2.78

[a]. replicate number (maximum is 3)

Table 10-4. List of Log_{10} population density for butyl-paraben at 58 °C

Time (sec)	Butyl-paraben Concentration (ppm)											
	0			31.25		62.5		75	100	125		
Rep[a]	R1	R2	R3	R1	R2	R1	R2	R1	R1	R1	R2	R3
0	8.84	8.84	8.61	9.06	8.84	8.80	8.74	8.73	8.85	8.00	8.63	8.82
90	7.92	8.40	8.09	7.82	7.87	7.40	7.54	7.83	7.22	-	6.55	6.98
180	7.81	8.25	8.03	7.77	-	7.13	7.12	7.29	5.40	4.53	4.83	-
270	7.57	8.38	-	7.56	7.27	6.82	6.56	6.47	4.71	-	4.42	4.82
360	7.33	7.86	7.47	7.31	6.58	6.41	6.09	5.31	4.37	3.34	4.12	4.82
450	7.29	7.77	-	7.14	6.32	5.54	5.31	4.71	4.23	2.78	3.95	3.88
540	7.19	7.53	6.78	6.75	5.87	4.81	4.84	4.44	4.10	-	3.75	3.73
630	6.83	7.27	6.78	6.32	5.42	4.34	4.26	4.36	4.00	2.78	3.83	3.68
720	6.77	7.29	6.37	5.93	5.00	4.04	3.86	4.09	3.86	2.30	3.65	3.45
810	6.42	6.97	-	5.31	4.46	3.82	3.60	4.11	3.80	2.30	3.35	3.45

[a]. replicate number (maximum is 3)

Table 10-5. List of Log_{10} population density for heptyl-paraben at 58 °C

Time (sec)	Heptyl-paraben Concentration (ppm)												
	0			5	7.8	15.5		31.25			62.5		125
Rep[a]	R1	R2	R3	R1	R1	R1	R2	R1	R2	R3	R1	R2	R1
0	8.84	8.78	8.59	8.31	8.75	8.80	8.74	8.71	8.87	8.64	8.83	8.63	8.50
90	7,92	7.86	8.06	7.49	7.63	5.90	-	3.64	4.16	-	5.00	4.24	3.26
180	7.81	7.84	7.64	7.05	7.14	4.60	6.14	2.60	-	4.93	2.78	2.60	1.80[b]
270	7.57	7.67	7.57	6.84	5.72	-	4.96	2.30	3.00	4.11	2.30	2.78	1.80[b]
360	7.33	-	7.39	6.54	4.71	4.24	4.27	2.30	2.90	3.66	2.78	2.90	1.80[b]
450	7.29	7.15	-	6.20	4.46	3.77	3.64	1.80[b]	2.30	3.60	2.78	1.80[b]	1.80[b]
540	7.19	6.99	-	5.91	4.25	3.70	3.51	1.80[b]	2.78	3.53	2.30	1.80[b]	1.80[b]
630	6.83	6.90	-	5.36	4.20	3.45	3.41	1.80[b]	2.30	3.00	1.80[b]	1.80[b]	1.80[b]
720	6.77	6.77	-	4.97	4.08	3.08	3.48	1.80[b]	2.60	3.08	1.80[b]	1.80[b]	1.80[b]
810	6.42	6.70	-	4.60	3.79	3.15	3.20	1.80[b]	1.80[b]	3.00	1.80[b]	1.80[b]	1.80[b]

[a]. replicate number (maximum is 3)
[b]. lower limit of detection = 2.0 log (CFU/ml). Samples where no viable cells were recovered were assigned a value of log CFU/ml =1.8.

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