Growth Kinetics and Model Comparison of Cronobacter sakazakii in Reconstituted Powdered Infant Formula

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Abstract: Cronobacter sakazakii is a life-threatening bacterium, infrequently implicated in illnesses associated with the consumption of powdered infant formula (PIF). It can cause rare but invasive infections in neonatal infants who consume contaminated PIF. The objective of this research was to investigate the growth kinetics and develop mathematical models to predict the growth of heat-injured C. sakazakii in reconstituted PIF (RPIF). RPIF, inoculated with a 6-strain cocktail of non-heat-treated (uninjured) or heat-injured C. sakazakii, was incubated at different temperatures to develop growth models. Except for storage at 6 °C, C. sakazakii grew well at all test temperatures (10 to 48 °C). Uninjured C. sakazakii exhibited no observable lag phase, while a lag phase was apparent in heat-treated cells. A simple 3-parameter logistic equation was used to fit growth curves for non-heat-treated cells, while both Baranyi and Huang models were suitable for heat-treated C. sakazakii. Calculated minimum and maximum growth temperatures were 6.5 and 51.4 °C for non-heat-treated cells, and 6.9 and 50.1 °C for heat-treated cells of C. sakazakii in RPIF, respectively. There was no significant difference between growth rates of non-heat-treated and heat-injured cells in RPIF. For heat-treated cells of C. sakazakii, the lag phase was temperature-dependent and very short (between 25 °C and 48 °C). These results suggest that both non-heat-treated and heat-injured C. sakazakii cells may present a risk to infants if the pathogens are not completely destroyed by heat in RPIF and then exposed to subsequent temperature abuse.

Keywords: C. sakazakii, growth kinetics, modeling, powdered infant formula, predictive microbiology

Practical Application: C. sakazakii is a life-threatening bacterium found in powdered infant formula (PIF). This study shows that the uninjured bacterium exhibits very short or no lag phase if not refrigerated and can grow well in reconstituted PIF (RPIF), while the heat-injured cells can multiply at an equivalent rate following metabolic recovery. Temperature abuse may allow C. sakazakii to grow and endanger infants fed with RPIF. Predictive models developed in this study can be used to estimate the growth and conduct risk assessments of this pathogen.

Introduction

Cronobacter sakazakii, formerly known as Enterobacter sakazakii (Iversen and others 2008), is a Gram-negative, nonsporforming, rod-shaped bacterium (Gurtler and others 2005). A ubiquitous microorganism, C. sakazakii has been isolated from a variety of foods, including meat and poultry, eggs, milk, fruits and vegetables, seafood, herbs and spices, and seed sprouts (Iversen and Forsythe 2003; Drudy and others 2006; Friedemann 2007; Kim and others 2008; Beuchat and others 2009; Molloy and others 2009), as well as from food production facility and household environments (Kandhai and others 2004). Powdered infant formula (PIF) and powdered milk have been identified as the most common sources and vehicles of C. sakazakii transmission (Weir 2002; FAO/WHO 2004; Kim and others 2008; Beuchat and others 2009; Chang and others 2009), associated with neonatal meningitis, septicemia, and enterocolitis, especially in premature infants (Urmenyi and Franklin 1961; Muytjen and Kollee 1982; Gallagher and Ball 1991; Bar-Oz and others 2001; Lai 2001; Gurtler and others 2005).

Since its discovery, first reported by Urmenyi and Franklin (1961), many clinical neonatal infections and outbreaks associated with C. sakazakii in PIF have been reported worldwide (Simmons and others 1989; van Acker and others 2001; Weir 2002; FAO/WHO 2008). The reported mortality rates of infections attributed to C. sakazakii vary significantly, ranging from 40% to 80% (Nazarowec–White and Farber 1997a; Bowen and Braden 2006). In the United States, sporadic cases of infant C. sakazakii infections have been reported (CDC 2001, 2009). The most recent cases of infant C. sakazakii infections, involving 4 infants less than 12–mo old, included 2 deaths reported between November and December of 2011 in the United States (CDC 2012). A nationwide voluntary recall of the suspect infant formula was issued as a result, and different strains of C. sakazakii were isolated from bottled water used to prepare infant formula as well as from an opened can of the suspect PIF.
C. sakazakii has been isolated from both PIF-processing environments and PIF itself. Reich and others (2010) collected environmental samples (867 samples in total) from various locations of a PIF-processing plant and reported that 14 of 35 (40%) of sampled locations, as well as 94.3% of the environmental powder samples, were positive for Cronobacter spp. This study accentuates the need for enhanced microbial monitoring, but also suggests that PIF products should not be considered sterile and may be potentially contaminated with C. sakazakii. While C. sakazakii does not grow in dehydrated PIF (Gurtler and Beuchat 2007c), reconstituted infant formula is an ideal medium for growth of the coliform (Gurtler and Beuchat 2007a, 2007b).

According to an international survey reported by Chap and others (2009), C. sakazakii was isolated from 24 of 199 samples (12%) of infant food and drinks, and three of 91 (3%) follow-up formulas. Compared to other Enterobacteriaceae in dairy products, C. sakazakii is frequently more heat-resistant, but can be effectively inactivated by standard pasteurization schedules (Nazarowec-White and Farber 1997b; Nazarowec-White and others 1999). Nevertheless, postshelflife contamination in the processing environment can occur. According to Edelson-Mammel and Buchanan (2004), rehydrating PIF with water at temperatures of > 70 °C can achieve > 4-log reductions of C. sakazakii, thus reconstituted PIF (RPIF) prepared by this procedure is highly unlikely to contain surviving Cronobacter (FAO/WHO 2004). To prevent C. sakazakii infections, both the Food and Agriculture Organization (FAO) and World Health Organization (WHO) of the United Nations recommended reconstituting PIF using hot water (> 70 °C) accompanied by sterile-thermometer temperature readings (FAO/WHO 2006; WHO 2007). Chap and others (2009) observed that Korea was the only country that clearly recommended using water at temperatures exceeding 70 °C to prepare RPIF, and many other countries did not follow the FAO/WHO recommendations. The preparation recommendations in these countries varied considerably, including a nonspecified temperature (lukewarm water) as well as a temperature of 40 °C, close to the optimum growth temperature for C. sakazakii (Kandai and others 2006; Chap and others 2009). According to the method of Ghassem and others (2011), the generation time of C. sakazakii in RPIF at temperatures between 25 °C and 45 °C is only 7 to 30 min, and growth curves do not exhibit a noticeable lag time. The relatively short generation time and lag time may allow C. sakazakii to grow to a dangerously high level, if the RPIF remains at room temperature for too long.

While most research concerning C. sakazakii has centered on its detection and prevalence in PIF, the objectives of this research were to investigate the growth kinetics of C. sakazakii, specifically of heat-injured cells, in RPIF and describe its behavior via predictive mathematical growth models. Results attained during the course of this investigation will be helpful for the PIF industry and regulatory agencies in conducting risk assessments of RPIF exposed to various temperature-abuse conditions, as well as for parents and other caretakers in properly storing leftover RPIF.

Materials and Methods
Preparation of bacteria
Six strains of C. sakazakii (ATCC 12868, ATCC 29004, ATCC 29544, HBP #2871, HBP #3439, and HBP #3437) were obtained from the culture collection at the USDA Agricultural Research Service (ARS) Eastern Regional Research Center (ERRC) located at Wyndmoor, Pa., U.S.A. The last 3 strains were food isolates previously reported by Gurtler and Beuchat (2007a). The stock cultures were regularly propagated and maintained on Tryptic Soy agar (TSA, BD/Difco Lab., Sparks, Md., U.S.A) plates and stored at 8 °C to prevent cell death. The purity of the cultures was periodically assessed using a chromogenic agar (AES CHEMUNE, Combourg, France).

One day prior to the experiment, a 1 μL loop of each respective strain was individually transferred to 10 mL of Brain Heart Infusion broth (BHI broth, BD/Difco) and held at 37 °C in an orbital shaker (ca. 100 rpm) for ca. 22 to 24 h. Bacterial cultures were harvested by centrifugation (2400 × g, 15 min, 4 °C), washed once with 10 mL of 0.1% peptone water (PW, BD/Difco), and resuspended in 10 mL of PW. A 30-μL cocktail suspension was created by combining the six 5 mL suspensions. The cocktail contained approximately 10^5 to 10^6 CFU/mL of C. sakazakii cells. A fresh cocktail was prepared the day of each experiment.

Preparation of heat-injured C. sakazakii
To prepare heat-injured C. sakazakii cells, 1 mL of the C. sakazakii suspension cocktail was added to 200 mL of PW preheated to 60 °C in a 500 mL beaker on top of a temperature-controlled hot plate. The bacterial solution was mixed in the beaker by a magnetic stirrer to allow uniform distribution and heating of bacterial cells. Thermal treatments lasted for 2.5 min, then the beaker was transferred to an ice–water bath for cooling. The surviving bacterial population following thermal treatment and cooling was determined to be ca. 10^4 to 10^5 CFU/mL of heat-injured C. sakazakii cells (plated on TSA plates).

Sample preparation and inoculation
Dehydrated milk-based IF was purchased from a local grocery store and prepared according to the manufacturer’s instructions in a sterile 500-mL Erlenmeyer flask. Briefly, sterile water (440 mL) was combined with 64 g of PIF and mixed by a magnetic stirrer until PIF was completely dissolved at room temperature. Ten milliliters of RPIF were then transferred to 16 mm × 150 mm sterile borosilicate glass culture tubes. A total of 36 test tubes were used for each temperature condition, as described subsequently. Twelve of the 36 culture tubes were set aside as controls for enumerating background microflora. A 0.1 mL aliquot of non-heat-treated C. sakazakii was added to each of the 12 culture tubes. To each of the remaining culture tubes, 0.1 mL of the heat-injured C. sakazakii cocktail was added. After inoculation, culture tubes were labeled and capped. The initial concentration of C. sakazakii (non-heat-treated or heat-injured) in RPIF was ca. 10^6 to 10^8 CFU/mL.

Growth study and enumeration of bacteria
Inoculated RPIF samples were placed in incubators maintained at 6, 10, 15, 20, 25, 30, 35, 40, 45, and 48 °C. Samples were removed from incubators at designated time intervals determined according to incubation temperature. RPIF samples were vortexed and plated onto TSA and Violet Red Bile Glucose agar (VRBGA, BD/Difco) plates directly or following appropriate serial dilutions with PW. TSA plates were used to enumerate total aerobic bacterial (TAB) counts and VRBGA plates were used to recover C. sakazakii from RPIF (Ghassem and others 2011). Both TSA and VRBGA plates were held overnight in an incubator maintained at 37 °C. Bacterial colonies were counted and converted to the logarithm of the natural base or base 10, and recorded as ln CFU/mL or log...
CFU/mL. Each growth experiment was conducted in duplicate for each temperature condition.

Primary models
Three primary growth models were used to describe bacterial growth curves. The first model was the Baranyi model (Baranyi and Roberts 1994, 1995):

\[ y(t) = y_0 + \mu_{\text{max}} A(t) - \ln \left( 1 + \frac{\exp[\mu_{\text{max}} A(t)] - 1}{\exp(y_{\text{max}} - y_0)} \right) \]  

In Eq. 1, \( y(t) \) represents the natural logarithm of bacterial counts at time \( t \); \( y_0 \) and \( y_{\text{max}} \) are the natural logarithms of the initial and the stationary phase bacterial counts; \( \mu_{\text{max}} \) is the specific growth rate (h^{-1}); and \( A(t) \) is defined by

\[ A(t) = \frac{t + \ln[\exp(-\nu t) + \exp(-h_0) - \exp(-\nu t - h_0)]}{\nu} \]  

In Eq. 2, \( h_0 \) is used to numerically define the bacterial physiological status, which is a virtual parameter, representing the effect of the prior exposure history on the growth of bacteria in a new environment.

The 2nd model was the Huang Model (Huang 2008), which is written as

\[ y(t) = y_0 + y_{\text{max}} - \ln[\exp(y_0) + \exp(y_{\text{max}}) - \exp(y_0)] \times \exp[-\mu_{\text{max}} \times B(t)] \]  

\[ B(t) = \frac{t + \frac{1}{\alpha} \ln \left( \frac{1 + \exp(-\alpha(t - \lambda))}{1 + \exp(\alpha\lambda)} \right)}{\alpha} \]  

In Eq. 4, \( \lambda \) is the lag phase duration (h). The parameter \( \alpha \) (25, for example) is a coefficient used to define the transition from the lag phase to exponential growth phase. The duration of the lag phase \( (1/\alpha) \) is explicitly defined in this model. The other variables, such as \( y_0, y_{\text{max}}, y(t), \) and \( \mu_{\text{max}} \), are identical to those used in the Baranyi model (Eq. 1).

The 3rd model was the logistic equation (Eq. 5), written as

\[ \frac{dC}{dt} = \mu_{\text{max}} C \left( 1 - \frac{C}{C_{\text{max}}} \right) \]  

In Eq. 5, \( C \) is the bacterial population (CFU/mL), and \( C_{\text{max}} \) is the maximum cell concentration. This equation can be solved analytically, yielding

\[ y(t) = y_0 + y_{\text{max}} - \ln[\exp(y_0) + \exp(y_{\text{max}}) - \exp(y_0)] \times \exp(-\mu_{\text{max}}t)] \]  

Equation 6 is a logistic equation, but it is different from the 4-parameter sigmoidal logistic model conventionally used in predictive microbiology (Gibson and others 1988), and has not been used to describe the bacterial growth in the literature before. This 3-parameter logistic equation does not have a lag phase, so it is more suitable for describing bacterial growth with only exponential and stationary phases. Since most of the growth curves observed in this study, as well as those of Ghassem and others (2011), did not contain appreciable lag phases, the logistic equation was chosen to fit the growth curves.

Secondary models—effect of temperature on bacterial growth

To evaluate the effect of storage temperature on \( C. sakazakii \) growth in RPIF, specific growth rates \( \mu_{\text{max}} \) of each model were fitted to a modified Ratkowsky square-root equation (Eq. 7) (Zwietering and others 1991) and an equation modified from Huang (2010) (Eq. 8). In Eq. 7 and 8, \( T \) is the incubation temperature (°C), \( a \) and \( b \) are coefficients, \( T_0 \) is the nominal minimum growth temperature for the Ratkowsky square-root model, \( T_{\text{min}} \) is the estimated minimum growth temperature, and \( T_{\text{max}} \) is the estimated maximum growth temperature (°C).

\[ \sqrt{\mu_{\text{max}}} = a(T - T_0)(1 - \exp[b(T - T_{\text{max}})]) \]  

\[ \sqrt{\mu_{\text{max}}} = a(T - T_{\text{min}})^{0.75}(1 - \exp[b(T - T_{\text{max}})]) \]  

A cardinal model (Rosso and others 1993) was utilized (Eq. 9) in the work reported by Kandhai and others (2006). To compare the kinetic parameters obtained from the present study with Kandhai and others (2006), the specific growth rates were also fitted to the cardinal model. In Eq. 9, \( \mu_{\text{opt}} \) and \( T_{\text{opt}} \) are the optimum specific growth rate and temperature.

\[ \mu_{\text{max}} = \frac{\mu_{\text{opt}}(T - T_{\text{max}})(T - T_{\text{min}})^2}{[(T_{\text{opt}} - T_{\text{min}})(T - T_{\text{opt}}) - (T_{\text{opt}} - T_{\text{max}}) \times (T_{\text{opt}} - T_{\text{min}} - 2T)(T_{\text{opt}} - T_{\text{min}})]} \]  

Data analysis and modeling

Growth data of all the growth curves were analyzed using an open-source statistical analysis package R (Version 2.13.2). Nonlinear regression and data analysis were accomplished using the “nls” (nonlinear least squares) package of R. To compare the accuracy among the primary models, the residual standard error (RSE) of each model, which is the standard deviation of the residual errors divided by the degree of freedom, was analyzed using ANOVA (analysis of variance). In addition, the AIC, or Akaike information criterion, produced after nonlinear regression of each model, was also analyzed using ANOVA. A model with smaller RSE and AIC would suggest that it has a better fit for the data. All statistical analyses were performed using R.

Results and Discussion

Growth of \( C. sakazakii \) in RPIF

The uninoculated PIF samples were not sterile, thus, as expected, low levels of background flora were detected in RPIF samples. No \( C. sakazakii \) cells were recovered from control samples by direct plating onto VRBGA plates at any storage temperature. Furthermore, no background microbiota at time zero was found.
Growth of C. sakazakii in infant formula . . .

by direct plating (1 mL of sample) at any incubation temperature; however, this is not an indication that there were no viable cells of bacteria in RPIF. These results merely demonstrate that the population of background flora was below the minimum detection limit, as determined by direct plating. Populations of background microbiota increased with incubation time; nevertheless, the growth was very slow, and the population of the background microbiota, in general, was 2 to 4 orders of magnitude lower than that of C. sakazakii (Figure 1). Population increases in background microbiota within RPIF appeared to be so slow that it did not affect the growth of C. sakazakii inoculated to RPIF, which corroborates the findings of Gurtler and Beuchat (2007a, 2007b).

No C. sakazakii growth was observed in RPIF samples incubated at 6 °C for the entire length of incubation; in fact, the population of C. sakazakii began to decline, particularly for heat-treated cells (Figure 2). Nazarowec-White and Farber (1997c) reported that the minimum growth temperature for C. sakazakii in BHI ranged from 5.5 °C to 8 °C. Our observation was well within this range. Based on this observation, it is safe to conclude that the minimum growth temperature for the growth of these 6 strains of C. sakazakii in milk-based RPIF is higher than 6 °C.

Inoculated RPIF samples that were exposed to incubation temperatures above 6 °C (viz., 10 to 48 °C) elicited exponential growth of C. sakazakii, with or without thermal injury (Figure 3). Populations of non-heat-treated cells of C. sakazakii, exhibiting no or very short lag phases, immediately transitioned into the exponential growth phase and subsequently to the stationary phase of growth (Figure 3). The lack of a lag phase for non-heat-treated cells, observed in this case, is in agreement with observations previously reported in the literature. For example, Kandhai and others (2006) reported that preculturing conditions did not affect the growth rate or lag time of C. sakazakii in RPIF, and the lag times were short. In the study reported by Ghassem and others (2011), no noticeable lag phases were observed at elevated temperatures (25, 37, and 45 °C), although the lag phase was evident during storage at 10 °C. Gurtler and Beuchat (2007a) reported that the growth of C. sakazakii did not exhibit lag times, regardless of high or low inoculum levels in RPIF, and the growth was not greatly affected by the composition of PIF.

Although the effect of heat treatment on the development and duration of the lag phase of heat-treated cells of C. sakazakii in RPIF varies according to incubation temperature, heat-treatments, without question, are known to induce lag phases, as heat-injured cells require time to repair thermal injury prior to shifting into exponential growth (Figure 3). While no differences were observed in C. sakazakii growth curves obtained from TSA versus VRBGA plates in RPIF samples inoculated with non-heat-treated cells, growth curves obtained from VRBGA plates lagged slightly behind those from TSA plates in samples inoculated with heat-treated cells (Figure 3).

It has been well established that the selection of plating media may affect the enumeration of heat-injured cells of C. sakazakii (Gurtler and Beuchat 2005). Microbiologically selective ingredients contained in VRBGA (viz., 1.5 g/L bile salts #3 and 2 mg/L crystal violet) can cause additional injury and impede the recovery of heat-injured cells of C. sakazakii. In the present study, heat-injured C. sakazakii cells recovered better on TSA plates than on VRBGA, which corroborates Gurtler and Beuchat (2005) who reported that 1-log CFU/mL fewer thermally injured C. sakazakii were recovered on VRBGA than on TSA + 0.1% sodium pyruvate. The authors also reported that, although a statistically greater number of C. sakazakii recovered on TSA and LBDC agar, VRBGA recovered equivalent populations of thermally injured cells as Fecal Coliform Agar, Druggan-Forsythe-Iversen (DFI) agar, Oh and Kang (OK) agar, and agar-solidified Enterobacteriaceae Enrichment broth (Gurtler and Beuchat 2005). Accordingly, samples inoculated with heat-injured C. sakazakii in the present study yielded lower counts when recovered on VRBGA versus TSA plates for the first few data points in the growth curves, which was particularly true in the lag phase. As incubation...
progressed, however, differences in plate counts between VRBGA and TSA were negligible for heat-treated cells, which may be attributable to the requisite metabolic recovery of heat-injured \textit{C. sakazakii} cells from thermal injury. Based on this observation, the bacterial counts from TSA plates were used to develop the growth models for heat-injured \textit{C. sakazakii}.

Primary growth models for non-heat-treated \textit{C. sakazakii}

All data obtained can be fitted into the 3 primary models, as illustrated in Figure 4, with the exception of growth curves at 6 °C. Results of ANOVA revealed that there was no significant difference in the means of $\mu_{max}$ determined by all 3 models for cells recovered on VRBGA or TSA plates ($P > 0.7$). There were also no significant differences in the means of RSE and AIC ($P > 0.90$), suggesting that all 3 models were equally suitable for describing the growth of non-heat-treated \textit{C. sakazakii} in RPIF.

The averaged $h_0$ value for the Baranyi model was 0.66 ± 0.78 h (mean ± standard deviation, $n = 16$); although, three $h_0$ values were negative, which resulted in negative lag phases. The average lag phase for the Huang model was 8.2 h at 10 °C, which was insignificant for the long incubation time at this temperature; however, at 15 and 20 °C, the average lag phase was 1.62 ± 1.16 h ($n = 4$). The average lag phase for the Huang model was 0.38 ± 0.40 h ($n = 10$) for the growth curves obtained at 25, 32, 35, 45, and 48 °C. Since non-heat-treated \textit{C. sakazakii} exhibited no or negligible lag phases, the growth curves were conveniently fitted to the 3-parameter logistic equation (Figure 4). Compared to the Baranyi and Huang models, the logistic model has only 3 parameters, is simpler to use, and, therefore, is recommended for describing the growth of uninjured \textit{C. sakazakii} in RPIF.

Primary growth models for heat-treated \textit{C. sakazakii}

Growth curves of heat-treated \textit{C. sakazakii} obtained at temperatures above 6 °C were also analyzed using Baranyi, Huang, and logistic models (Figure 4). Statistically, there was no significant difference in means of $\mu_{max}$, as estimated by all 3 models ($P = 0.243$). Due to the observed lag phase, Baranyi and Huang models, both containing 4 parameters, were more suited for describing the bacterial growth of heat-treated \textit{C. sakazakii} (Figure 4). The 3-parameter logistic equation, on the other hand, is not fit for describing the growth curves of heat-treated \textit{C. sakazakii} in RPIF, and can lead to overestimation of bacterial growth early in the incubation period.

Both Baranyi and Huang models were equally suitable for describing the growth of heat-treated \textit{C. sakazakii} in RPIF, as there was no significant difference in the means of $\mu_{max}$, RSE, and AIC between the 2 models ($P > 0.91$), according to the results of ANOVA. The $h_0$, for the Baranyi model ranged from 0.31 to 5.85, while the average $h_0$ was 2.75 ± 1.39 ($n = 15$, excluding one negative value). The $\lambda$ values estimated by the Huang model generally decreased with decreasing temperature (Figure 5). Because there is no difference between the Baranyi and Huang models and because the Huang model did not produce a negative lag phase, the Huang model was used to develop secondary models.

ANOVA was also used to compare growth rate means of non-heat-treated and heat-treated \textit{C. sakazakii} in RPIF. No significant difference ($P = 0.87$) was apparent in the $\mu_{max}$ means between growth curves of non-heat-treated and heat-treated \textit{C. sakazakii}, suggesting that heat-injured \textit{C. sakazakii} grew at an equivalent rate as non-heat-treated cells in RPIF, following metabolic recovery from thermal injury. Accounting for injury-recovery time, however, populations of non-heat-treated cells increased to higher levels in a less time, due to the observable lag phase of heat-injured cells (Figure 3).

Effect of temperature on growth rate and lag phase

The growth rates of both heat-treated and non-heat-treated cells of \textit{C. sakazakii} in RPIF were affected by temperature, and...
the temperature dependence can be described by 3 secondary models (Eq. 7, 8, and 9). Performance of the Ratkowsky square-root model, the modified Huang square-root model, and the cardinal model are essentially equivalent for fitting these data points (Figure 6). Table 1 and 2 list the results of nonlinear regression for the parameters of each model for non-heat-treated and heat-treated cells of C. sakazakii in RPIF, respectively.

Iversen and others (2004) reported that C. sakazakii (6 clinical and food strains) grew well in various media as well as RPIF between temperatures of 6 °C and 45 °C. In the present study, however, no growth of C. sakazakii in RPIF was observed at 6 °C. Therefore, the parameter $T_{0}$ (3.1 °C for non-heated and 3.9 °C for heat-injured cells) in the Ratkowsky square-root model is not the minimum temperature for C. sakazakii in RPIF. The $T_{0}$ estimated by the cardinal model was 3.2 °C for non-heated and 4.7 °C for heat-injured cells, respectively, which was also below 6 °C. Thus, both the Ratkowsky and cardinal models underestimated the minimum growth temperature for C. sakazakii in RPIF.

The $T_{\text{min}}$ estimated by the modified Huang square-root model, on the other hand, was 6.5 °C for non-heat-treated and 6.9 °C for heat-injured cells of C. sakazakii in RPIF, respectively. These $T_{\text{min}}$ values were slightly higher (<1 °C) than 6 °C, the point at which no growth was observed in this study. According to the method of Nazarowec-White and Farber (1997c), the minimum growth temperature for C. sakazakii in BHI ranges from 5.5 °C to 8 °C, which is within the range of the modified Huang square-root model. The $T_{\text{min}}$ estimated by Eq. 8 agreed well with our experimental observation and, therefore, can be used to represent the minimum growth temperature of C. sakazakii in RPIF.

Although Iversen and others (2004) observed that the maximum growth temperature for C. sakazakii was 47 °C, growth of C. sakazakii was observed in RPIF at 48 °C in this study. Farmer and others (1980) tested 57 strains of E. sakazakii and found that 50 strains grew at 47 °C, but no growth occurred either at 4 °C or 50 °C. The estimated maximum growth temperatures ($T_{\text{max}}$) in the present study, as estimated by the Ratkowsky square-root...
model, the modified Huang square-root model and the cardinal model, were 52.1, 51.4, and 49.9 °C for non-heat-treated and 50.6, 50.1, and 49.0 °C for heat-injured C. sakazakii, respectively. The maximum temperatures estimated in this study were very close to data reported in the literature.

The parameters for the Ratkowsky square-root model obtained in this study are also very close to data reported by Kandhai and others (2006). For non-heat-treated C. sakazakii in this study, parameters $a$ and $b$ are 0.040 and 0.179. These values are 0.044 and 0.186 for heat-treated C. sakazakii. In Kandhai and others (2006), the $a$ and $b$ values are 0.047 and 0.239, respectively. The smaller $b$ value in our study leads to a slightly higher estimate of $T_{\text{opt}}$. The $T_0$ and $T_{\text{max}}$ are 2.2 °C and 48.9 °C in Kandhai and others (2006), which are similar to data obtained in the current study.

To a certain degree, the parameters of the cardinal model obtained in this study are also comparable to the data reported by Kandhai and others (2006); namely, the optimum temperature ($T_{\text{opt}}$) estimated in this work is 41.2 °C for non-heat-treated and 40.3 °C for heat-treated C. sakazakii, while $T_{\text{opt}}$ was reported as 39.4 °C in Kandhai and others (2006). The optimum growth rate ($\mu_{\text{opt}}$) reported by Kandhai and others (2006) was 2.31 (h$^{-1}$), which is 38.3% higher than the $\mu_{\text{opt}}$ of non-heat-treated C. sakazakii calculated in this work, but only 21.4% higher than the $\mu_{\text{opt}}$ of heat-treated cells. The minimum temperature ($T_{\text{min}}$) is 3.6 °C in Kandhai and others (2006), while our $T_{\text{min}}$ reported herein is 3.2 °C for non-heat-treated and 4.7 °C for heat-treated cells of C. sakazakii, respectively. The maximum temperature ($T_{\text{max}}$) reported by Kandhai and others (2006) was 42.0 °C for non-heat-treated and 40.3 °C for heat-treated C. sakazakii, respectively. In Kandhai and others (2006), the $T_{\text{opt}}$, $T_{\text{max}}$, and $T_{\text{min}}$ values were 48.9 °C and 40.3 °C, respectively.

Table 1 – The effect of temperature on the growth rate of non-heat-treated C. sakazakii in RPIF and parameters for Ratkowsky square-root, modified Huang square-root, and cardinal models.

| Parameters | Estimate | Std. Error | $t$ value | Pr ($>|t|$) |
|------------|----------|------------|-----------|-------------|
| $a$        | 0.040    | 0.002      | 18.8      | 2.93e-10    |
| $b$        | 0.179    | 0.034      | 5.14      | 2.47e-4     |
| $T_0$      | 3.07     | 0.914      | 3.37      | 5.60e-3     |
| $T_{\text{max}}$ | 52.07   | 0.744      | 69.7      | <2e-16      |
| Modified Huang square-root model | | | | |
| $a$        | 0.099    | 0.003      | 33.2      | 3.54e-13    |
| $b$        | 0.250    | 0.043      | 5.76      | 9.05e-5     |
| $T_0$      | 6.49     | 0.57       | 11.5      | 8.11e-8     |
| $T_{\text{max}}$ | 51.39   | 0.60       | 84.4      | <2e-16      |
| Cardinal model | | | | |
| $T_{\text{opt}}$ | 3.22     | 1.548      | 2.09      | 0.06        |
| $T_{\text{max}}$ | 49.85    | 0.38       | 131.6     | <2e-16      |
| $T_{\text{opt}}$ | 41.19    | 0.44       | 93.5      | <2e-16      |
| $\mu_{\text{opt}}$ | 1.62     | 0.04       | 37.7      | 7.68e-14    |

Table 2 – The effect of temperature on the growth rate of heat-treated C. sakazakii in RPIF and parameters for Ratkowsky square-root, modified Huang square-root, and cardinal models.

| Parameters | Estimate | Std. Error | $t$ value | Pr ($>|t|$) |
|------------|----------|------------|-----------|-------------|
| $a$        | 0.044    | 0.002      | 20.2      | 1.23e-10    |
| $b$        | 0.186    | 0.027      | 6.84      | 1.78e-5     |
| $T_0$      | 3.92     | 0.63       | 4.72      | 5e-4        |
| $T_{\text{max}}$ | 50.64   | 0.39       | 129.8     | <2e-16      |
| Modified Huang square-root model | | | | |
| $a$        | 0.107    | 0.004      | 20.10     | 1.69e-12    |
| $b$        | 0.276    | 0.042      | 6.68      | 3.01e-5     |
| $T_0$      | 6.86     | 0.646      | 10.63     | 1.85e-7     |
| $T_{\text{max}}$ | 50.10   | 0.365      | 137.4     | <2e-16      |
| Cardinal model | | | | |
| $T_{\text{opt}}$ | 4.70     | 1.32       | 3.57      | 3.85e-2     |
| $T_{\text{max}}$ | 49.04    | 0.19       | 231.6     | <2e-16      |
| $\mu_{\text{opt}}$ | 40.26    | 0.38       | 107.1     | 2.68e-14    |
| $\mu_{\text{opt}}$ | 1.90     | 0.05       | 41.2      | <2e-16      |
was 47.6 °C, which is also very close to the $T_{max}$ (49.9 °C for non-heat-treated and 49.0 °C for heat-treated cells) obtained in this work.

All 3 secondary models (that is, Ratkowsky, Huang, and cardinal) can be used to fit the data points of $\mu_{max}$ and $T$ with similar or equivalent accuracy; however, both the Ratkowsky square-root and cardinal models underestimate the minimum growth temperature of *C. sakazakii* in RPIF, suggesting that *C. sakazakii* is capable of growth at temperatures below the minimum growth temperature. The major advantage of the modified Huang model (Eq. 8) is that it appears to provide a more realistic estimate of both minimum and maximum growth temperatures. Therefore, this model is recommended as the secondary model to describe the effect of temperature on the growth rates of *C. sakazakii* in RPIF.

Since heat-injured cells of *C. sakazakii* exhibited lag phase in RPIF, it is appropriate to identify the relationship between the lag phase and growth temperature. Figure 5 illustrates the log-log relationship between $\mu_{max}$ and $\lambda$, where the relationship between $\mu_{max}$ and $\lambda$ can be expressed as

$$\ln (\lambda) = 0.829 − 1.01 \ln(\mu_{max}), R^2 = 0.88$$  \hspace{1cm} (10)

**Conclusions**

This study investigated the effect of thermal injury on the growth of *C. sakazakii* in RPIF and compared the differences in the growth kinetics between healthy non-heat-treated and heat-treated, thermally injured cells at temperatures ranging from 6 °C to 48 °C. No growth of *C. sakazakii* in RPIF was observed at 6 °C. Based on this experimental evidence, normal, uninjured cells of *C. sakazakii* inoculated into RPIF did not exhibit an appreciable lag phase and commenced exponential growth shortly after inoculation at incubation temperatures higher than 10 °C. The thermally injured cells of *C. sakazakii*, however, exhibited substantial lag phases prior to exponential growth. Nevertheless, following metabolic recovery, there was no significant difference between the growth rates of non-heat-treated and heat-treated cells of *C. sakazakii* in RPIF. The growth curves of non-heat-treated *C. sakazakii* can be fitted to a simple 3-parameter logistic equation. For heat-treated *C. sakazakii*, both Baranyi and Huang models were sufficient to describe the isothermal bacterial growth. The effect of temperature on the growth rates of *C. sakazakii* in RPIF was also investigated, and 3 secondary models were compared. Both the Ratkowsky and cardinal models underestimated the minimum growth temperature for *C. sakazakii* in RPIF, while the modified Huang square-root model provided more realistic estimates of both minimum and maximum growth temperatures. According to the modified Huang square-root model, the minimum and maximum growth temperatures were 6.4 and 51 °C for non-heat-treated cells, and 6.9 and 50.1 °C for heat-treated cells of *C. sakazakii* in RPIF. For heat-treated cells of *C. sakazakii*, a log-log relationship between the lag phase and growth rate was established.

The growth kinetics of *C. sakazakii* in RPIF, according to our results, compared favorably to data published in the literature, although we have provided additional relevant findings not heretofore reported, specifically concerning heat-injured *C. sakazakii*. This research documents that sublethal heating during RPIF preparation induces a lag phase for *C. sakazakii*; however, the lag phase can be very short for incubation temperatures between 25 °C and 48 °C, where the average lag phase is 0.4 h. These temperatures may be representative of conditions encountered by the bacteria in vivo or either during intraperitoneal feeding in neonatal intensive care wards. *C. sakazakii*, following metabolic recovery from thermal injury, is capable of growth at rates equivalent to that of uninjured cells, suggesting that sublethally injured *C. sakazakii* in RPIF may present a risk to infants if not completely destroyed during thermal treatments. The results of the present study can be used to predict the growth of both non-heat-injured and heat-injured *C. sakazakii* in RPIF; as well as for conducting risk analyses of this microorganism in RPIF.

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**References**


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