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Modeling Growth of *Cronobacter sakazakii* IFST082014 in Reconstituted Powdered Infant Milk as Function of Temperature

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Authors' contributions

This work was carried out in collaboration between all authors. Authors MF and MMA designed the study. Authors MF, MMR and MNH performed all the experiments. Author MMA supervised the study. Author MF wrote the first draft of the manuscript. All authors read and approved the final manuscript.

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Original Research Article

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ABSTRACT

Aims: *Cronobacter sakazakii* has been associated most frequently with illness in neonates. This study aims to model effect of temperature on growth of a *C. sakazakii* isolate (IFST082014).
Methodology: Reconstituted powdered infant milk formulas (RIMFs) inoculated with *C. sakazakii* were incubated at 10, 20, 30 and 40℃.
Results: The primary model showed a good fit (r^2 = 0.9714–0.9821) to a Gompertz equation to obtain growth rates and lag times (LTs) at each temperature. The specific growth rate (SGR) of *C. sakazakii* in the RIMF increased, and the LT decreased with increasing temperature. The secondary model was "ln SGR = -0.05879+(0.00588 x temperature)+(0.00045 x temperature^2)." The SGR predicted using this model increased with an increasing temperature. This secondary polynomial model was judged as appropriate based on the mean square error (MSE of the SGR model = 0.00016), the coefficient of determination (r^2 of the SGR model = 0.9845), the bias factor (B_f of the SGR model = 1.0125) and the accuracy factor (A_f of the SGR model = 1.0007).
Conclusion: These results will be useful for industry and regulatory agencies.

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1. INTRODUCTION

Cronobacter sakazakii is considered an opportunistic pathogen and has been implicated in outbreaks causing meningitis or bacteremia, especially in neonates and infants, [1,2], with mortality rates of 20 to 50%. Although *C. sakazakii* has been detected in various types of food, only powdered infant formula has been linked to outbreaks of disease [3].

Mathematical modeling has been used to predict the effects of combinations of preservative factors (e.g., water activity [*A*w], pH, temperature and oxygen availability) on growth in many foods [4]. The aims of predictive microbiology are quantitative estimation of microbial growth in foods, prediction of microbial safety and determination of shelf life for food products using mathematical modeling [5]. This study was designed to develop a mathematical model for prediction of the growth of *C. sakazakii* in reconstituted powdered infant milk formula (RIMF) leading to development of effective control methods for *C. sakazakii*.

Predictive microbiology, or "the quantitative microbial ecology of foods" [4] attempts to provide mathematical models of microbial growth under a variety of environmental conditions- e.g. temperature, pH, aw and the effect of preservatives. Predictive modelling can be seen, therefore, as the quantification of hurdle technology [6].

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 [7,8], as well as from food production facility and household environments [9]. Powdered infant formula (PIF) and powdered milk have been identified as the most common sources and vehicles of *C. sakazakii* transmission [10,11], associated with neonatal meningitis, septicemia, and enterocolitis, especially in premature infants [3,12].

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.

2. MATERIALS AND METHODS

2.1 Bacterial Culture

An infant milk formula isolate of *Cronobacter sakazakii* resistant to ampicillin (AMP) and nalidixic acid (NA) was used in the study [13]. Tryptic soy broth (Difco, USA) was used for maintenance and growth of the bacterial strain. For bacterial culture maintenance, AMP (25 µg/ml) and NA (25 µg/ml) were added for the *C. sakazakii* isolate. A stock culture was maintained at -70℃ in tryptic soy broth (TSB) (Difco Laboratories, Detroit, MI) containing 50% glycerol.

2.2 Experimental Design

A central composite design was used for incorporating the variable and levels which include- temperature: 10, 20, 30 & 40℃.

2.3 Preparation of the Bacterial Suspension

C. sakazakii was cultured by transferring 10 µL of the stock culture into 10 mL of TSB at 35℃ for 24 h, harvested by centrifugation at 7,000 rpm for 10 min at 4℃ and washed with 0.1% peptone water. The bacterial suspension was resuspended in 0.1% peptone water and diluted to 10^4 cfu/mL.

2.4 Reconstitution of Powdered Infant Milk Formula (RIMF) and Inoculation

Powdered infant milk formula was bought in a retail market and screened for *C. sakazakii*, and found to be pathogen free. The formula was reconstituted according to the manufacturer's instructions printed on the label. *C. sakazakii* strain was sub-cultured in 5 mL TSB and incubated at 37℃ for 16-17 hr and centrifuged at 2800 x g for 25 min and then the cell pellets were suspended in 10 mL reconstituted PIF. Prior to inoculation, the reconstituted PIF was pre-heated in a water bath to the appropriate test temperatures of 10, 20, 30 and 40℃. Each reconstituted PIF were inoculated with 1 mL of cell suspensions to give a final inoculum of 10^7 cfu/mL and incubated at the required temperature.

2.5 Growth Temperature and Growth Rate Measurement

Inoculated RIMF was incubated at appropriate temperature of 10, 20, 30 and 40℃. To measure viable *Cronobacter* strains, at various time intervals, 1 mL aliquots of each heating menstruum was serially diluted in 1% peptone water and plated on TSA plates containing 1% sodium pyruvate, and then incubated at 37℃ for 24-48 hr using the surface drop method [14]. The observed values were natural log-transformed to homogenize variances.

2.6 Data Analysis and Modeling

A primary Gompertz equation model was used for interpreting changes in the microbial count with time. The lag time (LT) and the specific growth rate (SGR) at each incubation temperature were analyzed by nonlinear regression (Prism, version 4.0, GraphPad Software, San Diego, CA) to produce the equation:

$$Y = N_o + C \times \exp\{\exp[2.718 \times \text{mue}/C) \times (\text{Lag-X})+1]\}$$

The Gompertz parameter values were log cell number (Y), incubation time (X) and log initial number of cells (N_0). Measured values included the difference between the initial and final cell numbers, the LT before growth (Lag) and the maximum SGR (mue).

A secondary polynomial model based on temperature was used to predict growth rates. The Gompertz parameters for *C. sakazakii* growth in reconstituted powdered infant formula were determined using the least square analysis (PROC GLM) of SAS version 8.1 (SAS Institute 2002).

$$\ln(\text{Growth rates}) = b_0 + b_1 T + b_2 T^2 + \varepsilon$$

The polynomial model parameter values were incubation temperature (T), regression coefficient (b_0–b_2) and random error (ε).

2.7 Evaluation of Mathematical/Statistical Adequacy

The mean square error (MSE), calculated as the residual sum of squares divided by the number of degrees of freedom, is a measure of the remaining variability that is not accounted for by deliberate changes in factors, such as temperature.

$$\text{MSE} = [\sum(\text{observed growth rates} - \text{predicted growth rates})^2]/\text{number of observations}$$

The regression coefficient (r^2) is often used as an overall measure of the value of a prediction. This coefficient measures the fraction of the variation about the mean that is explained by a model.

The bias factor (B_f) determines whether, on average, the observed values lie above or below the line of equivalence and, if so, by how much. Structural deviations in a model can be thus identified.

$$B_f = 10^{[\Sigma\log(\text{predicted growth rates/observed growth rates})]/\text{number of observations}}$$

The accuracy factor (A_f) averages the distance between each point and the line of equivalence as a measure of how close, on average, predictions are to observations.

$$A_f = 10^{[\Sigma\log(\text{predicted growth rates/observed growth rates})]/\text{number of observations}}$$

3. RESULTS AND DISCUSSION

3.1 Survivality of *C. sakazakii*

Survival analysis showed that the *C. sakazakii* IFST082014 grows well at low temperature (Fig. 1).

3.2 Primary Modeling

Observed *C. sakazakii* growth curves in RIMF at 10, 20, 30 and 40℃ are shown in Fig. 2. Temperature had a significant effect on the growth of *C. sakazakii*. We used the Gompertz equation to fit growth curves for *C. sakazakii*. Growth rate data indicated a good fit for the Gompertz equation model with a high degree of goodness of fit (r^2 = 0.9714–0.9821) for all temperatures. Temperature also had a significant effect on both specific growth rate (SGR) and lag time (LT). With increasing temperature, the SGR strongly increased and the LT decreased (Table 1). SGR values for *C. sakazakii* in infant milk formula were 0.0251±0.0011 h^{-1} at 10℃, and 0.4474±0.0112 h^{-1} at 30℃. The longest LT observed was 59.4±3.24 h at 10℃, and the shortest LT was 2.34±0.47 h observed at 40℃. Our values for SGR increased from 0.0251±0.0011 h^{-1} at 10℃ to 0.7861±0.0027 h^{-1} at 40℃, and the LT decreased from 59.41±3.24 h^{-1} at 10℃ to 2.34±0.47h^{-1} at 40℃.

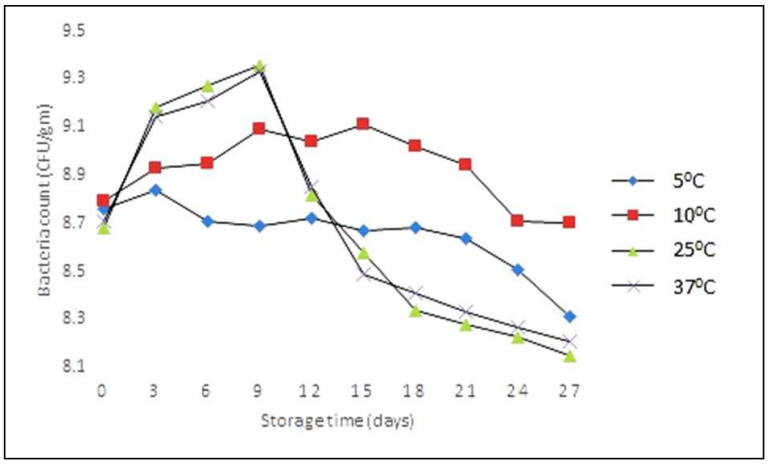

Fig. 1. Survivality of *C. sakazakii* IFST082014 in reconstituted milk formula

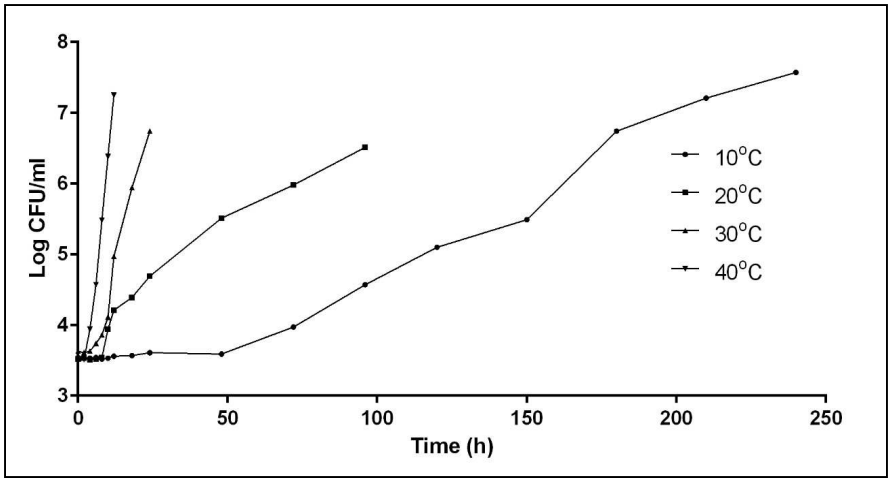

Fig. 2. Observed growth curves of *C. sakazakii* in RIMF at 10, 20, 30 and 40°C

Table 1. Observed values of specific growth rate (SGR) and lag time (LT) for *C. sakazakii* in RMIF as a function of temperature using a modified gompertz equation for primary modeling

Temperature (C)	SGR (h^{-1})	LT (h)	r^2
10	0.0251±0.0011	59.41±3.24	0.9821±0.0017
20	0.2016±0.0006	7.81±0.71	0.9813±0.0019
30	0.4474±0.0112	3.47±0.22	0.9751±0.0021
40	0.7861±0.0027	2.34±0.47	0.9714±0.0142

3.3 Secondary Modeling

Growth curves were transformed to natural logarithms to stabilize model variance [15] and were subjected to response surface analysis using the SAS general linear model. The following equation was determined:

$$LnSGR=-0.06581 + (0.00575 \times temperature) + (0.00039 \times temperature^2)$$

The predicted SGR increased with increasing temperature. We compared our results with the growth of predictive models for other microorganisms, including *Salmonella* Typhimurium, *Listeria monocytogenes*, *Staphylococcus aureus* and *C. sakazakii* in TSB [16,17,18,19] (Table 2). The predicted SGR of *C. sakazakii* in TSB [19] varied from 0.028 h^{-1} at 10℃ to 0.159 h^{-1} at 40℃, while our values varied from 0.029 h^{-1} at 10℃ to 0.741 h^{-1} at

40℃. The predicted SGR of *C. sakazakii* in RIMF is higher because RIMF is a food rich in nutrients and vitamins needed for microorganism growth.

3.4 Evaluation of Mathematical/Statistical Adequacy

Evaluation of the mathematical/statistical adequacy of our predictive model for *C. sakazakii* growth in RIMF is presented in Table 3. Lower MSE values result in better adequacy of the model to describe the data. Because the MSE of the SGR of our model was low, the predictive capability of our model was high. Higher r^2 ($0<r^2<1$) values result in better model predictions. The r^2 of our SGR model was 0.9845. A $B_f<1$ indicates a "fail safe" model, and $B_f>1$ indicates a "fail dangerous" model. Ross [21] also noted, for models describing pathogen growth rates, that a B_f in the range of 0.9–1.05 can be considered good, in the range 0.7–0.9 or 1.06–1.15 can be considered acceptable and <0.7 or >1.5 can be considered unacceptable. Higher A_f values result in a decrease in the accuracy of the average estimate [22]. An A_f value in the range 1.3–1.5 can be considered good. When $A_f = B_f = 1$, the predictive model is perfect. The B_f and A_f values of our SGR model were in the good range. We applied our model at a temperature of 25℃. The model predicted SGR was 0.2852 log cfu/h with an observed SGR of 0.2972±0.041 log cfu/h. The similarity of these results indicates good model performance. Fig. 2 shows the predicted and observed growth curves of *C. sakazakii* in RIMF at 25℃.

Table 2. Predicted specific growth rates of *C. sakazakii* in RIMF and comparisons with other microorganisms based on predictive models

Bacteria	SGR (h^{-1}) at				References
	10℃	20℃	30℃	40℃	
Enterobacter sakazakii	0.031	0.205	0.458	0.788	[20]
Listeria monocytogenes	0.055	0.219	0.488	0.600	[16]
Staphylococcus aureus	0.057	0.180	0.330	0.494	[18]
Salmonella typhimurium	0.051	0.129	0.209	0.291	[17]
E. sakazakii	0.028	0.111	0.107	0.159	[19]
C. sakazakii	0.029	0.195	0.427	0.741	This study

Table 3. Statistical indices of the secondary modeling step for the growth rate of *C. sakazakii* in RIMF

Indicator	r^2	MSE	B_f	A_f
SGR	0.9845	0.00016	1.0125	1.0007

MSE= mean square error; SGR=specific growth rate

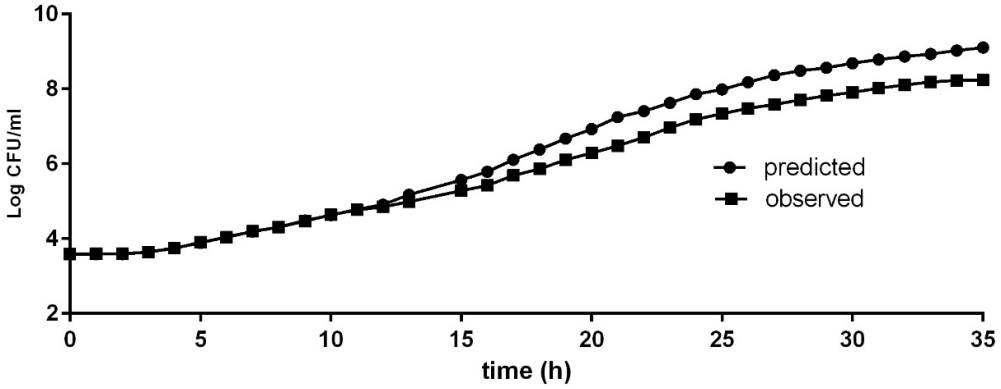

Fig. 3. Comparison of predicted and observed growth curves of *C. sakazakii* IFST082014 in RIMF at 25℃

We compared our model with models for other microorganisms. Kobayashi and Hayashi [23] used a polynomial model to predict the growth of *Zygosaccharomyces rouxii* in soy sauce mash based on temperature. The model produced a high degree of goodness of fit between the predicted and observed values (MSE = 0.002–0.011, r^2 = 0.62–0.97). Te Giffel and Zwietering [21] evaluated polynomial models for predicting the growth of *L. monocytogenes* on various foods, such as meat, fish, egg, milk, dairy products, cheese and vegetables. MSE values were 0.0034–0.6437, and r^2 values were 0.13–0.90. Milk exhibited the highest r^2 value. Our results showed that our model provided reliable predictions for the growth rate of *C. sakazakii* in RIMF based on temperature. Processing plant managers, hospital administrators and homemakers can use our model when RIMF is contaminated with *C. sakazakii*. The risk of *C. sakazakii* contamination in powdered infant milk formula for neonates and children can be significantly reduced.

4. CONCLUSION

Predictive models allow quantitative estimation of microorganism growth. Predicted specific growth rates (SGRs) using our secondary model were similar to measured SGRs, and evaluation of mathematical/statistical adequacy of the predictive model showed reliable results (r^2 = 0.9845, MSE = 0.00016, B_f = 1.0125, A_f = 1.0007). This model may be of use to dairy producers and regulatory authorities.

COMPETING INTERESTS

Authors have declared that no competing interests exist.

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