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Temperature effect on kinetic parameters of *Cronobacter sakazakii* in powdered infant formula

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***Abstract*— Since the early 1980 *Cronobacter sakazakii* is a food-borne pathogen that has increasingly raised interest among the scientific community, health care providers, and the food industry. Detection of *Cronobacter* spp. from samples at the retail market of dry powdered infant formulae has been constantly reported in EU Rapid Alert System. Even if the contamination of *Cronobacter spp*. occurring in dry powdered infant formulae are generally very low, reconstituted infant formula is a good medium for growth.**

The aim of the study was to describe the effect of temperature on the specific growth rate and lag time by using secondary growth models Gompertz and Ratkowsky, as well as evaluate relationship between physiological growth phase versus specific growth rates and lag time. The resulting minimum and maximum temperature are estimated with the secondary Rosso equation. The estimated optimal growth temperature was 37ºC, whereas the maximum specific growth rate was 2.23 h^{-1} at 39 ºC.

The growth rates and lag times at various temperatures obtained in this study may help in calculation of the period for which reconstituted infant formula can be stored at a specific temperature without impact on health.

Keywords*— *Cronobacter sakazakii, specific growth rate, lag time, secondary growth model

I. INTRODUCTION

Cronobacter sakazakii is a motile, rod shape and Gram negative bacteria that excrete cellulose during biofilm production and occasionally cause neonatal meningitis, sepsis in the population at risk (neonates, newborns or immunocompromised infants). *Cronobacter* spp. can be present, although in low levels, in dry powdered infant formulae and it has been linked to cases of meningitis in neonates, especially those born prematurely [1].

Cronobacter spp. may contaminate infant formulae either during production or during bottle preparation. In factory environments *Cronobacter* spp. may grow in wet spots and survive in dust containing residues of infant formulae and contaminate the product after the drying process. Infant formulae may be subject of contamination in health care unit and household kitchens by utensils and via environmental vectors [3].

To keep contamination to a minimum in the finished product depends on knowledge of the lag time, specific growth rate and maximum population density.

In dry powdered infant formula *Cronobacter* spp. stays in lag phase, but after the addition of water, the reconstituted formula becomes a good medium for bacterial growth and the barriers which prevent bacterial growth are short storage and low temperature.

The experiment was carried out in reconstituted powdered infant formulae with tap boiled water when bacterial cells of *C. sakazakii* were in different phases of growth.

II. MATERIAL AND METHODS

A. Bacterial and growth conditions

In our experiment the *Cronobacter sakazakii* ATCC 29544 stock cultures were maintained at -80ºC in cryo-vials (Microbank 20 Mulral Sreeet, Unit 4 Richmond Hill ON, Canada). After the working culture were maintained in a mixture which contained 0.7 mL of a stationary-phase culture suspension in Trypticase Soy Broth with 0.6% Yeast Extract (Himedia; L.B.S. Marg, Mumbai - 400 086, India.) broth with 0.3 ml 87% glycerol (Fluka-Chemica, GmbH CH9471 Buchs, Switzerland).

B. Preparation of bacterial suspension

Cronobacter sakazakii ATCC 29544 was cultured by transferring 250 *µ*L of the stock culture into 250 mL BHI followed by incubation at 37ºC. Bacterial cells were incubated for 3, 6, 16, 24 and 72 hours to obtain respectively the exponential phase, early stationary phase, middle stationary phase, stationary phase and late stationary phase cells. In order to obtain enough cells in the lag phase, 1 mL of a stock culture of ATCC 29544 maintained in –20ºC was diluted in 2 mL glycerol, whereupon the suspension was centrifuged (2524 R model; Eppendorf AG 22331 Hamburg

Germany) for 10 minutes at 10,000 rfc . Cells harvested were transferred into 30 mL BHI and incubated for 1.5 hour at 37ºC. In order to obtain cells for spiking dry infant formula, these BHI grown cultures were centrifuged for 5 minutes at 20ºC at 3000 rfc (5804 R model; Eppendorf AG 22331 Hamburg Germany). Cells were washed in physiological salt solution (1%) and suspended in 30 mL physiological salt solution (1%) for the lag phase cells. Cell suspension concentration is about 1.0+E4 CFU/mL for lag phase grown cells and between 1.0+E5 and 1.0+E7 CFU/mL for the other growth phases.

C. *Artificial contamination of infant formula*

Infant formula was bought in drug store; the numbers of the viable bacteria in the infant formula were less than LOD of the method (1.0+E3 CFU/g). The obtained bacterial suspension was pipetted directly to the powdered infant formula and then was minced aseptically in order to homogenize the matrix. After 3-4 days spiking, the final bacterial concentration was 1.0+E2 - 1.0+E2 CFU/g of dry powder and the infant formula had a water activity (a_w) less than 0.22. All growth infant formula experiment were performed after 7 days spiking.

D. *Design*

The prediction of specific growth rates at various temperatures requires the lowest temperature supporting growth (T_{min}), highest temperature supporting growth (T_{max}) and the specific growth rate (μ_{opt})bat optimal growth temperature (T_{opt}), as cited in table 1. The lag time at each temperature was initially estimated by the k value, which is the product of the specific growth rate (μ_m) as well as the lag time (λ) which is known for large variability, but anyhow can be considered constant over a wide range of temperatures [3].

E. *Growth experiments*

Ten-gram portions of artificial contaminated infant formula were reconstituted in 100 mL sterilized tap water. Each contaminated glass bottle was divided in 10 mL glass tubes in order to determine the effect of various growth phases. Bottles with PIF and strain ATCC 29544 were incubated as follows (the number of tubes incubated is given in parentheses: 8ºC (n=17); 14ºC (n=17); 37ºC (n=16); 39ºC (n=14); Early stationary phase grown cells of strain ATCC 29544 were additionally used to assess growth in reconstituted infant formula at the following temperatures (ºC): 4, 8, 14, 37, 39, 41.5, 43, 45, 46, 47, 48, 51. All samples were surface plated onto Plate Count Agar (PCA) (Himedia; L.B.S. Marg, Mumbai - 400 086, India.) with a spread plate technique. Inoculated plates were incubated for 20 to 24 h at 30ºC (UVP DOC it Colony Counter; UVP, LLC 2066 W. 11th St. Upland, CA).

F. *Data analysis*

Kinetic parameters (maximum population density, lag time and specific growth rate) were estimated with a probability model as modified by Gompertz[10] and two secondary models of Rosso [7] and Ratkowsky [6].

The results were estimated in lag time (λ; [h]), specific growth rate (μ_m; [h^{-1}]) and the asymptotic value was estimated (A; [-] in tested temperature for each growth curve.

In order to obtain homogeneity of variance, lag time data were log transformed and specific growth rate data were square root transformed. A significance level of 5% was used. All data analyses and visualization were performed using JMP 10.0.0 SAS Institute Inc. 2014.

III. RESULTS AND DISCUSSION

The secondary growth model of Rosso was used to describe the effect of temperatures on growth rate. This model contains four interpretable parameters μ_{opt}, T_{min}, T_{max} and T_{opt} (table 1).

TABLE I. GROWTH PARAMETERS OF *CRONOBACTER* SPP. IN RECONSTITUTED INFANT FORMULA: INITIAL ESTIMATION IS BASED ON PREVIOUS PUBLISHED DATA.

Parameter	Table Column Head	
	Initial estimate	***Reference(s)***
T_{min}	5.5ºC	Nazarowec-White, M., and J. M. Farber(1997)
T_{max}	47ºC	Farmer et al.(1980); Iversen, et al. (2004)
T_{opt}	37 ºC	Iversen, et al. (2004)
μ_{opt}	2.5 h^{-1}	Iversen, et al. (2004)
k	3.75	Juneja et al. (2003)

If,

$$T_{min} < T < T_{max}$$

then,

$$\mu_m(T) = \left(\frac{(T-T_{max})(T-T_{min})^2}{[(T_{opt}-T_{min})(T-T_{opt})-(T_{opt}-T_{max})(T_{opt}+T_{min}-2T)](T_{opt}-T_{min})}\right)\mu_{opt} \quad (1)$$

and if,

$$\begin{cases} T \le T_{min} \\ T \ge T_{max} \end{cases}$$

then,

$$\mu_m = 0$$

where:

T_{min} and T_{max} are defined in equation, T_{opt} [ºC] is the temperature at which the specific growth rate μ_m [h^{-1}] is optimal, and μ_{opt} is the μ_m at optimal temperature.

In order to meet the specified requirements of the experiment, theoretical growth curves was carried out

at 4, 8, 14, 37, 39, 41.5, 43, 45, 46, 47, 48 and 51. Initial estimation of Rosso equation indicate that the incubation temperature (ºC): 4, 47, 48, 49 and 51 have no growth rates (μ_m=0). Visualization of the μ_m estimation using Rosso equation is shown in figure 1.

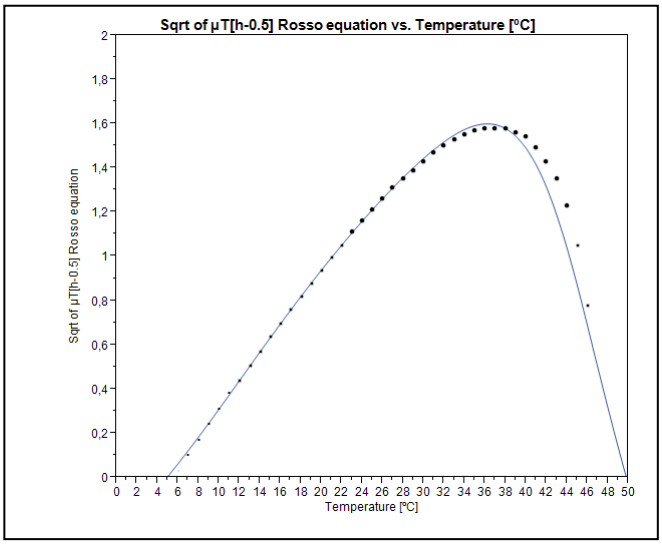

Fig. 1. *Square root of μm distribution (Theoretical Rosso model)*

The second predictive model labeled modified Gompertz (equation 2) was used to find the specific growth rate on experimental findings.

Modified Gompertz equation:

$$ln\left(\frac{N_t}{N_0}\right) = A\ exp\left\{-\exp\left[\frac{\mu_m e}{A}(\lambda - t) + 1\right]\right\} (2)$$

where:

N_0 [CFU/mL] is the initial bacterial concentration at time t = 0;

N_t [CFU/mL] is the bacterial concentration at time t;

A [CFU/mL] is the asymptote at a specific physiological stage;

λ [h] is the lag time;

μ_m [h^{-1}] specific growth rate.

Microbial behavior under different conditions was defined by using modified Gompertz in order to estimate the population of *Cronobacter sakazakii* ATCC cells in lag, in early and stationary phase. Graphical visualization of modified Gompertz is shown in figure 2.

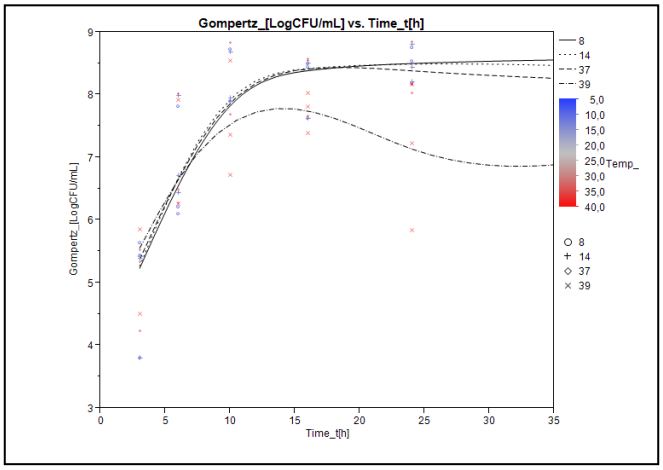

Fig. 2. *Rate of response of log transformed Nt according to modified Gompertz (Sigmoid Model)*

Determination of lag time λ was computed using the logarithm the inverse of secondary Ratkowsky model (equation 3)(data not shown).

$$ln(\lambda(Ti)) = ln\left[\left((b(T_i - T_{min})).\{1 - exp[c(T_i - T_{max})]\}\right)^2\right] (3)$$

where b [ºC^{-1} h$^{-0.5}$], and c [ºC^{-1}] are named Ratkowsky parameters. The T_{min} and T_{max} values were assumed to be equal to the T_{min} and T_{max} of equation Secondary Rosso growth model describes the specific growth rate.

Statistical evaluations (p-values) of univariate analysis of variance were done in order to keep significant records related to the effects of the physiological growth phase, specific growth rates of *Cronobacter sakazakii* ATCC 29544 cells (on lag time), and both products (k) at temperatures (ºC): 8, 14, 37 and 39. (table 2).

TABLE II. . RELATIONSHIP BETWEEN PHYSIOLOGICAL GROWTH PHASE AND SPECIFIC GROWTH RATES (μM) AND LAG TIME (λ)

Parameter	***p*-value at:**			
	T=8°C	***T=14°C***	***T=37°C***	***T=39°C***
Lag time (λ)[h]	5,00E-06	2,90E-08	1,10E-07	6,30E-07
Specific growth rate (μ_m)[h^{-1}]	0,0028	0,00059	0,00064	0,0037
k-value (λ x μ_m)	0,0173	0,00382	0,002	0,0044

According to p-value (p<0.05) founded there is a significant positive relationship between physiological growth phase versus specific growth rates (μ_m) and lag time (λ) at all temperatures.

Since statistical analysis showed that the physiological growth phases of strain ATCC 29544 at ºC): 8, 14, 37, 39 did not influence significantly to specific growth rates and lag times, further experiments were performed over a wide range of

temperatures, from 4 up to 51ºC with early stationary spiked cells of strain ATCC 29544 (figure 3).

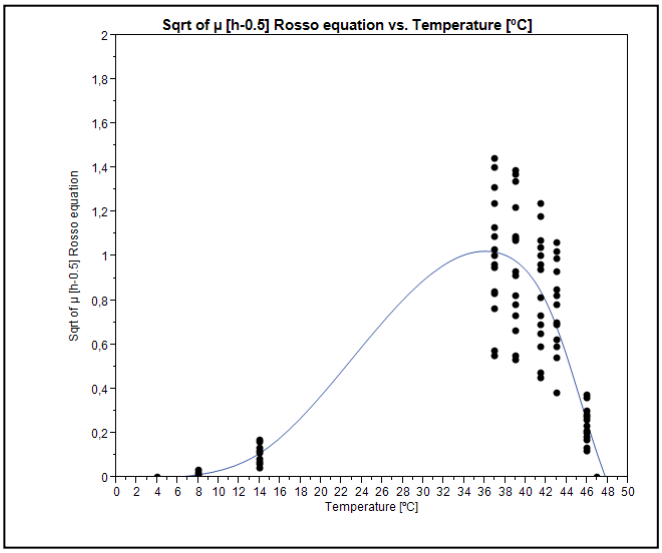

Fig. 3. Square root of measured and fitted specific growth rates μ as function of the temperature (experiment data only).

Although growth was observed up to 47ºC, no reliable estimates of the specific growth rate and lag time could be derived from the models.

In general was observed growth in all cases and in each temperature. With increasing temperature, μ_m is increased to 39ºC. A clear rising value can be observed for cells in stationary phase compare to lag phase and early stationary phase. Mean values of μ_m at 4, 8, 14 and 37ºC incubation temperature were shown in table 3.

TABLE III. . MEAN VALUES OF SPECIFIC GROWTH RATE (μ_M) FOR VARIOUS PHYSIOLOGICAL GROWTH STATES IN DIFFERENT TEMPERATURES OF STRAIN *C. SAKAZAKII* ATCC 29544

Physiological growth phases	***Mean values of μ_m[h^{-1}] at***			
	T=8°C	***T=14°C***	***T=37°C***	***T=39°C***
Cells in lag phase	0.75± 0.31	0.77± 0.35	0.82± 0.32	0.6± 0.35
Cells in early stationary phase	1.01± 0.44	1.05± 0.46	0.98± 0.52	0.82± 0.32
Cells in stationary phase	1.41± 0.56	1.39± 0.56	1.42± 0.6	1.39± 0.69

IV. CONCLUSIONS

The effect of temperature on kinetic factors was described in detailed by using statistically significant experiment on different physiological stage of *C. sakazakii* ATCC cells. The specific growth rate, lag time and *k* coefficient were extrapolated according to Rosso, Ratkowsky and modified Gompertz equation[3].

The findings of this study indicate that optimum growth temperature was met on 37ºC, whereas the maximum specific growth rate was 2.23 h^{-1} at 39ºC.

The effect of strain variability related to growth parameters was studied in different physiological phase of *Cronobacter sakazakii* cells (table 2).

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