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Prediction of growth kinetics of *Bacillus tequilensis* in nutrient broth under isothermal and non-isothermal conditions

Giti EMTIAZI[1], Fatemeh Sadat GHOREISHI[2], Kianoush Khosravi DARANI[3]*, Özgün YÜCEL[4], Fatih TARLAK[5]*

Abstract

The main objective of the present study was to develop and validate a new alternative modelling method to predict *Bacillus tequilensis* count in nutrient broth under iso-thermal and non-isothermal conditions. The bacterial growth data of *Bacillus tequilensis* was obtained experimentally in aerobically-kept nutrient broth medium and described with two-step and one-step modelling approaches by different primary models (the modified Gompertz, logistic, Baranyi and Huang models) under isothermal temperatures. Temperature-dependent kinetic parameter (maximum specific growth rate 'μ_{max}') was described as a function of temperature via the Ratkowsky model integrated with each primary model. The Huang model based on the one-step modelling approach yielded the best fitting results (RMSE = 0.143 and adjusted-R^2 = 0.977) for all isothermal conditions. Therefore, the Huang model was also used to check it's prediction capability under non-isothermal conditions. The differential form of the Huang model provided satisfactorily statistical indexes (B_f = 1.07 and A_f = 1.08) indicating reliably being able to use to describe the growth behaviour of *Bacillus tequilensis* in broth subjected to non-isothermal conditions.

Keywords: dynamic condition; microbiological quality; *Bacillus tequilensis*; growth kinetic; predictive microbiology.

Practical Application: In real-world scenarios, environmental conditions are subject to change, and it is essential to consider these changes when modelling dynamic systems. One of the most significant factors that can vary is temperature, which can significantly impact the growth behaviour of microorganisms. Thus, dynamic models that consider the effect of changing temperature are crucial to predict microbial growth under non-isothermal conditions. The predictive models used in this work have the potential to be utilized as a simulation tool.

1 Introduction

Microbial load can be determined with traditional microbiological measuring techniques. But the traditional enumeration techniques of microorganisms are time- consuming and expensive. Even more, the results of these techniques give us only information about specific time and condition. But the growth behaviour of microorganisms depends on changing environmental factors. Therefore, the traditional enumeration techniques are not adequately practical. Because of these disadvantages, mathematical microbial models have aroused increasingly interest to evaluate growth behaviour of microorganisms (Bovill et al., 2001).

The main objective of predictive microbiology is to predict microbial behaviour by employing mathematical models. Primary and secondary models are commonly used in predictive microbiology (Tarlak et al., 2020; Buchanan et al., 1997; Whiting, 1995). For the first class, the modified Gompertz, logistic, Baranyi and Huang models are the most popular ones describing microbial growth data as a function of time at constant environmental conditions. The secondary models indicate how obtained parameters from primary models change with respect to one or more environmental or cultural factors (*e.g.*, gas composition, pH, temperature and salt level). Temperature is one of the most important environmental factors directly affecting the growth behaviour of microorganisms, and its effect is commonly simulated using the Ratkowsky model (Tarlak, 2020; Ratkowsky et al., 1982).

Under real life conditions, environmental factors are not always constant (Zwietering et al., 1994). Therefore, dynamic models are essential models taking into account the changing environmental conditions (Pérez-Rodríguez & Valero, 2013). The most likely variable environmental factor is the temperature that considerably affects the growth behaviour of microorganisms. Dynamic models considering the effect of changing temperature are important to model the effect of the temperature on microbial growth under non-isothermal conditions.

Generally, the primary and secondary models are separately fitted to the growth data and kinetic parameters, respectively, which is the most popular modelling procedure followed in the predictive microbiology. But there are some drawbacks

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[1] *Department of Biotechnology, Faculty of Biological Science and Technology, Shahid Ashrafi Esfahani University, Isfahan, Iran*
[2] *Department of Biology, Faculty of Science, Naghshejahan Higher Education Institute, Isfahan, Iran*
[3] *Department of Food Sciences and Technology, National Nutrition and Food Technology Research Institute, Faculty of Nutrition Sciences and Food Technology, Shahid Beheshti University of Medical Sciences, Tehran, Iran*
[4] *Department of Chemical Engineering, Gebze Technical University, Kocaeli, Turkey*
[5] *Department of Nutrition and Dietetics, Faculty of Health Sciences, Istanbul Gedik University, Istanbul, Turkey*
*Corresponding author: k.khosravi@sbmu.ac.ir; ftarlak@gtu.edu.tr

concerning about this modelling approach. The major drawback is to lead to be accumulation and propagation of errors due to being sequentially performed nonlinear regression two times (Huang, 2017). To avoid these disadvantages of two-step modelling approach, alternatively, a one-step modelling approach can be applied while simulating microbial data and kinetic parameters. In this approach, primary and secondary modelling for the growth and temperature (as a changing environmental factor) data is performed simultaneously. Therefore, the use of this approach frequently provides better prediction performance, lower uncertainty, more precise coefficients and robust confidence interval than the two-step modelling approach (Jewell, 2012; Martino & Marks, 2007; Tarlak & Pérez-Rodríguez, 2022).

In the present study, the growth behaviour of *Bacillus tequilensis* in nutrient broth at isothermal storage temperatures (30, 37, 45 and 50 °C) were simulated using two-step modelling approaches with various primary models (the modified Gompertz, logistic, Baranyi and Huang models). The fitting capability of both approaches used was compared, and in the second step validation of one-step modelling approach was done under non-isothermal conditions.

2 Materials and methods

2.1 Experimental data

Microorganism

The feces of a 70-year-old total healthy man were collected in order to isolate halotolerant Bacillus strains. The sample diluted by sterile 0.9% sodium chloride solution and heat shocked (80 °C for 10 minutes) to isolate Spore-bearing bacteria on a nutrient agar medium containing 6% sodium chloride salt. The plates incubated for 24 to 48 hours at 37 °C. After purification, the isolate was stored on a salt-free nutrient agar medium at 4 °C.

Preparation of test cultures

Vials were partially thawed at room temperature and 0.1 mL of them was transferred to 10 mL of nutrient broth in 50 mL tubes and incubated at 37° C for 24 h, to propagate the cultures. This culture contained the freeze-damaged cells, so was not used in growth experiments. The bacterial suspension for the experiments were prepared by transferring 0.1 mL of each culture to the tubes of nutrient broth (10 mL) and incubating aerobically at 37 °C for 24 h. These cultures were then stored in nutrient broth at 4 °C for 2 weeks. A new series of cultures was initiated from the frozen stock on a biweekly basis. The bacterial suspensions were prepared by transferring 0.1 mL of each culture to 50 mL of nutrient broth in 250 mL flasks and incubated aerobically at 37 °C for 18 h to provide late stationary phase cells. Then, each culture was centrifuged (5000 g, 15 min, 4 °C), and the bacterial biomass in each cell suspension were enumerated by the mean of three replicates.

Incubation temperatures, sampling times and bacterial enumeration

Growth rates were determined by inoculation of 10% from a half McFarland bacterial suspension in salt free nutrient broth pH =6 in three replicates. The cultured were incubated at the desired temperatures (30, 37, 45 and 50 °C) at 160 rpm and every two hours, the cells forming units were detected. Also, the growth at dynamic temperature 30, 37 and 45 °C obtained with 6 hours incubation at variable temperature for 48 hours duration. The cell forming unit was determined from the mean of three replicates.

2.2 Modelling

Four different primary models namely the modified Gompertz equation (Zwietering et al., 1990), logistic (Zwietering et al., 1990), Baranyi & Roberts (1994) and Huang (2017) models were fitted with the two-step and one-step modelling approaches as they are the most used sigmoid functions describing the bacterial growth behaviour at constant environmental conditions which are defined by Equations 1, 2, 3 and 4, respectively:

$$y(t) = y_0 + (y_{max} - y_0).\exp\left\{-\exp\left[\frac{\mu_{max}.e}{(y_{max} - y_0)}.(\lambda - t) + 1\right]\right\} \quad (1)$$

$$y(t) = y_0 + \frac{(y_{max} - y_0)}{\left\{1 + \exp\left[\frac{4.\mu_{max}}{(y_{max} - y_0)}.(\lambda - t) + 2\right]\right\}} \quad (2)$$

$$y(t) = y_0 + \mu_{max} F(t) - \ln\left(1 + \frac{e^{\mu_{max} F(t)} - 1}{e^{(y_{max} - y_0)}}\right) \quad (3)$$

$$y(t) = y_0 + y_{max} - ln(e^{y_0} + \left[e^{y_{max}} - e^{y_0}\right].e^{-\mu_{max} B(t)} \quad (4)$$

where t is the time (h), y(t) is the concentration of bacterial populations (ln CFU/mL) at time t, y_0 is the initial concentration of bacterial populations (ln CFU/mL), y_{max} is the maximum concentration of bacterial populations (ln CFU/mL), μ_{max} is the maximum specific growth rate (1/h), λ is the duration of lag phase (h), F(t) and B(t) are the adjustment functions that are respectively described by Baranyi & Roberts (1994) and Huang (2017).

The Ratkowsky model (Juneja et al., 2007; Ratkowsky et al., 1982) was employed for the determination of relationship between storage temperature and μ_{max} using the Equation 5:

$$\mu_{max} = b_1 (T - T_0)^2 \left\{1 - \exp(b_2 (T - T_{max})\right\} \quad (5)$$

where T is the storage temperature (°C), T_0 and T_{max} are the theoretical lowest and highest bacterial growth temperatures (°C), μ_{max} is the maximum specific bacterial growth rate (1/h), b_1 and b_2 are the regression coefficients.

For the two-step and one-step modelling approaches, each of the parameters was calculated by means of NonLinearModel command which uses Levenberg Marquardt algorithm in the Matlab 8.3.0.532 (R2014a) software (MathWorks Inc., Natick, MA, USA). Determination of suitable starting values in nonlinear regression procedure is a critical step to improve the regression process. The starting values for the parameters, y_0 and y_{max} were selected based on the observed minimum and

maximum concentrations considering the entire temperature range, respectively. For the parameters, b_1, b_2, T_0 and T_{max}, no observation or prior knowledge could be applied. Using a randomly selected starting value for these parameters could suffice for finding solutions that are locally optimal, but it could not lead to the global optimal point of the objective function. To improve the performance of the optimization procedure and increase the probability of finding globally optimal solutions, the starting points of the parameters for both modelling approaches were selected by using ga command which uses genetic algorithm in Global Optimization Toolbox of Matlab software.

Goodness of fit for model comparisons

The comparison of the performance of the tested models and approaches was carried out by using the root mean square error (RMSE) and the adjusted coefficient of determination and (adjusted-R^2) values using Equations 6 and 7, respectively:

$$RMSE = \sqrt{\sum_{i=1}^{n} \frac{\left(observed_i - fitted_i\right)^2}{n-s}} \quad (6)$$

$$\text{adjusted-}R^2 = 1 - \left(\frac{n-1}{n-s}\right)\left(\frac{SSE}{SST}\right) \quad (7)$$

where $observed_i$ is the experimental bacterial growth, n is the number of experiments, s is the number of parameters of the model, SSE is the sum of squares of errors and SST is the total sum of squares.

Statistical analysis

RMSE and adjusted-R^2 values obtained from the two-step and one-step modelling approaches were subjected to one-way analysis of variance (ANOVA) using the Matlab 8.3.0.532 (R2014a) software (MathWorks, Natick, Massachusetts, USA). Statistical differences between the modelling approaches were determined by post hoc analysis using Tukey's test. The differences between the means were regarded as statistically significant when the $p \leq 0.05$.

Model validation

Verification of the developed models in the predictive food microbiology is necessary to be reliably employed as a simulation tool. The prediction performance of the global model that gave the best fitting capability to model the growth behaviour of *Bacillus tequilensis* was assessed by considering the growth data obtained from non-isothermal storage conditions. The comparison was done using the bias (B_f) and accuracy (A_f) factors (Ross, 1996) given in Equations 8 and 9, respectively:

$$B_f = 10^{\frac{\sum_{i=1}^{n} \log\left(y_{predicted}/y_{observed}\right)}{n}} \quad (8)$$

$$A_f = 10^{\frac{\sum_{i=1}^{n} \left|\log\left(y_{predicted}/y_{observed}\right)\right|}{n}} \quad (9)$$

where $y_{predicted}$ refers to predicted maximum growth rate (log CFU/h), $y_{observed}$ refers to experimental maximum growth rate (log CFU/h), n refers to the number of data.

The B_f and A_f shows how close simulated data to experimental data, and a value of 1 for B_f and A_f means that there is a perfect agreement between experimental and maximum growth rate data.

3 Results and discussion

Bacillus tequilensis growth data in nutrient broth were fitted using two-step and one-step modelling approaches (Tables 1-4),

Table 1. Observed and fitted growth data of *Bacillus tequilensis* in nutrient broth at 30 °C.

Time (h)	Observed data (log CFU/mL)[a]	Two-step modelling approach Fitted data (log CFU/mL)				One-step modelling approach Fitted data (log CFU/mL)			
		Modified Gompertz model	Logistic model	Baranyi model	Huang model	Modified Gompertz model	Logistic model	Baranyi model	Huang model
0	7.0 ± 0.3	7.0	6.9	7.0	7.0	7.0	7.0	7.0	7.0
2	7.0 ± 0.2	7.1	7.1	7.3	7.1	7.1	7.2	7.2	7.0
4	7.2 ± 0.2	7.5	7.5	7.7	7.6	7.4	7.5	7.5	7.5
6	7.6 ± 0.3	8.2	8.0	8.3	8.1	7.9	7.9	7.9	7.9
8	8.4 ± 0.4	8.7	8.6	8.8	8.6	8.3	8.3	8.3	8.3
10	8.8 ± 0.3	9.0	9.0	9.2	9.0	8.7	8.7	8.7	8.7
12	9.1 ± 0.3	9.2	9.2	9.3	9.3	9.0	9.0	9.0	9.0
14	9.4 ± 0.4	9.3	9.3	9.4	9.4	9.2	9.2	9.3	9.2
16	9.5 ± 0.2	9.4	9.4	9.4	9.4	9.3	9.3	9.4	9.4
18	9.5 ± 0.1	9.4	9.4	9.4	9.4	9.4	9.4	9.4	9.4
20	9.4 ± 0.2	9.4	9.4	9.4	9.4	9.4	9.4	9.4	9.4
26	9.4 ± 0.4	9.4	9.4	9.4	9.4	9.4	9.4	9.4	9.4
28	9.3 ± 0.2	9.4	9.4	9.4	9.4	9.5	9.4	9.4	9.4
30	9.3 ± 0.1	9.4	9.4	9.4	9.4	9.5	9.4	9.4	9.4
32	9.3 ± 0.1	9.4	9.4	9.4	9.4	9.5	9.4	9.4	9.4

[a]Observed data in the table are given as average values ± standard deviations.

Table 2. Observed and fitted growth data of *Bacillus tequilensis* in nutrient broth at 37 °C.

Time (h)	Observed data (log CFU/mL)[a]	Two-step modelling approach Fitted data (log CFU/mL)				One-step modelling approach Fitted data (log CFU/mL)			
		Modified Gompertz model	Logistic model	Baranyi model	Huang model	Modified Gompertz model	Logistic model	Baranyi model	Huang model
0	7.0 ± 0.3	7.0	6.9	7.0	7.0	7.0	7.0	7.0	7.0
2	7.2 ± 0.2	7.2	7.2	7.4	7.3	7.3	7.3	7.3	7.3
4	7.7 ± 0.2	8.0	7.9	8.0	8.0	8.0	8.0	8.0	8.0
6	8.7 ± 0.3	8.7	8.7	8.7	8.7	8.7	8.7	8.7	8.7
8	9.1 ± 0.4	9.1	9.2	9.2	9.2	9.1	9.1	9.2	9.1
10	9.3 ± 0.3	9.3	9.3	9.4	9.4	9.3	9.3	9.4	9.4
12	9.3 ± 0.3	9.4	9.4	9.4	9.4	9.4	9.4	9.4	9.4
14	9.5 ± 0.4	9.4	9.4	9.4	9.4	9.4	9.4	9.4	9.4
16	9.6 ± 0.2	9.4	9.4	9.4	9.4	9.4	9.4	9.4	9.4
18	9.6 ± 0.1	9.4	9.4	9.4	9.4	9.5	9.4	9.4	9.4
20	9.6 ± 0.2	9.4	9.4	9.4	9.4	9.5	9.4	9.4	9.4
26	9.6 ± 0.4	9.4	9.4	9.4	9.4	9.5	9.4	9.4	9.4
28	9.6 ± 0.2	9.4	9.4	9.4	9.4	9.5	9.4	9.4	9.4
30	9.6 ± 0.1	9.4	9.4	9.4	9.4	9.5	9.4	9.4	9.4
32	9.6 ± 0.1	9.4	9.4	9.4	9.4	9.5	9.4	9.4	9.4

[a]Observed data in the table are given as average values ± standard deviations.

Table 3. Observed and fitted growth data of *Bacillus tequilensis* in nutrient broth at 45 °C.

Time (h)	Observed data (log CFU/mL)[a]	Two-step modelling approach Fitted data (log CFU/mL)				One-step modelling approach Fitted data (log CFU/mL)			
		Modified Gompertz model	Logistic model	Baranyi model	Huang model	Modified Gompertz model	Logistic model	Baranyi model	Huang model
0	7.0 ± 0.3	7.0	6.9	7.0	7.0	7.0	7.0	7.0	7.0
2	8.1 ± 0.2	7.3	7.3	7.4	7.4	7.7	7.7	7.7	7.7
4	8.7 ± 0.2	8.2	8.2	8.1	8.3	8.8	8.7	8.7	8.7
6	9.2 ± 0.3	8.9	9.0	8.9	9.0	9.3	9.3	9.3	9.3
8	9.4 ± 0.4	9.3	9.3	9.3	9.3	9.4	9.4	9.4	9.4
10	9.3 ± 0.3	9.4	9.4	9.4	9.4	9.4	9.4	9.4	9.4
12	9.3 ± 0.3	9.4	9.4	9.4	9.4	9.5	9.4	9.4	9.4

[a]Observed data in the table are given as average values ± standard deviations.

Table 4. Observed and fitted growth data of *Bacillus tequilensis* in nutrient broth at 50 °C.

Time (h)	Observed data (log CFU/mL)[a]	Two-step modelling approach Fitted data (log CFU/mL)				One-step modelling approach Fitted data (log CFU/mL)			
		Modified Gompertz model	Logistic model	Baranyi model	Huang model	Modified Gompertz model	Logistic model	Baranyi model	Huang model
0	7.0 ± 0.3	7.0	6.9	7.0	7.0	7.0	7.0	7.0	7.0
2	7.9 ± 0.2	7.2	7.3	7.4	7.4	7.6	7.6	7.6	7.6
4	8.5 ± 0.2	8.1	8.2	8.0	8.2	8.6	8.6	8.6	8.6
6	9.1 ± 0.3	8.9	9.0	8.7	8.9	9.2	9.2	9.3	9.2
8	9.3 ± 0.4	9.2	9.3	9.2	9.3	9.4	9.4	9.4	9.4
10	9.4 ± 0.3	9.3	9.4	9.4	9.4	9.4	9.4	9.4	9.4
12	9.3 ± 0.3	9.4	9.4	9.4	9.4	9.5	9.4	9.4	9.4
14	9.3 ± 0.3	9.4	9.4	9.4	9.4	9.5	9.4	9.4	9.4

[a]Observed data in the table are given as average values ± standard deviations.

and the statistical indicates were given in Table 5. RMSE and adjusted-R^2 values shown in Table 5 indicate the overall fitting potential for two-step modelling approach, which means that RMSE and adjusted-R^2 values were calculated after consecutively done primary and secondary model fitting for entire data sets for each temperature. The statistical indices showed the Huang

model gave the best fitting performance for all temperatures. The logistic model was the second for providing fitting performance. The Baranyi model could not estimate the growth behaviour of *Bacillus tequilensis* as good as other primary models when the two-step modelling approach was employed.

It is known that the degrees of freedom is important factor for reliability of the model parameters while employing nonlinear regression procedure (Huang, 2017). While doing simulation with one-step modelling approach, primary and secondary modelling is performed simultaneously considering whole experimental data set, which means that the simulation with one-step modelling approach has always higher degrees of freedom than the simulation with two-step modelling approach. Therefore, the improvement obtained from one-step modelling approach can be attributed to higher degrees of freedom in one-step modelling approach.

One-step modelling approach, an alternative way to traditionally used two-step modelling one, was employed to quantitatively detect *Bacillus tequilensis* count. The statistical indices, RMSE and adjusted-R^2 values, showing the fitting capability of one-step modelling approach were presented for each model in Table 5. The RMSE and adjusted-R^2 values of each of the primary models based on one-step modelling approach were calculated maximum 0.156 and minimum 0.970, respectively. These results showed that no matter which primary model was used; the one-step modelling approach gave considerably better prediction performance when the one-step modelling approach was employed.

When the one-step modelling approach was used, the minimum counts of *Bacillus tequilensis* were found to be round 7 log CFU/mL for each temperature (Tables 1-4). The experimental minimum count was an average of 7.0 ± 0.3, which showed that each model perfectly estimated the minimum *Bacillus tequilensis* populations.

The Huang model based on the one-step modelling approach showed that maximum counts of *Bacillus tequilensis* were 9.4 CFU/mL for each temperature (Tables 1-4), while the maximum counts were experimentally found to be of 9.3 ± 0.1, 9.6 ± 0.1, 9.3 ± 0.3 and 9.3 ± 0.3 for the temperature of 30, 37, 45 and 50 °C, respectively. This indicated that the Huang model provided suitable prediction performance for maximum counts of *Bacillus tequilensis* in each temperature.

μ_{max} is the most important critical parameter to describe the growth behaviour of microorganisms, and temperature has a key role in affecting directly μ_{max} (Huang, 2008). The kinetic parameter of μ_{max} belonging to *Bacillus tequilensis* for each temperature and each primary model (the modified Gompertz, logistic, Baranyi and Huang models) were shown for two-step and one-step modelling approach in Figure 1. The figures demonstrate that as the storage temperature increased, the μ_{max} values also increased up to a certain point, which is consistent with the findings of the study conducted by Juneja et al. (2007)

Table 5. The comparison of statistical indices for two-step and one-step modelling approaches.

Modelling approach	Temperature (°C)	Modified Gompertz model	Logistic model	Baranyi model	Huang model
Two-step modelling	RMSE	0.259^{c}	0.235^{c}	0.290^{d}	0.238^{c}
	adjusted-R^2	0.917^{c}	0.932^{c}	0.896^{d}	0.930^{c}
One-step modelling	RMSE	0.154^{b}	0.155^{b}	0.156^{b}	0.133^{a}
	adjusted-R^2	0.971^{b}	0.971^{b}	0.970^{b}	0.987^{a}

RMSE: root mean square error and adjusted; R^2: adjusted coefficient of determination calculated using overall total viable count (log CFU/mL) for 30 °C, 37 °C, 45 °C and 50 °C. Different letters show the results are statistically significant considering the $p \leq 0.05$.

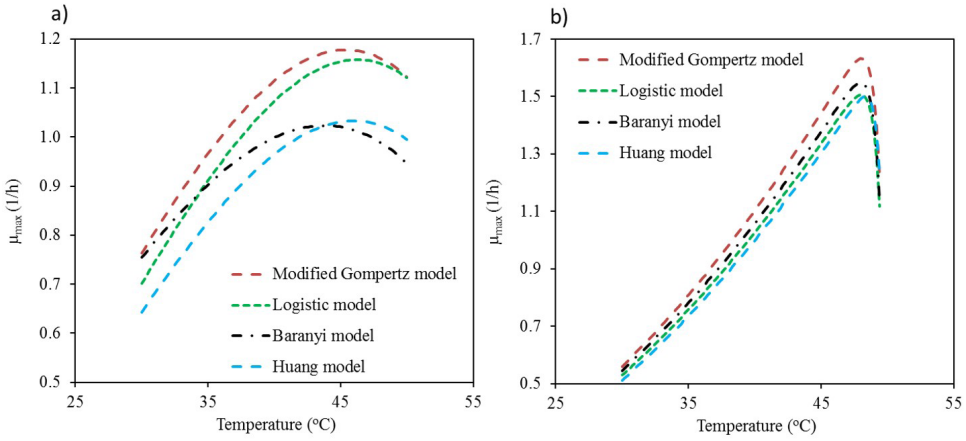

Figure 1. The kinetic growth parameters of *Bacillus tequilensis* for each isothermal temperature and each primary model (the modified Gompertz, logistic, Baranyi and Huang models) for a) two-step b) one-step modelling approach.

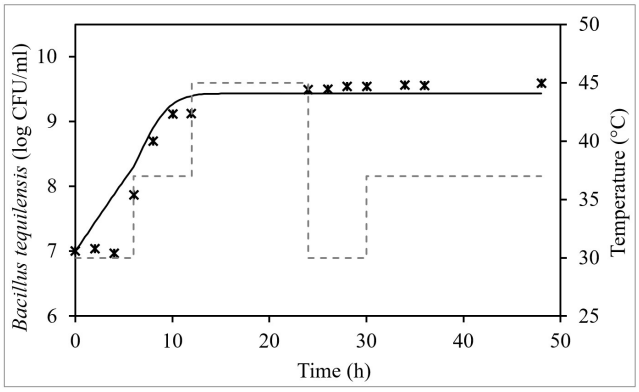

Figure 2. The predicted and observed count of *Bacillus tequilensis* for non-isothermal condition. Dashed line (---) shows the change in dynamic temperature conditions. Symbol (×) indicates observed values. Continues line (—) represents the predicted values using Huang model.

who observed a similar pattern of increasing μ_{max} values followed by a decrease at higher temperatures.

One of the important steps to check how well the developed models are working is validation. The Huang model is the best primary model simulating the growth behaviour of *Bacillus tequilensis* in nutrient broth, therefore, the Huang model was used to predict capability of the *Bacillus tequilensis* concentration under non-isothermal storage conditions (Figure 2). The statistical values for validation B_f and A_f were calculated as 1.07 and 1.080, respectively. A B_f and A_f of 1 indicates no structural deviation of the model. The B_f factor of 1.07 indicated that the model overestimates less than 7% whereas the A_f factor of 1.08 showed that on average the predicted value was less than 8% different (either smaller or larger) from the observed value for the dynamic condition. All these statistical indexes show that the Huang model can be reliably used to predict the growth behaviour of *Bacillus tequilensis* in nutrient broth.

4 Conclusion

No matter which primary model was used, the one-step modelling approach considerably improved the prediction capability of the models for *Bacillus tequilensis* concentration in nutrient broth. The successfully confirmed differential form of the Huang model merged with the Ratkowsky model provided valuable data to evaluate and simulate the growth behaviour of *Bacillus tequilensis* in aerobically-stored nutrient broth under non-isothermal conditions. The predictive models used in this study have a high potential to be used as a simulation tool.

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