J. Microbiol. Biotechnol. (2007), **17**(4), 644–649

JOURNAL OF MICROBIOLOGY AND BIOTECHNOLOGY

A Response Surface Model Based on Absorbance Data for the Growth Rates of *Salmonella enterica* Serovar Typhimurium as a Function of Temperature, NaCl, and pH

PARK, SHIN-YOUNG[1], KYO-YOUNG SEO, AND SANG-DO HA*

Department of Food Science and Technology, Chung-Ang University, Anseong 456-756, Korea
[1]Institute of Biomedical Science, Hanyang University, Seoul 133-791, Korea

Received: October 12, 2006
Accepted: January 2, 2007

Abstract Response surface model was developed for predicting the growth rates of *Salmonella enterica* sv. Typhimurium in tryptic soy broth (TSB) medium as a function of combined effects of temperature, pH, and NaCl. The TSB containing six different concentrations of NaCl (0, 2, 4, 6, 8, and 10%) was adjusted to an initial of six different pH levels (pH 4, 5, 6, 7, 8, 9, and 10) and incubated at 10 or 20°C. In all experimental variables, the primary growth curves were well (r^2=0.900 to 0.996) fitted to a Gompertz equation to obtain growth rates. The secondary response surface model for natural logarithm transformations of growth rates as a function of combined effects of temperature, pH, and NaCl was obtained by SAS's general linear analysis. The predicted growth rates of the *S.* Typhimurium were generally decreased by basic (9, 10) or acidic (5, 6) pH levels or increase of NaCl concentrations (0–8%). Response surface model was identified as an appropriate secondary model for growth rates on the basis of coefficient determination (r^2=0.960), mean square error (MSE= 0.022), bias factor (B_f=1.023), and accuracy factor (A_f=1.164). Therefore, the developed secondary model proved reliable predictions of the combined effect of temperature, NaCl, and pH on growth rates for *S.* Typhimurium in TSB medium.

Keywords: *Salmonella* Typhimurium, response surface model, growth rates

Salmonella spp. are facultative, Gram-negative, and non-sporing rods in the family Enterobacteriaceae [18]. This organism is of great concern in the food industry and public because of its ability to grow over a wide range of temperature (7 to 45°C), pH (4.5 to 9.5), and water activity (0.94 to 1.00) [12]. This organism can cause human salmonellosis, mainly caused by contaminated food products such as egg and egg products [19, 23, 40], poultry, other meat products [24, 40], and dairy products [15, 20, 40].

Mathematical quantitative models that can predict the growth of *Salmonella* spp. as a function of the main environmental controlling factors will improve the shelf life and safety of foods [4, 6, 8, 9, 21, 22, 27, 29, 36–38, 41, 42]. However, earlier studies of predictive models describing the effects of environmental factors such as temperature, pH, water activity, and preservative agents on the growth, survival, or inactivation of *Salmonella* spp. are still limited.

Therefore, the objective of this study was to investigate the combined effects of temperature, NaCl, and pH on the growth kinetics of *S.* Typhimurium in a broth system with the goal of developing a model that could be used to predict the maximum growth rates of the organisms in any combination of the variables.

Materials and Methods

Bacterial Culture

A poultry isolate of *Salmonella enterica* serovar Typhimurium (*S.* Typhimurium) resistant to novobiocin (NO) and nalidixic acid (NA) was used in the study [44]. Tryptic soy broth (TSB, Difco Laboratories, Detroit, MI, U.S.A.) was used for maintenance and growth of the bacterial strain. For bacterial culture maintenance, NO (25 g/ml) and NA (25 g/ml) were added for the *S.* Typhimurium poultry isolate (NO/NA) strain.

*Corresponding author
Phone: 82-31-670-4831; Fax: 82-31-675-4853;
E-mail: sangdoha@cau.ac.kr

Experimental Design
A central composite design was used for incorporating the following variables and levels. A total of 84 factor combinations were tested. These included

- Temperature: 10, 20°C
- NaCl: 0, 2, 4, 6, 8, 10% (wt/vol)
- pH: 4, 5, 6, 7, 8, 9, 10

Preparation and Inoculation of Culture Media
TSB containing two different concentrations of sodium chloride (NaCl, 0, 2, 4, 6, 8, 10% (wt/vol)) was autoclaved at 121°C for 15 min and allowed to cool. The pH of the media was then adjusted to an initial of either 4, 5, 6, 7, 8, 9, or 10 using 1 N NaOH or 1N HCl solution. Microplate wells were filled with 150 μl of each medium condition to which 50 μl of inoculum containing 10^7 CFU/ml of *S.* Typhimurium was added. Control wells containing 200 μl of uninoculated medium were used as blanks and also to check the sterility of the medium.

Growth Temperature and Growth Rate Measurement
Growth rates of *S.* Typhimurium in microplate wells incubated at either 10 or 20°C were measured every 2 h at optical density of 600 nm by an Automated Microplate Reader (ELx808, Biotech Ltd., Winooski, VI, U.S.A.), using Microplate Data Analysis Software. The observed values were natural log-transformed to homogenize variances.

Primary Modeling
Growth curves of the resulting absorbance versus time values were iteratively generated using the Gompertz equation and fitted to a nonlinear regression model (Prism, version 4.0, GraphPad Software, San Diego, CA, U.S.A.) to determine maximum growth rates (GR, in $\log_{10}$ CFU/ml per hour) at each incubation temperature. The Gompertz equation was described by Gibson *et al.* [15].

$$Y=N_0+C*\exp(\exp((2.718*GR/C)*(LT-X)+1))$$

Y= Log cell number
X= Incubation time
N_0= Log initial number of cells
C= Difference between initial and final cell numbers
LT= Lag time before growth, same units as X
GR= Maximum growth rates

Secondary Modeling
Response surface model in terms of temperature, sodium chloride concentration, and pH was calculated on the growth rates. The Gompertz parameter for *S.* Typhimurium growth data was determined by the least squares analysis of PROC GLM of the SAS version 8.1 [35]. The response surface model was described by Gibson *et al.* [15].

$$\ln \text{Growth Rates}=b_0+b_1A+b_2B+b_3C+b_4A^2+b_5B^2+b_6C^2+b_7AB+b_8AC+b_9BC+\varepsilon$$

A= Incubation temperature
B= Initial pH
C= Sodium chloride concentration
b_0–b_9= Regression coefficients
ε= Random error

Evaluation of Model Performance
The coefficient of determination (r^2) provided by GraphPad [16] is often used as an overall measure of the prediction attained. It measures the fraction of the variation about the mean that is explained by a model.

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

$$MSE=(\sum \log(\text{predicted growth rates}/\text{observed growth rates})^2)/n$$

n=the number of observations

The bias factors (B_f) answers the question whether, on average, the observed values lie above or below the line equivalence and, if so, by how much. It gives the structural deviations of a model.

$$B_f=10^{(\sum \log(\text{predicted growth rates}/\text{observed growth rates})/n)}$$

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 observe.

$$A_f=10^{(\sum |\log(\text{predicted growth rates}/\text{observed growth rates})|/n)}$$

RESULTS AND DISCUSSION

Primary Modeling
Absorbance measurement is regarded as an alternative method of viable count measurement because the traditional viable count measurement for collecting the growth data is time-consuming and labor intensive, However, the absorbance measurement is typically used to determine only growth rates [10, 11, 25, 26, 43] because of its high detection levels, which make it difficult to measure the lag time [25]. Therefore, the absorbance measurement was used for the development of a predictive growth model for the growth rates of *S.* Typhimurium in TSB medium in the current study.

The growth rates of *S.* Typhimurim in the seven different pH levels of TSB medium in the presence of six different concentrations of NaCl at the incubation temperature of 10 and 20°C are shown in Table 1. No growth of *S.*

Table 1. Best-fit growth rates (GR) of *Salmonella* Typhimurium in TSB medium obtained from the Gompertz equation for the primary modeling.

T (°C)	NaCl (%)	pH	GR (h^{-1})	r^{2a}	T (°C)	NaCl (%)	pH	GR (h^{-1})	r^2
10	0	4	NG[b]	NA[c]	20	0	4	NG	NA
10	0	5	0.011	0.963	20	0	5	0.074	0.968
10	0	6	0.047	0.968	20	0	6	0.106	0.962
10	0	7	0.059	0.952	20	0	7	0.129	0.970
10	0	8	0.059	0.956	20	0	8	0.126	0.943
10	0	9	0.051	0.955	20	0	9	0.116	0.921
10	0	10	0.031	0.957	20	0	10	0.111	0.906
10	2	4	NG	NA	20	2	4	NG	NA
10	2	5	0.011	0.996	20	2	5	0.072	0.952
10	2	6	0.032	0.953	20	2	6	0.093	0.958
10	2	7	0.036	0.946	20	2	7	0.126	0.973
10	2	8	0.030	0.966	20	2	8	0.121	0.974
10	2	9	0.026	0.932	20	2	9	0.105	0.923
10	2	10	0.023	0.943	20	2	10	0.096	0.909
10	4	4	NG	NA	20	4	4	NG	NA
10	4	5	0.014	0.924	20	4	5	0.057	0.930
10	4	6	0.019	0.935	20	4	6	0.085	0.966
10	4	7	0.023	0.931	20	4	7	0.103	0.960
10	4	8	0.023	0.948	20	4	8	0.102	0.960
10	4	9	0.019	0.903	20	4	9	0.102	0.947
10	4	10	0.017	0.954	20	4	10	0.087	0.915
10	6	4	NG	NA	20	6	4	NG	NA
10	6	5	NG	NA	20	6	5	0.044	0.960
10	6	6	0.018	0.944	20	6	6	0.057	0.948
10	6	7	0.020	0.985	20	6	7	0.064	0.942
10	6	8	0.015	0.968	20	6	8	0.063	0.932
10	6	9	0.007	0.946	20	6	9	0.060	0.911
10	6	10	NG	NA	20	6	10	0.060	0.900
10	8	4	NG	NA	20	8	4	NG	NA
10	8	5	NG	NA	20	8	5	0.004	0.947
10	8	6	NG	NA	20	8	6	0.027	0.944
10	8	7	NG	NA	20	8	7	0.034	0.951
10	8	8	NG	NA	20	8	8	0.020	0.950
10	8	9	NG	NA	20	8	9	NG	NA
10	8	10	NG	NA	20	8	10	NG	NA
10	10	4	NG	NA	20	10	4	NG	NA
10	10	5	NG	NA	20	10	5	NG	NA
10	10	6	NG	NA	20	10	6	NG	NA
10	10	7	NG	NA	20	10	7	NG	NA
10	10	8	NG	NA	20	10	8	NG	NA
10	10	9	NG	NA	20	10	9	NG	NA
10	10	10	NG	NA	20	10	10	NG	NA

[a]r^2, coefficient of determination.
[b]NG, no growth.
[c]NA, no application.

Typhimurium was observed in the combination of all experimental variables of either 10% NaCl or pH 4 at the incubation of 10 and 20°C. At the incubation temperature of 10°C, the growth of *S.* Typhimurium was not observed in 8% NaCl of pH 5, 6, 7, 8, 9, and 10 of TSB medium and in 6% NaCl of pH 5 and 10 of TSB medium. At the incubation temperature of 20°C, the growth of *S.* Typhimurium was not observed in 8% NaCl of pH 9 and 10 of TSB medium. Therefore, the model development of the current study involved 50 growth curves conducted with 84 combinations of temperature, NaCl, and pH in TSB medium.

The Gompertz equation is typically used to fit bacterial growth curves for estimating lag time and maximum growth rates in the U. S. Department of Agriculture

(USDA) [2, 6, 7, 30]. Therefore, the current study used the Gompertz equation to fit growth curves for *S.* Typhimurium. Best-fit values of growth rates on 50 growth curves in the primary model were also shown in Table 1. The data of growth rates for TSB medium fitted the Gompertz equation model well, with a high degree of goodness of fits (r^2=0.900 to 0.996) at all treatment factors (Table 1).

Secondary Modeling

Salmonella enterica serovar Typhimurium (*S.* Typhimurium) is one of the most common serotypes among 21,449 known serotypes of the *Salmonella.* This serotype is also responsible for environmental contamination and the incidence of infections. Since *S.* Typhimurium is regarded as an important cause of foodborne salmonellosis in humans [3], there are several developed mathematical quantitative models of *S.* Typhimurium growth in predictive food microbiology. However, no predictive models have been constructed describing the effect of temperature in combination with NaCl and acidic to basic pH. Therefore, in the current study, the model development phase of this involved 50 growth curves conducted under 84 combinations of temperature, NaCl, and acidic to basic pH in TSB medium. The growth rates from these 50 growth curve fits were transformed to their natural logarithm to stabilize model variance [15] and were subjected to response surface analysis using SAS's general linear model. The following equation was given:

$$\ln \text{Growth Rate} = -0.2323 + (0.0046 * \text{Temp}) + (0.0617 * \text{pH}) + (0.0084 * \text{NaCl}) + (0 * \text{Temp}^2) + (-0.0042 * \text{pH}^2) + (-0.0010 * \text{NaCl}^2) + (0.0004 * \text{Temp} * \text{pH}) + (-0.0003 * \text{Temp} * \text{NaCl}) + (-0.0007 * \text{pH} * \text{NaCl})$$

r^2=0.960

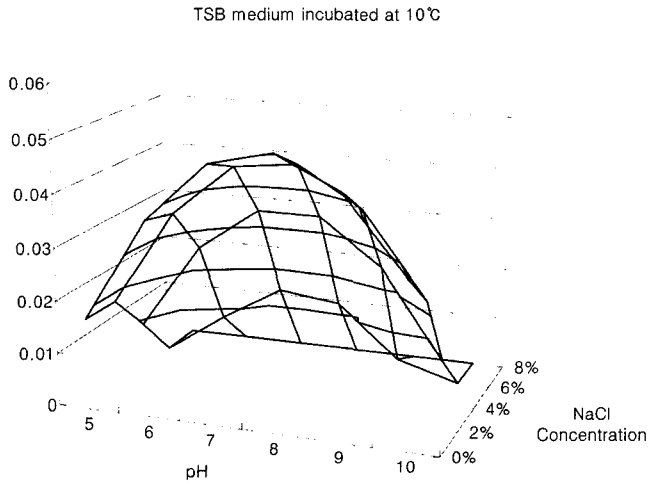

Fig. 1. Response surface models to predict growth rates of *Salmonella* Typhimurium in the presence of sodium chloride in different pH levels of TSB medium incubated at 10°C.

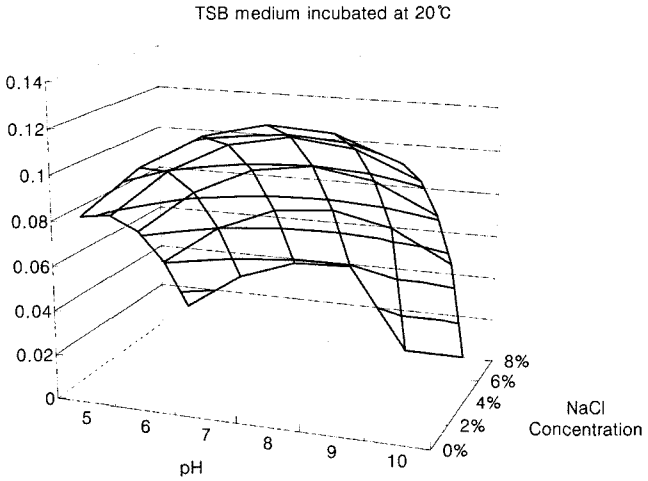

Fig. 2. Response surface models to predict growth rates of *Salmonella* Typhimurium in the presence of sodium chloride in different pH levels of TSB medium incubated at 20°C.

This equation estimated the predicted growth rate of *S.* Typhimurium in combinations of temperature, NaCl, and pH in TSB medium, shown in Figs. 1 and 2. When the overall main effects of NaCl concentrations or pH levels in TSB media incubated at 10 or 20°C were compared, the predicted growth rates of the *S.* Typhimurium were generally decreased by either basic (9, 10) or acidic (5, 6) pH levels and increase of NaCl concentrations (0–8%) (Figs. 1 and 2). However, the predicted growth rates of the *S.* Typhimurium was not inhibited in the presence of pH 8 compared with that of pH 7 of TSB medium incubated at 10 or 20°C. At the incubation of 10°C, the predicted growth rates of *S.* Typhimurum in the pH 5, 6, 7, 8, 9, and 10 were 0.014, 0.013, 0.026, 0.035, 0.036, and 0.028 (h^{-1}), respectively and the predicted growth rates of *S.* Typhimurum in 0, 2, 4, and 6% of NaCl were 0.038, 0.034, 0.023, and 0.010 (h^{-1}), respectively (data not shown). At the incubation of 20°C, the predicted growth rates of *S.* Typhimurum in the pH 5, 6, 7, 8, 9, and 10 were 0.055, 0.076, 0.088, 0.092, 0.101, and 0.089 (h^{-1}), respectively and the predicted growth rates of *S.* Typhimurum in 0, 2, 4, 6, and 8% of NaCl were 0.114, 0.104, 0.087, 0.061, and 0.029 (h^{-1}), respectively (data not shown). Although the effects of the incubation temperature for the predicted growth rates were not compared, the predicted growth rates in the combination of all experimental variables appeared to be generally less at the storage of 10°C than that of 20°C.

Evaluation of the Model Performance

Table 2 presents four different statistical indices of the secondary modeling step for the predicted growth rates of *S.* Typhimurium in TSB medium. The higher the value of r^2 ($0<r^2<1$), the better is the prediction by the model [13, 17, 39]. The lower the value of MSE, the better is the

Table 2. Statistical indices of the secondary response surface modeling step for growth rates of *Salmonella* Typhimurium in TSB medium.

Model	r^{2a}	MSE[b]	B_f[c]	A_f[d]
Response surface model	0.960	0.022	1.023	1.164

[a]r^2, coefficient of determination.
[b]MSE, mean square error.
[c]B_f, bias factor.
[d]A_f, accuracy factor.

adequacy of the model to describe the data [1, 39]. $B_f<1$ indicates a "fail safe" model [32]. $B_f>1$ indicates a "fail dangerous" model [32]. Ross [33] also noted that for models describing pathogen growth rates, B_f in the range 0.9–1.05 could be considered good, in the range 0.7–0.9 or 1.06–1.15 considered acceptable, and <0.7 or >1.5 considered unacceptable. The larger the value of A_f, the less accurate is the average estimate. An acceptable model that predicts the growth rates of *Listeria monocytogenes* as a function of temperature, NaCl, and pH could be expected to have A_f in the range 1.3–1.5 [31]. If there is a display of $A_f=B_f=1$, the predictive model is perfect.

Based on the above statement about four different statistical indices, our results indicated that the developed response surface model proved reliable for predictions of the combined effects of temperature, NaCl, and pH on the growth rates of *S.* Typhimurium in TSB medium.

However, for risk management, further work is necessary to confirm the prediction of the growth rate model for *S.* Typhimurium in food products. Furthermore, there is an urgent necessity in developing models of growth and death, and survival and transmission of *S.* Typhimurium occurred in diverse food matrices and food processing plants exposed to various environmental conditions. Therefore, the developed model of growth rates will reduce the uncertainty against *S.* Typhimurium in food production, processing, and distribution and thus will ensure food safety.

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