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ORIGINAL ARTICLE

Growth kinetics of a native toxigenic isolate of *Yersinia enterocolitica* CFR 2301 under the influence of incubation temperature, pH, sodium chloride and sodium nitrite

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Abstract Growth attributes such as lag phase duration (LPD) and growth rate (GR) estimates of a native toxigenic isolate of *Yersinia enterocolitica* CFR 2301 were studied in a broth medium under the influence of incubation temperature, pH, sodium chloride and sodium nitrite. The experimental growth curves derived from the central composite design based on 5 levels of factors namely incubation temperature (12–40 °C), pH level (5.5–8.5), sodium chloride (0.5–5.5 %) and sodium nitrite (0–200 ppm) were fitted using Baranyi and Gompertz functions, individually. With both these functions, the observed LPD values were similar ranging from 2.0 to 20.0 h and with GR values ranging from 0.1 to 0.5/h. The predicted values of LPD and GR estimates related well with observed values. The experimental culture broths with GR estimates of 0.4 and 0.5/h revealed positive phospholipase activity that is associated with the toxigenic trait of phospholipase A in the culture of *Y. enterocolitica* CFR 2301. The generated response surface plots for LPDs and GR estimates show a definite behavioral pattern under the influence of a combination of selected factors and variables. Higher LPDs and lower GR estimates as the indices of microbial safety could be of significance in developing effective food safety protocols in the food chain.

Keywords *Yersinia enterocolitica* · Lag phase duration · Growth rate · Baranyi function · Gompertz function · Quadratic responses

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Introduction

In the present scenario of food chain establishment, microbial safety is of major public health concern. Foodborne pathogenic bacterial species which occur in a diverse range of foods can result in health hazards, if the culture harbors toxigenic trait(s) and can elaborate toxin(s) in food system, if factors are favorable for the culture (Ramesh et al. 2003). Among the significant foodborne pathogenic bacterial species, *Yersinia enterocolitica* appears to be implicated in several foodborne outbreaks (Ackers et al. 2000; Sakai et al. 2005; Grahek-Ogden et al. 2007). There have been several studies focusing on the growth behavior of *Y. enterocolitica* under the influence of extrinsic and intrinsic factors, both in culture broth and food systems (Jones et al. 1994; Sutherland and Bayliss 1994; Bhaduri et al. 1994, 1995; Geer et al. 1995; Pin et al. 2000; Bozkurt and Erkmen 2001; Wei et al. 2001; Virto et al. 2005; Bari et al. 2011). The intrinsic factors that greatly influence microbial growth are pH, moisture content, oxidation-reduction potential (Eh), nutrients and antimicrobials. Most of the bacterial pathogens can grow best in the pH range of 6.5 to 7.5, although they can survive at extremes of pH 4 and 9. The important effect of lowering water activity below optimum is to increase the lag phase. The major constituents of food commodity as well as the presence of naturally occurring antimicrobials are known to influence microbial growth. The extrinsic factors affecting microbial growth are storage temperature, relative humidity and presence/concentration of gases in the environment. The storage temperatures of finished products influence bacterial species that could behave as psychrophiles, mesophiles and thermophiles. Few factors such as incubation temperature, pH, salt concentration and water activity are predominant in that they affect the growth of organisms (Buchanan et al. 1993; Duffy et al. 1999). However, in those bacterial organisms that can behave both as psychrophile and mesophile, time-temperature combinations become an important and deciding factor for the level

of microbial populations reached (Zwietering et al. 1994; Bari et al. 2011).

The relationship between growth curve parameters and combination of environmental factors are most frequently described by response surface methodology (RSM), wherein growth parameters are measured under a range of conditions and polynomial equation derived for the data. These equations can be used in computer programmes that allow the prediction of growth kinetics under untested conditions within the range of variables tested (Buchanan 1993; McKellar and Lu 2003). Growth models based on Gompertz and Baranyi functions for *Y. enterocolitica* in relation to temperature, pH, sodium chloride and sodium nitrite concentrations have been described in earlier studies (Adams et al. 1991; Little et al. 1992; Hudson 1993; Bhaduri et al. 1994; Guerzoni et al. 1994; Jones et al. 1994; Sutherland and Bayliss 1994; Pin et al. 2000). In the Indian scenario, few studies have revealed the prevalence of *Y. enterocolitica* and other *Yersinia* spp. in foods including traditional fast foods (Warke et al. 2000; Satishbabu and Rati 2003).

In an earlier study by the authors (Divya and Varadaraj 2011), it was established that *Y. enterocolitica* did occur in heat processed traditional food product of complex profile and this native isolate of *Y. enterocolitca* did harbor toxigenic traits. It is quite likely that the potential of such toxigenic culture to reach risk causing population levels under given time-temperature combinations would depend upon the lag phase duration and growth rate of the culture. Considering the significance of microbial safety in the food chain, the present study was aimed at determining lag phase duration (LPD) and growth rate (GR) estimates in a toxigenic culture of *Y. enterocolitica* in a broth medium across the range of selected cultural and nutritional parameters.

Materials and methods

All glasswares, media and other materials used in the present study were either wet or dry sterilized. Wet sterilization was carried out at 121 °C for 20 min in an autoclave and dry sterilization at 180 °C for 4 h in a Hot Air Oven. All bacteriological media used were those of dehydrated media procured from Hi-Media Lab., Mumbai, India. The media were prepared as per manufacturer's instructions. The water used in the experimental trials was Milli-Q water (A10 Elix 3, Millipore Corporation, Billerica, USA).

Bacterial culture and inoculum preparation This included a native toxigenic food isolate of *Y. enterocolitica* CFR 2301 that harbored toxigenic traits of regulator of virulence, mucoid Yersinia factor regulator, attachment invasion locus, heat stable enterotoxin, Yersinia type II secretory system and phospholipase A (Divya and Varadaraj 2011). The culture was maintained at 6 °C on brain heart infusion (BHI) agar slant in the Culture Collection Stock of this Department and propagated twice successively in BHI broth for 18 h at 30 °C prior to use in experimental trials. A loopful of previously propagated culture broth of *Y. enterocolitica* CFR 2301 was inoculated in to 10 ml aliquot of nutrient broth and incubated for 24 h at 30 °C in an orbital shaker incubator (Alpha Scientific Co., Bangalore, India) at 140 rpm. The culture broth was centrifuged at 8500 rpm for 20 min at 15 °C (Superspin R-V/F_M, Plasto Crafts, Mumbai, India). The supernatant was discarded and the resulting cell pellet was washed with saline and resuspended in sterile 10 ml aliquots of 0.85 % saline and stored in sterile screw-capped (25×125 mm) tubes at 4 °C till further use. Prior to use in the experimental trials, the cell titers were enumerated by surface plating of serial dilutions of the cell suspensions on pre-poured nutrient agar plates with incubation for 24 h at 30 °C. Appropriate serial dilution in sterile saline was used so as to obtain the final desired inoculum level of 2.3 $\log_{10}$ CFU/ml in aliquots of pH adjusted broth tubes.

Sodium chloride and sodium nitrite solutions and pH adjusted nutrient broth Requisite quantity of sodium chloride stock solution in Milli-Q water was prepared using an appropriate concentration of NaCl, from which aliquots when added to broth tubes gave the desired concentrations of NaCl as per the experimental design. Similarly, requisite quantity of sodium nitrite stock solution in Milli-Q water was prepared using an appropriate concentration of $NaNO_2$. The prepared stock solution was filter sterilized (0.22 μm membranes, Millipore, Bangalore, India). Aliquots of filter sterilized stock solution when added to sterilized broth tubes gave the desired concentrations of $NaNO_2$ as per the experimental design. Further, requisite numbers of tubes containing nutrient broth in aliquots of 10 ml were prepared with individual pH levels adjusted as desired in the experimental design.

Experimental design Keeping in line with the prevailing scenario of commercial food chain establishment, the range of individual factors in the experimental design was selected. The experimental design was a central composite design (CCD) based on 5 levels of factors namely incubation temperature (12–40 °C), pH level (5.5–8.5), sodium chloride (0.5–5.5 %) and sodium nitrite (0–200 ppm). The experimental design consisted of 31 treatment sets with 7 centre point repeats. Multiple tubes of pH adjusted nutrient broth (10 ml aliquots) with requisite levels of NaCl and $NaNO_2$ as per the experimental design were prepared. As 10 ml aliquot of nutrient broth contains 0.5 % NaCl, the requisite level was added from the prepared stock solution. The individual tubes were inoculated with aliquots of 0.1 ml inoculum of test culture to give a final concentration of 2.3 $\log_{10}$ CFU/ml and incubated

at desired temperatures of 10, 19, 26, 33 and 40 °C in a BOD Incubator (Sub-Zero, Industrial and Laboratory Tools Corporation, Chennai, India). Experimental culture broths were enumerated for the viable counts of inoculated *Y. enterocolitica* by surface plating in duplicate of 0.1 ml aliquots of appropriate serial dilutions on pre-poured nutrient agar plates at pre-determined intervals. Inoculated plates were incubated for 24–48 h at 30 °C and characteristic colonies of *Y. enterocolitica* appearing in the incubated plates were counted and expressed as average $\log_{10}$ CFU/ml.

Determination of lag phase duration and growth rate The derived average viable populations of *Y. enterocolitica* in CFU/ml obtained from the experimental trials were transformed to $\log_{10}$ values. At each combination of treatment variables, the log values were plotted against time (h) to obtain growth curves using DMFit curve fitting software programme version 2.0 (Institute of Food Research, Norwich, UK) as a function of Baranyi model (Baranyi and Roberts 1994) to determine lag phase duration (LPD) and growth rate (GR) values. Similarly, the data was also analyzed using Gompertz function, which was also available in the DMFit programme. This would give a comparative output.

Model development and response surface plots The generated LPD and GR values from the DMFit curve fittings were transformed to natural logarithms (Ln) and used to perform multiple regression analysis with Microsoft Excel Software Programme, 2010 (Microsoft Corporation, Redmond, WA, USA). Separate quadratic models for LPD and GR, respectively, were developed with Ln values and expressed as a quadratic function of incubation temperature, pH level, NaCl concentration and $NaNO_2$ concentration using the following equation:

$$\begin{aligned} LnR = {} & \mathbf{x}_1 + \mathbf{x}_2(T) + \mathbf{x}_3(P) + \mathbf{x}_4(C) + \mathbf{x}_5(N) + \mathbf{x}_6(T)^2 \\ & + \mathbf{x}_7(TxP) + \mathbf{x}_8(TxC) + \mathbf{x}_9(TxN) + \mathbf{x}_{10}(P)^2 \\ & + \mathbf{x}_{11}(PxC) + \mathbf{x}_{12}(PxN) + \mathbf{x}_{13}(C)^2 \\ & + \mathbf{x}_{14}(CxN) + \mathbf{x}_{15}(N)^2 + e \end{aligned}$$

Where R is any one of the growth parameters of Baranyi model (Gompertz function); $\mathbf{x}_n$ (n=1, 2, 3……..15) are the coefficients; T is the incubation temperature (C); P is the pH; C is the concentration of sodium chloride (%), N is the concentration of sodium nitrite (ppm) and e is the random error.

Statistical testing of the model was performed using analysis of variance (ANOVA), which was used to test the significance and adequacy of the model. The coefficient of determination (R^2) value near to 1.0 indicates a high degree of correlation between observed and predicted values and also the effectiveness of equation to derive model fits for predicting the growth behavior of *Y. enterocolitica* in terms of LPD and GR. Further, observed and predicted values were subjected to Chi-Square Test to assess the goodness of the fit, wherein value of 1.0 could establish a high degree of correlation between observed and predicted values.

The values of the coefficients were estimated by least square regression method of Bechmann et al. (1998). Statistical significance was determined based on Fischer (*F)* test. The value of significance of F (P<0.05) indicate that model terms are significant, whereas values greater than 0.10 indicate no significance. The feasibility to predict growth responses depends on these statistical indices (Duffy et al. 1994). The derived equations for LPD and GR were simplified by reducing the model parameters, which were not statistically significant (*21*). Three dimensional response surface plots were generated to depict the interaction of the dependent and independent variables. The effects of independent variables on LPD and GR were evaluated using these three-dimensional plots obtained by imposing a constant value to one variable at a time.**Assay for phospholipase activity** This assay was carried out as a qualitative related attribute of the toxigenic trait of phospholipase A in the culture of *Y. enterocolitica*. Individual culture broth tubes of the experimental design were assayed for phospholipase activity by spot inoculation on nutrient agar plates containing 0.2 % egg yolk. Incoulated plates were incubated for 24 h at 30 °C. Phospholipase production was indicated by a zone of precipitation around the growth area of respective spots in the inoculated agar plates.

Statistical analysis All the experimental trials were carried out independently, in triplicates and the mean values were presented after carrying out requisite statistical analysis. All calculations and statistical analyses were performed in Microsoft Excel Programme, 2010 (Microsoft Corporation, Redmond, WA, USA).

Results and discussion

Lag phase duration and growth rate in Y. enterocolitica Limited studies have focused on the survival of microbial contaminants to different pasteurization temperatures as well as metabolic and kinetic aspects of a yeast in relation to temperature effect (Dumalisile et al. 2005; Brandam et al. 2008). Although the public health significance of *Y. enterocolitica* is high, only limited studies relate to the behavior of this bacterial species under probable conditions of those factors and variables, which tend to prevail in a food chain specifically of traditional foods. Reliable criteria of intrinsic and extrinsic factors as effective combination of factors need to be evaluated in terms of behavior of microbial pathogens as related to the food matrices through RSM studies (Heo et al. 2009).

The observed and predicted LPD and GR estimates based on Baranyi and Gompertz functions are presented in Tables 1 and 2, respectively. The observed LPD values based on Baranyi function ranged from 2.0 to 17.8 h. The lowest of 2.0 h was recorded with the treatment set of incubation temperature of 33 °C, pH of 6.25, NaCl level of 4.25 % and $NaNO_2$ of 50 ppm, while the highest of 17.8 h was observed with 26 °C, 7.0 pH, 5.5 % NaCl and 100 ppm $NaNO_2$. The same LPD values derived by Gompertz function revealed the range from 2.0 to 20.0 h. However, the lowest of 2.0 h was with treatment set of 26 °C, pH of 7.0, NaCl of 3 % and $NaNO_2$ of 100 ppm and the highest of 20.0 h were with the same variables of pH, NaCl and $NaNO_2$, except for the incubation temperature of 12 °C. The predicted LPD values did relate well with the observed values (Fig. 1). In the case of GR estimates of *Y. enterocolitica*, both with Baranyi and Gompertz functions, the lowest and highest observed estimates ranged from 0.1 to 0.5/h (Table 2). Both the lowest and highest GR estimates were obtained with different treatment sets. Similarly, predicted GR estimates did relate well with observed estimates (Fig. 2).

Many of the model parameters were not statistically significant based on the *P* values. The relevant coefficients for LPD and GR estimates in *Y. enterocolitica* CFR 2301 are shown in Tables 3 and 4, respectively. The R^2 values of 0.87 and 0.88 obtained for LPD based on the models of Baranyi and

Table 1 Observed and predicted lag phase duration of *Yersinia enterocolitica* CFR 2301 based on Baranyi and Gompertz functions

Factors / Variables				Baranyi function		Gompertz function	
Temp (°C)	pH level	NaCl conc. (%)	$NaNO_2$ conc. (ppm)	Observed LPD (h)±SE	Predicted	Observed LPD (h)±SE	Predicted
19	6.25	1.75	50	6.3±0.8	6.7	7.7±0.5	8.0
33	6.25	1.75	50	2.8±0.6	2.3	3.5±0.4	2.9
19	7.75	1.75	50	4.9±0.6	3.5	6.4±0.5	4.6
33	7.75	1.75	50	2.5±0.4	2.1	3.3±0.5	2.7
19	6.25	4.25	50	9.6±1.8	9.6	8.4±2.5	8.8
33	6.25	4.25	50	2.0±0.9	2.7	2.8±1.3	3.3
19	7.75	4.25	50	8.5±1.7	10.0	7.5±2.0	9.7
33	7.75	4.25	50	5.9±0.7	5.2	7.1±0.8	6.0
19	6.25	1.75	150	7.6±0.9	8.2	8.8±0.5	9.2
33	6.25	1.75	150	3.7±0.5	2.8	4.1±0.4	3.2
19	7.75	1.75	150	5.6±1.0	3.6	5.7±1.6	4.8
33	7.75	1.75	150	2.3±0.6	2.2	3.2±0.3	2.7
19	6.25	4.25	150	10.6±1.3	11.0	10.2±2.0	12.6
33	6.25	4.25	150	2.3±0.8	3.1	3.8±0.9	4.6
19	7.75	4.25	150	8.6±3.2	10.0	12.0±3.4	12.7
33	7.75	4.25	150	6.3±0.7	5.1	7.6±0.6	7.5
12	7.0	3.0	100	14.7±3.3	15.6	20.0±2.6	17.5
40	7.0	3.0	100	2.4±0.8	2.7	3.0±1.0	3.8
26	5.5	3.0	100	5.6±1.2	4.4	6.9±1.6	5.7
26	8.5	3.0	100	2.5±1.2	3.7	4.0±1.3	5.4
26	7.0	0.5	100	2.1±0.5	3.6	2.5±0.5	4.2
26	7.0	5.5	100	17.8±2.7	11.9	19.4±3.6	12.6
26	7.0	3.0	0	2.4±0.5	2.5	2.4±0.9	2.6
26	7.0	3.0	200	2.7±0.5	3.0	3.8±0.5	3.8
26	7.0	3.0	100	2.5±0.6	2.5	3.2±0.6	2.8
26	7.0	3.0	100	2.4±0.5	2.5	2.5±0.8	2.8
26	7.0	3.0	100	2.6±0.6	2.5	2.8±0.9	2.8
26	7.0	3.0	100	2.6±0.9	2.5	3.1±0.6	2.8
26	7.0	3.0	100	2.5±0.5	2.5	2.9±0.5	2.8
26	7.0	3.0	100	2.4±0.5	2.5	3.2±0.7	2.8
26	7.0	3.0	100	2.5±0.7	2.5	2.0±1.2	2.8

Table 2 Observed and predicted growth rate estimates of *Yersinia enterocolitica* CFR 2301 based on Baranyi and Gompertz functions

Factors / Variables				Baranyi function		Gompertz function	
Temp (°C)	pH level	NaCl conc. (%)	$NaNO_2$ conc. (ppm)	Observed GR (/h)±SE[a]	Predicted	Observed	Predicted
19	6.25	1.75	50	0.2	0.2	0.3	0.2
33	6.25	1.75	50	0.4	0.4	0.5	0.4
19	7.75	1.75	50	0.2	0.2	0.2	0.2
33	7.75	1.75	50	0.4	0.4	0.4	0.4
19	6.25	4.25	50	0.1	0.1	0.1	0.1
33	6.25	4.25	50	0.2	0.2	0.2	0.2
19	7.75	4.25	50	0.1	0.1	0.1	0.1
33	7.75	4.25	50	0.2	0.2	0.2	0.2
19	6.25	1.75	150	0.2	0.2	0.2	0.2
33	6.25	1.75	150	0.4	0.3	0.4	0.4
19	7.75	1.75	150	0.1	0.1	0.1	0.2
33	7.75	1.75	150	0.4	0.4	0.4	0.4
19	6.25	4.25	150	0.1	0.1	0.1	0.1
33	6.25	4.25	150	0.2	0.2	0.2	0.2
19	7.75	4.25	150	0.1	0.1	0.1	0.1
33	7.75	4.25	150	0.2	0.2	0.2	0.2
12	7.0	3.0	100	0.1	0.1	0.1	0.1
40	7.0	3.0	100	0.3	0.3	0.3	0.4
26	5.5	3.0	100	0.1	0.1	0.1	0.1
26	8.5	3.0	100	0.1	0.1	0.2	0.1
26	7.0	0.5	100	0.4	0.4	0.4	0.5
26	7.0	5.5	100	0.1	0.1	0.1	0.1
26	7.0	3.0	0	0.2	0.3	0.2	0.3
26	7.0	3.0	200	0.2	0.2	0.2	0.2
26	7.0	3.0	100	0.2	0.2	0.2	0.2
26	7.0	3.0	100	0.2	0.2	0.2	0.2
26	7.0	3.0	100	0.2	0.2	0.2	0.2
26	7.0	3.0	100	0.2	0.2	0.2	0.2
26	7.0	3.0	100	0.2	0.2	0.2	0.2
26	7.0	3.0	100	0.2	0.2	0.2	0.2
26	7.0	3.0	100	0.2	0.2	0.2	0.2

In all the experimental treatments, SE was±0.0

Gompertz functions, respectively, indicate a fairly reasonable degree of correlation between observed and predicted values. Based on the value of linear coefficients and associated *P* value ($P<0.05$) with both Baranyi and Gompertz functions, it appears that LPD was more influenced by temperature and pH level, followed by sodium chloride concentrations. Similarly, the R^2 for GR estimates based on Baranyi and Gompertz functions was 0.93. With Baranyi function, the primary influencing factor appeared to be incubation temperature, while with Gompertz function, the linear coefficient of pH alone was significant and hence was the primary influencing factor on GR in *Y. enterocolitica* CFR 2301. Further, results of Chi Square test (0.99 to 1.0) showed a goodness of the fit and a high degree of correlation between observed and predicted values for both LPD and GR with both functions (data not shown).

The individual culture broth tubes assayed for phospholipase activity revealed positive activity in the plates with those broth tubes that had GR estimates of 0.4 and 0.5/h (Fig. 3). This activity was not observed with LPDs and also GR estimates of less than 0.4/h.

The factors and variables included in this study have been more or less the same with those of earlier studies (Adams et al. 1991; Hudson 1993; Bhaduri et al. 1994;

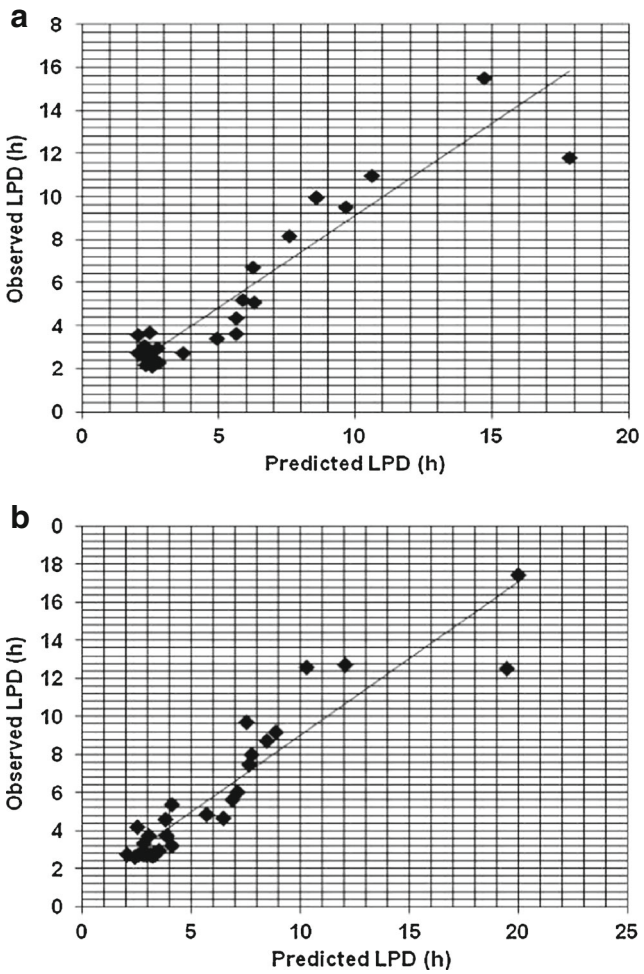

Fig. 1 Comparison of the observed and predicted LPD values of *Yersinia enterocolitica* CFR 2301 in broth system under various pH levels, incubation temperatures, NaCl and $NaNO_2$ concentrations as derived with Baranyi (**a**) and Gompertz (**b**) functions

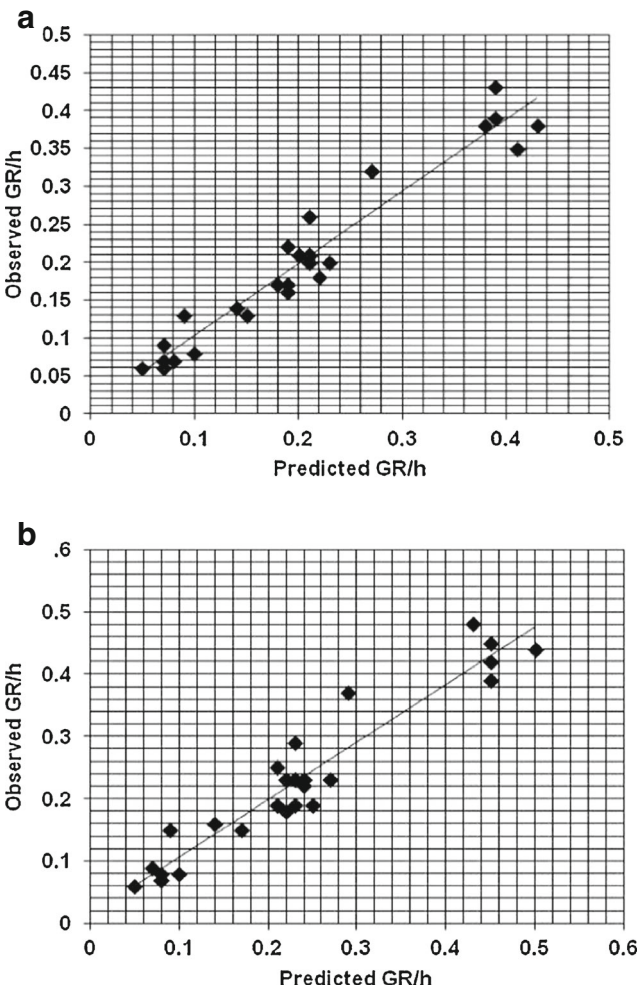

Fig. 2 Comparison of the observed and predicted GR estimates *of Yersinia enterocolitica* CFR 2301 in broth system under various pH levels, incubation temperatures, NaCl and $NaNO_2$ concentrations as derived with Baranyi (**a**) and Gompertz (**b**) functions

Sutherland and Bayliss 1994). These studies had used Gompertz parameters and its derivatives. In the present study, both LPD and GR estimates (Tables 1 and 2) were derived using Baranyi and Gompertz functions, wherein the values obtained with Gompertz function were slightly higher. The Gompertz curve has a definite curvature around the inflection point of the sigmoidal growth curve, which causes too high estimates of the maximum specific growth rate and lag times (Baranyi et al. 1993). Further, modification of Gompertz equation has been used to model non-linear survival curves in foodborne bacterial pathogens (Linton et al. 1995). Earlier studies with foodborne bacterial pathogens have shown that growth related values obtained with Gompertz and logistic models were more or less same, but differed from those estimated by Baranyi model (Perni et al. 2005). Further, Baranyi function has given better parameter estimates as against over-estimated specific GR values by Gompertz function (Membré et al. 1999). Suitability of mathematical functions for microbial growth curves of *Y. enterocolitica* under the influence of pH, temperature and carbon dioxide revealed that Baranyi and other models had better ability to fit experimental values (López et al. 2004).

The GR and LPD values obtained in the present study were comparable with a few of the earlier studies (Bhaduri et al. 1994; Sutherland and Bayliss 1994). The model of Adams et al. (1991) dealt with growth of *Y. enterocolitica* in tryptone soy broth (TSB) and GR was defined in terms of time to a given value of absorbance. As such, the findings could not be related with the present experimental trials. Similarly, the model proposed in the studies of Hudson (1993) was based on turbidity measurements that could not be related with

Table 3 Coefficients derived by regression analysis for the LPD values of *Yersinia enterocolitica* CFR 2301 based on Baranyi and Gompertz functions

Source	Baranyi function Coefficient	SE	*P*	Gompertz function Coefficient	SE	*P*
Intercept	25.34	6.37	0.001	29.54	5.94	0.0001
T	−0.50	0.12	0.001	−0.49	0.11	0.0006
P	−4.24	1.52	0.01	−5.34	1.42	0.002
C	−1.86	0.67	0.01	−2.04	0.63	0.005
N	0.007	0.01	0.69	0.002	0.01	0.91
T^2	0.005	0.001	0.0007	0.005	0.001	0.0001
T x P	0.03	0.01	0.07	0.023	0.01	0.11
T x C	−0.005	0.009	0.57	0.001	0.01	0.87
T x N	−0.00001	0.0002	0.95	−0.00003	0.0002	0.87
P^2	0.21	0.10	0.05	0.301	0.09	0.006
P x C	0.19	0.08	0.03	0.17	0.08	0.038
P x N	−0.001	0.002	0.64	−0.0006	0.002	0.75
C^2	0.15	0.04	0.0007	0.15	0.03	0.0004
C x N	−0.0002	0.0001	0.85	0.0009	0.001	0.44
N^2	−0.00001	0.00002	0.68	0.00001	0.00002	0.56
R^2	0.87			0.88		

T incubation temperature (°C), *P* pH level, *C* NaCl concentration (%), *N* $NaNO_2$ concentration (ppm), R^2 Coefficient of determination

the viable counts used as parameters to determine LPD and GR values in this study.

Although, the range of factors tested in few of the earlier studies were slightly different from those used in the present study, a comparison was possible with regard to the derived values. The predicted GR and LPD values obtained for combinations of treatment variables in this study related well with those observed by earlier research investigators. Further, as it could be seen in the present study, it is unreliable to make predictions outside the matrix of conditions used in the preparation of the models (Bhaduri et al. 1994; Sutherland and Bayliss 1994; Sutherland et al. 1995). However, in some treatment sets, wherein the temperature and/or salt concentrations were near extreme, the LPD values were slightly higher in the model derived in study of Bhaduri et al. (1994) that appeared to be similar to the values obtained in the present experimental model.

Attempts to fit the data of present model to combinations that fall outside the range of present study showed no correlation between the observed and predicted values. A similar observation was also reported in earlier studies while attempting to predict growth parameters outside the matrix of experimental range. Therefore, it is unreliable to make predictions outside the matrix of conditions used in the preparation of the model (Bhaduri et al. 1994; Sutherland and Bayliss 1994).

Further in this study, visualization of phospholipase activity (related to toxigenic trait of phospholipase A) in *Y. enterocolitica* grown culture broths with GR estimates of 0.4 and 0.5/h did indicate the probable risk that could be associated in the food chain under favorable conditions of extrinsic and intrinsic factors. The associated toxigenic trait was being observed with just lower GR estimates need to be viewed with serious concern as potential health hazards could occur as and when favorable factors exist in the food chain.

Response surface plots for LPD and GR estimates based on Baranyi and Gompertz functions In the present study, response surface plots were derived for LPDs and GR estimates of *Y. enterocolitica* under the influence of incubation temperature, pH levels and concentrations of NaCl and $NaNO_2$, which were based on Baranyi and Gompertz functions. The coefficients derived by multivariate analysis were utilized to generate response surface plots for LPD and GR estimates in *Y. enterocolitica* CFR 2301 as a function of varying NaCl levels and incubation temperatures. The derived quadratic responses for LPD and GR, respectively, were as follows:

LPD with Baranyi function

$$\begin{aligned} Ln\,LPD = {} & 25.34 - 0.50T - 4.24P - 1.86C + 0.007N + 0.005T^2 \\ & + 0.03TxP - 0.005TxC - 0.00001TxN + 0.21P^2 \\ & + 0.19PxC - 0.001PxN + 0.15C^2 - 0.0002CxN \\ & - 0.00001N^2 \end{aligned}$$

Table 4 Coefficients derived by regression analysis for the GR estimates of Yersinia enterocolitica CFR 2301 based on Baranyi and Gompertz functions

Source	Baranyi function			Gompertz function		
	Coefficient	SE	*P*	Coefficient	SE	*P*
Intercept	−9.87	4.16	0.03	−8.78	4.36	0.06
T	0.09	0.08	0.29	0.078	0.08	0.38
P	2.33	1.0	0.03	2.15	1.04	0.05
C	−0.57	0.44	0.21	−0.68	0.46	0.16
N	−0.01	0.01	0.41	−0.01	0.01	0.37
T^2	−0.002	0.00	0.01	−0.002	0.00	0.01
T x P	0.009	0.01	0.35	0.01	0.01	0.32
T x C	0.005	0.00	0.44	0.006	0.01	0.31
T x N	0.00009	0.00	0.52	0.00009	0.00	0.57
P^2	−0.2	0.07	0.01	−0.18	0.07	0.02
P x C	0.02	0.05	0.65	0.031	0.06	0.60
P x N	0.0003	0.00	0.82	0.0004	0.00	0.80
C^2	−0.009	0.02	0.72	−0.01	0.02	0.65
C x N	0.0002	0.00	0.83	0.0004	0.00	0.61
N^2	0.00002	1.51 E-05	0.32	0.00002	1.58 E-05	0.31
R^2	0.93			0.93		

T incubation temperature (°C), *P* pH level, *C* NaCl concentration (%), *N* $NaNO_2$ concentration (ppm), R^2 Coefficient of determination

LPD with Gompertz function

$$\begin{aligned} Ln\,LPD &= 29.54 - 0.49T - 5.34P - 2.04C + 0.002N \\ &+ 0.005T^2 + 0.023TxP + 0.001TxC - 0.00003TxN \\ &+ 0.301P^2 + 0.17PxC - 0.0006PxN + 0.15C^2 \\ &+ 0.0009CxN + 0.00001N^2 \end{aligned}$$

GR with Baranyi function

$$\begin{aligned} Ln\,GR &= -9.87 + 0.09T + 2.33P - 0.57C - 0.01N \\ &- 0.002\left(T^2\right) + 0.009TxP + 0.005TxC + 0.00009TxN \\ &- 0.2P^2 + 0.02PxC + 0.0003PxN - 0.009C^2 \\ &+ 0.0002CxN + 0.00002N^2 \end{aligned}$$

GR with Gompertz function

$$\begin{aligned} Ln\,GR &= -8.78 + 0.078T + 2.15P - 0.68C - 0.01N \\ &- 0.002\left(T^2\right) + 0.01TxP + 0.006TxC + 0.00009TxN \\ &- 0.18P^2 + 0.031PxC + 0.0004PxN - 0.01C^2 \\ &+ 0.0004CxN + 0.00002N^2 \end{aligned}$$

Individual response surface plots for LPDs and GR estimates with Baranyi and Gompertz functions at defined concentrations of NaCl and incubation temperatures over different pH levels and $NaNO_2$ concentrations were generated and only representative surface plots are presented in this study.

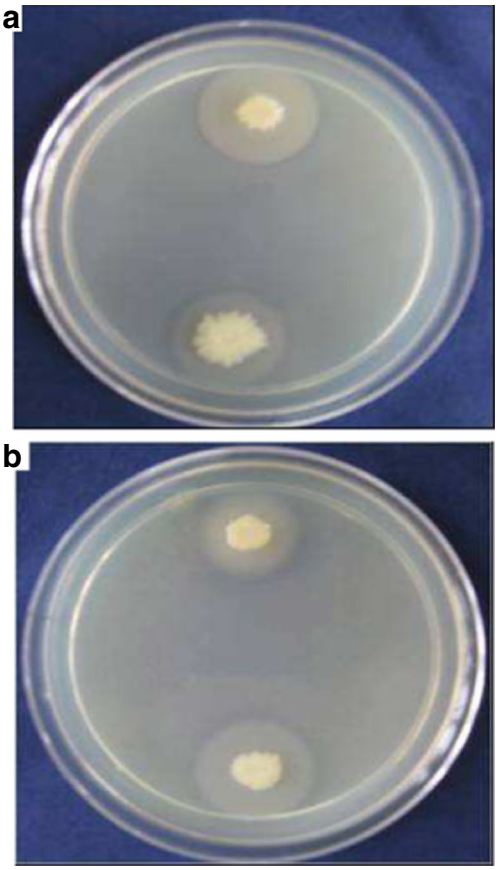

Fig. 3 Phospholipase activity of *Yersinia enterocolitica* CFR 2301 in representative culture broths with GR estimates of 0.4/h (**a**) and 0.5/h (**b**) in 0.2 % egg yolk containing nutrient agar plates

At a defined level of 0.5 % NaCl, the generated LPD values over the range of pH and $NaNO_2$ levels at 3 different incubation temperatures appeared to have certain relationship with the growth behavior of *Y. enterocolitica* CFR 2301 under varied cultural conditions. At 12 °C, the highest LPD of 18.5 h was observed with 200 ppm $NaNO_2$ and pH 5.5, while the lowest of 7.6 h was at pH 8.5 and 100 ppm $NaNO_2$. With incubation temperatures of 26 and 40 °C, lower LPD values were generated. An initial decrease, followed by increase over increasing pH levels was more prominent at 40 °C. The response surface plot pattern appeared to be different at 3 incubation temperatures with 2.5 % NaCl concentration. At 12 °C, the trend was similar to that observed with 0.5 % NaCl, except for lower LPD values. The pattern visualized at 26 and 40 °C was almost same. A decreasing trend in LPDs occurred between pH 5.5 and 7.5 with $NaNO_2$ levels of 0–200 ppm, followed by an increase till pH 8.5. The generated response surface plots at 5.5 % NaCl and incubation temperatures of 12, 26 and 40 °C revealed a varied growth behavior (Fig. 4). At 12 °C, all the LPD values obtained were higher than those

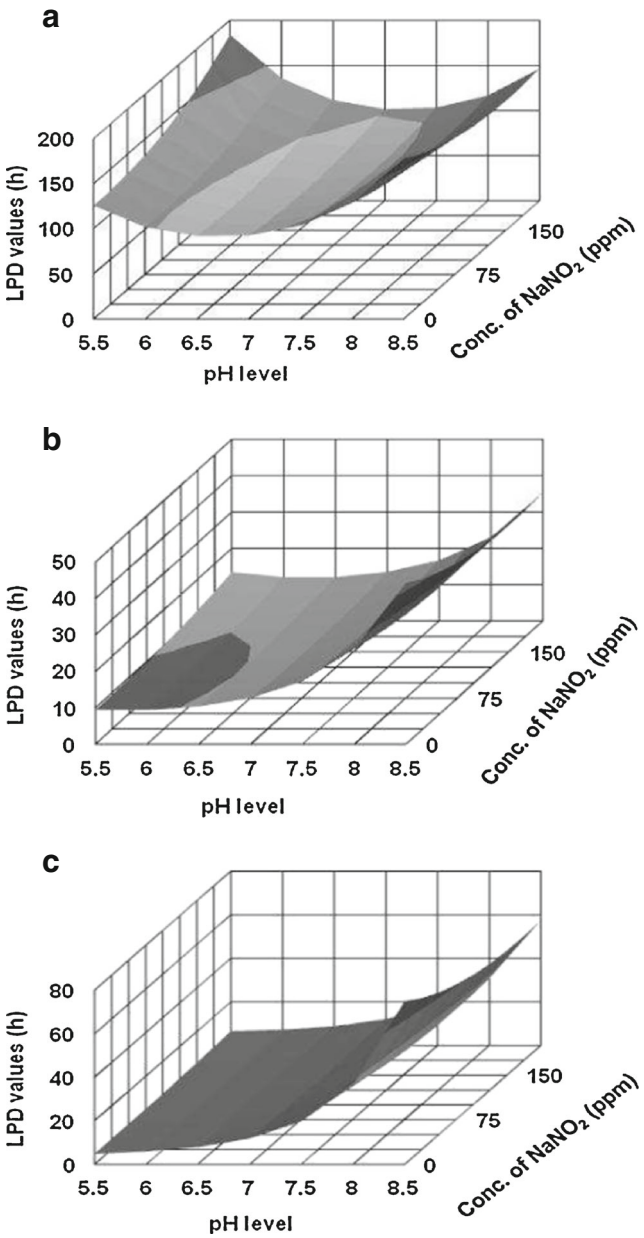

Fig. 4 Baranyi function based response surface plots for LPD values of *Yersinia enterocolitica* CFR 2301 under the influence of 5.5 % NaCl at individual incubation temperatures of 12 °C (**a**), 26 °C (**b**) and 40 °C (**c**)

obtained at 26 and 40 °C. At 26 °C, the LPD values at pH 5.7–7.0 were higher than that at 40 °C. However, at higher pH levels of 7.5 to 8.5, the LPD values were higher at 40 °C as against that of 26 °C.

The LPDs based on Gompertz function were higher than those values obtained with Baranyi function. At 0.5 % NaCl and incubation temperatures of 12 °C, the increasing $NaNO_2$ levels resulted in a decreasing pattern of LPDs, followed by an increasing pattern based on individual pH levels and $NaNO_2$ concentrations. A similar pattern to the above was visualized with incubation temperatures of 26 and 40 °C, except for lower LPD values. However, the limiting level of $NaNO_2$ was higher than that observed with Baranyi model. With 2.5 % NaCl, 12 °C incubation temperature revealed an initial decrease in LPD values over the range of 0–125 ppm of $NaNO_2$ levels and pH 5.5 to 7.5, followed by an increase. At $NaNO_2$ levels of 150–200 ppm, the initial decrease was observed till pH 8.0. A similar pattern with lower LPDs was evident at 26 °C. At 40 °C, over levels of 0–75 ppm of $NaNO_2$, the LPDs decreased marginally and later increased till 200 ppm. In contrast, with 5.5 % NaCl (Fig. 5), at 12 °C, LPDs generated under all pH conditions over the range of 0–100 ppm $NaNO_2$ were lower than that derived by Baranyi function. However, with increasing $NaNO_2$ levels, there was a progressive increase with all pH levels. The

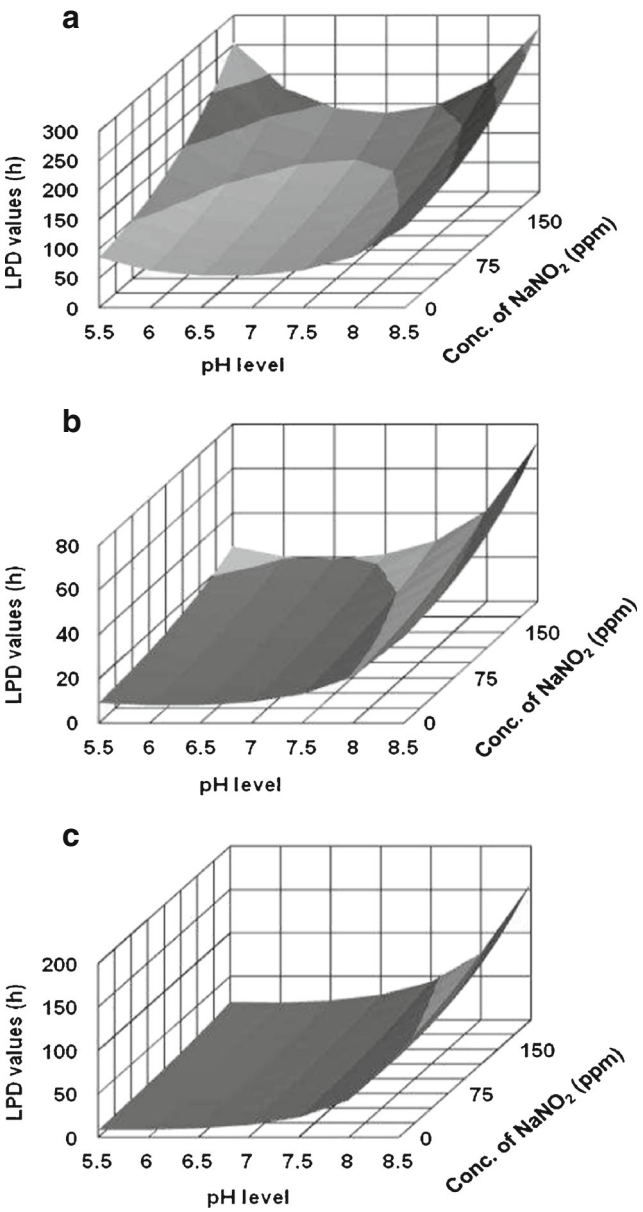

Fig. 5 Gompertz function based response surface plots for LPD values of *Yersinia enterocolitica* CFR 2301 under the influence of 5.5 % NaCl at individual incubation temperatures of 12 °C (**a**), 26 °C (**b**) and 40 °C (**c**)

pattern was same at 26 °C, except for lower values. At 40 °C, the LPDs revealed a progressive increasing pattern from pH 5.5 to 8.5 and also over the range of $NaNO_2$ levels of experimental design.

The response surface pattern for GR estimates based on Baranyi function appeared more or less same with minor differences at incubation temperatures of 12, 26 and 40 °C with 0.5 % NaCl concentration. At 12 °C, GR estimates decreased with increasing $NaNO_2$ levels, except for a marginal increase at pH 7.5–8.5 with 175–200 ppm $NaNO_2$. At 26 and 40 °C, GR estimates were higher than those visualized at 12 °C. With increasing $NaNO_2$ concentrations, GR estimates showed an initial decrease, followed by an increase at different pH and $NaNO_2$ levels. The response surface plots derived with 2.5 % NaCl (Fig. 6) exhibited a comparable pattern at 3 incubation temperatures. In case of 12 °C, individual $NaNO_2$ concentrations, GR estimates increased at pH levels of 5.5–6.5, followed by a decrease at 6.5–8.5. At 26 °C, with individual $NaNO_2$ concentrations, the GR estimates showed an initial increase till pH 7.0, followed by a decrease in the range of pH 7.0–8.5. At 40 °C, with individual pH levels, GR estimates showed an initial decrease, followed by an increase over increasing levels of $NaNO_2$. The response surface pattern with 5.5 % NaCl was quite different. Lower GR estimates were evident at 12 °C.

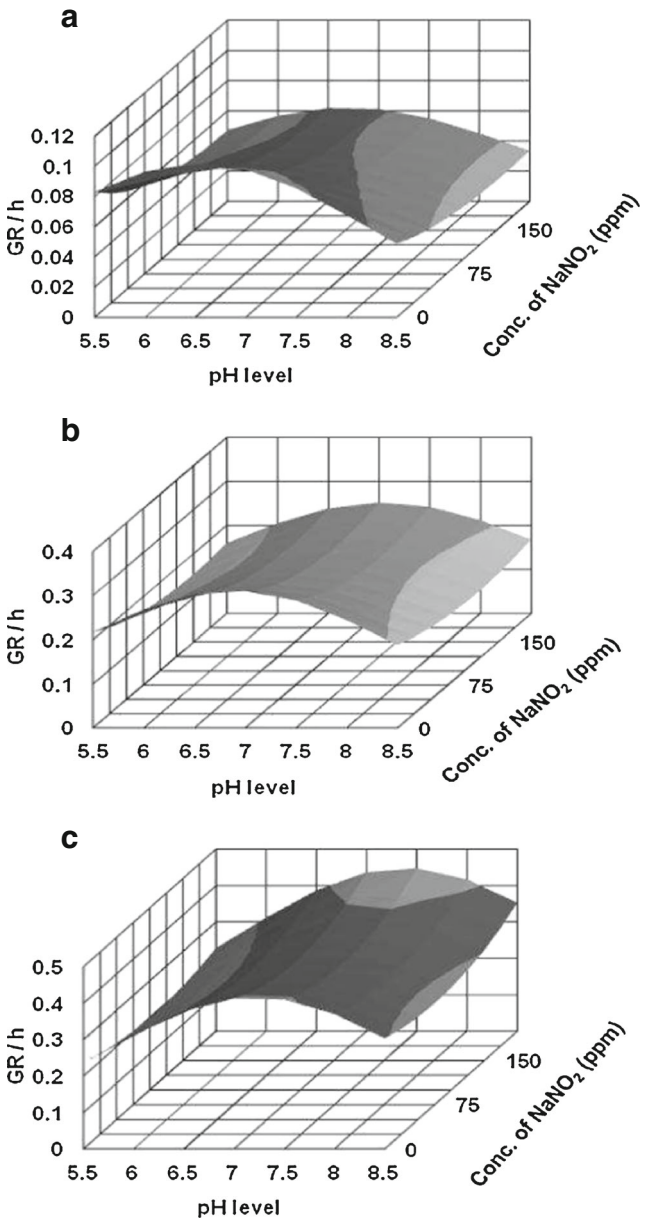

Fig. 6 Baranyi function based response surface plots for GR estimates of *Yersinia enterocolitica* CFR 2301 under the influence of 2.5 % NaCl at individual incubation temperatures of 12 °C (**a**), 26 °C (**b**) and 40 °C (**c**)

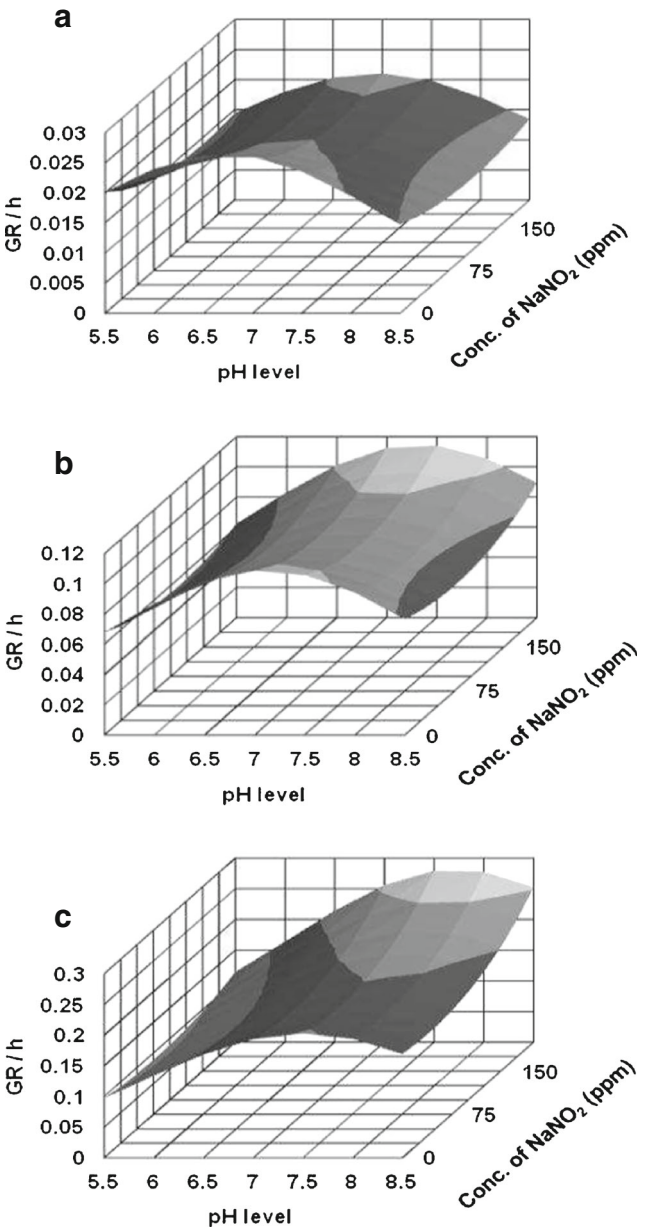

Fig. 7 Gompertz function based response surface plots for GR estimates of *Yersinia enterocolitica* CFR 2301 under the influence of 5.5 % NaCl at individual incubation temperatures of 12 °C (**a**), 26 °C (**b**) and 40 °C (**c**)

Considering individual pH levels, GR estimates showed an initial decrease, followed by an increase over increasing $NaNO_2$ levels. At 26 °C, the GR estimates were higher than visualized at 12 °C. Irrespective of $NaNO_2$ concentrations, GR estimates exhibited an initial increase in the pH range of 5.5–7.0, followed by a decrease till pH 8.5. The incubation temperature of 40 °C revealed still higher GR estimates. With individual pH levels of 5.5–6.0 and increasing $NaNO_2$ concentrations, GR estimates showed an initial decrease till 100 ppm, followed by an increase till 200 ppm.

The response surface plots derived for GR estimates by Gompertz function revealed a similar pattern to that of Baranyi function, except for higher values. With 0.5 % NaCl, at 12 °C, GR estimates revealed an initial increase at pH levels of 5.5–6.5, followed by a decrease with all levels of $NaNO_2$. At 26 and 40 °C, GR estimates were higher than those observed at 12 °C. With lower pH levels of 5.5–6.5, GR values decreased till 175 ppm of $NaNO_2$ level, followed by an increase at 200 ppm. In case of 40 °C, with higher levels of $NaNO_2$ (175–200 ppm), the increase in GR estimates extended till pH 7.5. The pattern obtained with 2.5 % NaCl and incubation temperature of 12 °C revealed a decreasing pattern of GR estimates over pH range of 6.0–7.5 till 175 ppm of $NaNO_2$, followed by an increase. Both at 26 and 40 °C, the pattern of variation in GR estimates was similar to that visualized with Baranyi function. However, with 5.5 % NaCl (Fig. 7), a contrasting pattern was visualized at 3 incubation temperatures. At 12 °C, most of the GR estimates were lower than that derived by Baranyi function. With pH levels of 5.5–6.5, an initial decrease was observed till 150 ppm $NaNO_2$, followed by an increase till 200 ppm. At 26 and 40 °C, GR estimates were higher than those derived by Baranyi function. At 26 °C, an initial decrease was evident till $NaNO_2$ levels of 125 ppm with pH of 5.5–6.0 and 100 ppm with pH 6.0–7.5. At 40 °C, with pH range of 5.5–6.5, the initial decrease in GR estimates extended till 75 ppm of $NaNO_2$, while in pH range of 7.5–8.5, the initial decrease was only till 50 ppm of $NaNO_2$, followed by an increase till 200 ppm.

Although LPD and GR values are the two attributes of a growth phase, the behavior of this native culture of *Y. enterocolitica* CFR 2301 may be related to the inherent characteristics. Similar variable behavior was observed in one of the earlier studies, wherein of the 3 strains of *Y. enterocolitica*, only 2 could survive and grow in tryptic soy broth at pH 4.5 and 25 °C only (Brackett 1986). Similar to this study, a mathematical equation was used in combination with second order polynomials to fit response surface data relating to growth, survival and death of *Y. enterocolitica* as affected by temperature, NaCl, pH and undissociated lactic acid. The resulting predictive model revealed a good correlation within the experimental design. In a few earlier studies, response surface models for *Y. enterocolitica* were derived to predict growth under fixed pH level and concentrations of NaCl and $NaNO_2$ and varying temperatures (Jones et al. 1994; Avery et al. 1996).

It appears that the behavioral pattern of *Y. enterocolitica* is dependent upon several factors, besides the inherent attributes of individual isolates. The response surface plots generated in this experimental study reveal a definite behavioral pattern under the influence of a combination of factors and its variables. The LPD values visualized indicate an appreciable range in their values, which could either lead to increase / decrease in cell numbers. In one of the earlier studies, the cell population of *Y. enterocolitica* in TSB showed a decreasing pattern with lower pH levels at 25 °C. Further at 5 °C, no growth could be visualized for any pH levels tested (*11*). The culture of *Y. enterocolitica* being a psychrophilic and also able to grow well at 25 °C gives an indication that it is not always true for an organism to behave in accordance with even the taxonomically established and documented cultural characteristics.

Conclusion

The findings of this study indicate that, both Baranyi and Gompertz functions could give meaningful leads towards understanding LPDs and GR estimates as attributes in the growth behavior of *Y. enterocolitica* under defined set of factors and variables such as incubation temperature, pH, NaCl and $NaNO_2$. The generated data in respect of culture broth system find suitability in a food matrix, wherein well defined approaches in food chain would be evolved through the selection of a combination of factors to monitor the growth of *Y. enterocolitica*, as LPD values could influence an increase and/or decrease in viable population, which finally matters in microbial food safety.

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Conflict of interest The authors declare that there is no conflict of interest, in terms of scientific, financial and personal.

Author contributions statement The first author has contributed towards undertaking of literature search, conduct of experimental trials and compilation of data as well as draft preparation of manuscript. The corresponding author has planned the experimental design and finalizing the manuscript by going through the compiled data and available literature.

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