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Article

Modeling the Growth of *Lactococcus lactis* NCIM 2114 under Differently Aerated and Agitated Conditions in Broth Medium

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Abstract: The study of growth of *Lactococcus lactis NCIM 2114*, a nisin producer, was modeled using continuously generated concentration data for growth in fermenter. The sigmoidal growth functions, Logistic, Gompertz, and Richards were used to fit the data. A nonlinear regression method was used to fit the data and estimate growth parameter values of *L. lactis*, using Marquardt algorithm with Statistical Software SPSS, version 20. Bacterial growth data from the exponential phase of the bacteria's growth was analyzed. An F test showed that the Gompertz and Logistic functions were acceptable 92% and 67% of times respectively in the batch fermenter runs where this particular application was used to derive the lag time, growth rates, and time to maximum growth rates of *L. lactis*. The maximal specific growth rate ranged between 0.23 h^{-1} to 0.30 h^{-1} and the lag time lasted up to a maximum of 1.63 h depending upon aeration conditions provided to the organism. This study will help to estimate specific growth rates and lag time of *L. lactis* under different growth conditions. Predicted values can be accurately determined.

Keywords: *Lactococcus lactis*; specific growth rate; lag time; Gompertz; Logistic; Richards; growth modeling

1. Introduction

The growth of an organism in a growth medium can be monitored by measuring absorbance, cell biomass (BM as cell dry weight) or cell counts per unit cell volume. Cell plate counts and increase in metabolites of interest, as well as indirect measures can also be correlated to growth rate of an organism. The lag time determined from absorbance units are inaccurate and of insufficient precision [1]. Growth of *Pseudomonas putida* has been modeled and found suitable when recorded in terms of absorbance units for different cell mass *versus versus* time [2] using a Logistic function. For the bacterium, *Lactobacillus plantarum*, the relative population size against time has been applied to predict growth dynamics by the Gompertz function [3]. Modeling cell population increase has been successfully utilized [4]. Modified equations for bacterial growth can be derived for conditions to determine the lag time and growth rate of an organism. Modeling can predict growth when microbial interactions are studied in common environments [5]. For instance, if a lactic bacterium first converts glucose to pyruvate followed by lactate production, a competing organism present in the medium may attempt to take up glucose and survive. Now, populations due to such interactions can then be compared and interpreted by the use of a statistical modeling approach. Other statistical software such as "grofit" available from Comprehensive R Archive Network (CRAN), have also been used to estimate parameters in different growth models and dose response growth curves [6,7].

In the present study, there was no nutrient limitation in the medium for growth of *L. lactis.* This organism also produced nisin as a primary metabolite [8]. Thus, to model the growth of this organism, we studied it during its exponential growth phase. The maximum specific growth rate of the organism and lag time to enter exponential growth was determined under the different levels of agitation and aeration. Growth modeled by (1) Logistic, (2) Gompertz, and (3) Richards sigmoidal functions were all found suitable for curve fits of bacterial concentration data. For growth of *L. lactis*, all the models were strong fits, with high multiple correlation coefficients (R^2 values at $p \leq 0.01$). Because the Gompertz function is a general (dynamic) deterministic model used with differential equations at constant temperature [9], it was used most successfully to determine lag time (λ) and specific growth rate maxima μ_m of the organism [3]. Our results also point to the acceptability of Gompertz function as compared to Logistic function.

2. Experimental Section

2.1. Growth of L. lactis *NCIM 2114*

Cultivation of *L. lactis* was carried out in a 2-L vessel (STR Sartorius A+, Germany), coupled to Multi Fermenter Control System with Data Acquisition Software, controlling the temperature, airflow rate (flow meter), and agitation (Rushton). A Hamilton Dissolved Oxygen probe [10] monitored O_2 % as DO [11,12] at 30 °C. Sterile silicone solution antifoam was added as required. *L. lactis* cell growth was monitored using an Optek NIR sensor probe mounted in the fermenter vessel detecting at 700 nm

in concentration units "CU". After completion of each of the 24 h batch runs, data collected on an Optek Model FC 20 data logger unit was downloaded to a PC via FC-PC transfer software.

2.2. Bacterial Strains, Media, and Culture Conditions

The bacterial strain was procured from National Collection of Industrial Microorganisms, NCL, Pune, India as *Streptococcus lactis* NCIM 2114, a nisin producer (now classified as *Lactococcus lactis* [13] and was maintained by bimonthly sub-culturing. A 48 h growth of culture in de-Mann Rogosa Sharpe (MRS) agar slants [14] (incubated at 30 °C), was preserved at 4 °C. The MRS medium used for growth and nisin production contained (g/L): proteose peptone 10; beef extract 10; yeast extract 5; dextrose 20; polysorbate-80 1; ammonium citrate 2; sodium acetate 5; magnesium sulfate 0.10; manganese sulfate 0.05; di-potassium phosphate 2.0; and agar 12.0 (when required for slants/plates), at a final pH 6.5 ± 0.2. The medium was autoclaved at 121 °C for 20 min. Stock cultures were maintained at −4 °C in nutrient agar and sub-cultured at bimonthly intervals. A uniformly dispersed inoculum of *L. lactis* NCIM 2114 (loopful in a 0.2 mL MRS broth) was inoculated into 3.5 mL MRS broth and incubated 3 h at 30 °C. Transfer of inoculum from 3.5 mL to 75 mL starter inoculum and then adding to 1500 mL fermenting medium ensured synchronous growth of inoculum with an absorbance of ~6.0 (at 600 nm). The working volume of fermenting medium was always 50% of total vessel capacity.

2.3. Experimental Runs

The experiments were randomized and designed using a Box Behnken design [15,16]. This approach required 15 design points/experimental runs, according to:

$$N = k^2 + k + cp \quad (1)$$

where k is the factor number which is 3 in this case and cp is the number of replications at the center point, which is also 3 in this design.The design is single block with the combination(s) of 3 factors x_1, x_2 and x_3, at three coded levels: −1, 0 and +1: x_1 {aeration: 0 vvm (−1); 0.2 vvm (0); 0.4 vvm (+1)}; x_2 {agitation: 25 rpm (−1); 50 rpm (0); 100 rpm (+1)}; and x_3 {harvest periods (h) during growth 4 h (−1), 7 h (0) and 24 h (+1)}, respectively. This configuration includes 12 factorial points and three center points (as replications of control). Three different harvest periods were used to obtain samples for production of nisin. This study reports the bacterial growth using concentration units (CU) data, collected in 15 min intervals over a 24 h period in each of the experimental runs. The mathematical parameters were obtained after statistical analysis of the sigmoidal growth functions, using SPSS version 20 software. Lag periods and specific growth rates of the bacteria under different growth conditions in batch scale were then calculated after analysis using SPSS version 20 software.

2.4. Fitting of Data and Modeling Growth of L. lactis *as a Function of Time*

The growth of *L. lactis* in CU units that was recorded in 15 min intervals in all the batch runs was plotted against time. The Logistic, Gompertz, and Richards sigmoidal functions used to model growth Equations (2)–(4) were:

$$\text{Logistic } y = \frac{a}{[1+\exp(b-ct)]} \quad (2)$$

$$\text{Gompertz } y = a * \exp\left[-\exp(b-ct)\right] \quad (3)$$

$$\text{Richards } y = a\{1+b*\exp[c*(d-t)]\}^{-1/b} \quad (4)$$

2.5. F Test

Using the SPSS software for fitting data, the RSS (residual sum of squares) values were calculated for the three functions. To discriminate among the three-parameter functions (Logistic/Gompertz) or four-parameter functions (Richards), they were compared statistically by the F test. As previously reported [3], Schnute's sigmoidal function encompassed all other sigmoidal functions for growth [3] and also defined four parameters as Richards functions. A function that can define more number of parameters to growth is always advantageous in modeling. The residual sum of squares RSS_1 (of Richards function) can be used as a measuring error [3], was taken as a reference, to compare Logistic and Gompertz functions, in this study.

Fitted data was analyzed and used in calculating f_d values (Equation (5)) as given below, for Logistic *vs. vs.* Richards and Gompertz *vs. vs.* Richards functions and then tested against F [3,16]:

Where DF_2—DF_1 was 1 and Table F values, of 1 as numerator and DF_1 denominator were used to obtain F distribution [15].

$$f_d = \frac{RSS_2 - RSS_1}{RSS_1 / DF_1} \text{ calculated and tested against } F_{\frac{DF_2 - DF_1}{DF_1}} \quad (5)$$

The terms RSS denotes the residual sum of squares where RSS_2 was from the three-parameter model/function (Gompertz or Logistic function) and RSS_1 was from the four-parameter model /function (Richards function). Similarly DF denotes the degree of freedom (regression), where DF_1 was from the four parameters Richards function and DF_2 was from three-parameters Logistic and Gompertz functions.

The f_d values also were then tested against F Table values for acceptability of the sigmoidal function(s) used to model growth of *L. lactis* [3].

In our study, MRS medium was the growth medium for *L. lactis* and substrate was not a limiting factor. Thus, growth was not based on Monod's equation where substrate is a limiting factor for growth.

2.6. Growth Characteristics

The three sigmoidal functions were employed to analyze data points obtained on bacterial growth in 24 h in MRS medium to give best-fit exponential curves for each set of data. For all batch runs, the organism entered the stationary phase by 24 h. Since nisin, a primary metabolite of practical significance, is produced by *L. lactis* NCIM 2114 during its exponential phase this phase was of significant interest to model its growth. Thus, the growth parameters studied for *L. lactis* were

- The specific growth rate maxima μ, the slope of the line when the organism grows exponentially and is drawn on the inflection point of the tangent drawn;
- The lag time, λ, as t, on X-axis intercept of this tangent passed through inflection point on exponential curve;

- The asymptote (A = "a" computed) as the maximal value of growth reached, t approaching infinity. The decline of growth was not studied.

To derive the above growth parameters, growth data were fitted by nonlinear regression on the original sigmoidal functions: Logistic, Gompertz, and Richards [3,17] with Marquardt algorithm [18] using software SPSS version 20. The algorithm calculated the set of parameter values a, b, c and d within the 95% confidence intervals of the lower and upper bound values of a, b, c and d [3]. The re-parameterized values: a, b, and c derived on the Gompertz function were further used to determine the growth parameters by conversion formulas (see (6), (7) and (8) below) on the modified Gompertz function. The ANOVA tables of the regression sum of squares and residual sum of squares were used for F test.

The conversion formulas for the Gompertz function selected to model growth were:

$$a = A \text{ (asymptotic level A of the growth curve)} \tag{6}$$

$$c = \frac{\mu_{max} e}{a} \tag{7}$$

$$b = \frac{\mu_{max} e}{a} \lambda + 1 \tag{8}$$

The Gompertz function was used to determine the lag time (λ) (elapsed time after inoculation and before the start of exponential growth) and specific growth rate maxima μ_m, (time from the exponential growth phase) of the organism in each of the batch runs using different agitation and aerations factors, as designed. The formulas used and derived values of the three parameters a, b, and c so obtained was calculated as:

Using Equation (6) $a = A$

Using Equation (7) $\mu_m = ac/e$ {Specific growth rate maxima, h^{-1}}

Using Equations (7) and (8) $\lambda = (b - 1)/c$ {lag time, h}

3. Results and Discussion

3.1. Modeling Growth of L. lactis

The exponential growth phase of *L. lactis* was of interest as it produces nisin, a primary antimicrobial metabolite. Growth was followed by recording "CU" as measured spectroscopically. By modeling the exponential phase of the growth period, the parameters a, b, c and d were determined as per the sigmoidal functions shown below (Table 1).

For the nonlinear functions (Logistic, Gompertz, and Richards) used here, the growth data was fitted to determine mathematical parameters, using a Marquardt algorithm with least-squares estimation. It removed the divergence of successive iterates while fitting data points, by the nearest neighborhood method, as it assumed local linearity at each iteration (as in Taylor series) [18]. This algorithm also had the ability to close in rapidly when the converged values were reached, which were usually extremely slow in converging after the first few iterations, if the gradient method was used. Thus this algorithm used for modeling growth by the three nonlinear sigmoidal functions will provide adequate representation of the nonlinear growth. The models fitted by nonlinear regression gave reliable estimates of the multiple correlation coefficients (R^2 values at $p \leq 0.01$) for the three functions used. They also

assess the degree of fit of the models, to each of the experimental data sets, that fell close to 1 (Table 2), where R^2 = 1 − (residual sum of squares)/(corrected sum of squares) on the growth curves modeled.

Table 1. Mathematical parameter estimates for all the sigmoidal functions used.

S. No	Factors Used in Different Batch Runs		Sigmoidal functions									
			Logistic			Gompertz			Richards			
	Agitation (rpm)	Aeration (vvm)	a	b	c	a	b	c	a	b	c	d
1	25	0.0	1.46	3.04	0.60	1.48	1.68	0.42	1.48	0.03	0.42	4.09
2	25	0.0	1.76	2.92	0.64	2.04	1.34	0.34	2.54	−0.55	0.18	3.30
3	25	0.2	1.68	3.00	0.58	1.70	1.62	0.40	1.75	−1.54	0.21	0.70
4	25	0.4	1.68	3.02	0.65	1.70	1.66	0.45	1.70	0.14	0.47	3.88
5	50	0.0	1.54	2.78	0.76	1.55	1.53	0.53	4.82	−3.41	0.06	0.13
6	50	0.0	2.05	2.27	0.43	2.09	1.16	0.30	2.09	−0.07	0.29	3.75
7	50	0.2	1.71	2.84	0.62	1.73	1.51	0.43	1.77	−2.09	0.22	−0.36
8	50	0.0	1.74	1.97	0.53	1.76	0.97	0.37	1.82	−1.17	0.22	0.04
9	50	0.4	2.03	2.04	0.44	2.05	1.02	0.31	2.05	0.33	3.53	0.14
10	100	0.0	1.97	2.61	0.49	2.00	1.34	0.33	1.99	0.14	0.35	4.32
11	100	0.2	1.82	2.28	0.56	1.85	1.14	0.38	1.84	0.32	0.44	3.42
12	100	0.4	1.82	2.67	0.54	1.84	1.40	0.37	1.88	−1.10	0.24	1.64

e = 2.71828; Among all the batch runs the maximum nisin content was observed at 4.55 h at S. No 1 run (25 rpm × 0 vvm).

Table 2. Multiple correlation coefficients for all the sigmoidal functions used in different batch runs.

S. No	Factors		R^2		
	Agitation (rpm)	Aeration (vvm)	*Logistic*	*Gompertz*	*Richards*
1	25	0.0	0.993	0.993	0.997
2	25	0.0	0.995	0.991	0.986
3	25	0.2	0.992	0.998	0.992
4	25	0.4	0.996	0.998	0.998
5	50	0.0	0.995	0.992	ND
6	50	0.0	0.994	0.999	0.999
7	50	0.2	0.997	0.995	0.967
8	50	0.0	0.994	0.998	0.989
9	50	0.4	0.883	0.885	0.885
10	100	0.0	0.996	0.998	0.998
11	100	0.2	0.997	0.998	0.999
12	100	0.4	0.996	0.999	0.992

3.2. F Test

Of the two three-parameter functions used, the Gompertz function showed a higher acceptance (92%), to model the growth of *L. lactis*, than the Logistic function (67%), when each was compared to the four-parameter Richards function on f_d values (Table 3).

Table 3. Statistical-analytical data for growth curves of *L. lactis* NCIM 2114.

S. No	Run with Factors		f_d Logistic	f_d Gompertz	*F* Value	*p* Value [#]
	Agitation (rpm)	Aeration (vvm)				
1	25	0.0	4.91	0	3.98	0.05
2	25	0.0	−2.07	−0.55	7.00	0.05
3	25	0.2	2.18	−2.32	3.98	0.05
4	25	0.4	6.12	0.25	6.64	0.22
5	50	0.0	5.07	10.04	6.97	0.05
6	50	0.0	12.44	0.15	3.96	0.05
7	50	0.2	−2.89	−2.14	3.98	0.05
8	50	0.0	−0.74	−2.93	7.00	0.05
9	50	0.4	0.07	0.003	3.89	0.05
10	100	0.0	6.27	0.30	3.97	0.05
11	100	0.2	5.94	2.06	3.96	0.05
12	100	0.4	−0.22	−2.53	3.98	0.05
% Acceptance of given model: among the 12 batch runs			67%	92%		

Boldface values of f_d indicate acceptance of Logistic and Gompertz functions used to model growth as tested against Richards function (at $p \leq 0.05$); [#] Calculated using *p* value calculator [19]. Among the 15 design points the 12 batch runs shown above: 2 control (center) points and 10 experimental runs, where design points at S. Nos 5, 8, 9, 10, have 2 harvest points in common.

3.3. Derivation of the Growth Characteristics of the Organism

The growth characteristics determined on the non-linear modified Gompertz function could help to calculate A, λ and μ accurately (Figure 1).

The specific growth rates maxima (μ_{max}) and lag time (λ) of the organism, derived by Gompertz function [3], under differently agitated and aerated conditions, were in the range of 0.23 h^{-1} to 0.30 h^{-1} and 0 h to 1.63 h respectively (Table 4). It was observed in a different study [20] that the specific growth rate maxima of *L. lactis* can be as high as 0.91 h^{-1} under low aeration conditions. They observed more than 90% of the glucose converted to lactate, whereas our study showed about 36% glucose utilization up to five hours and ~57% utilization by eight hours after inoculation during exponential growth (details not reported). The growth of *L. lactis* modeled by the Gompertz function thus facilitated estimation of the lag time "λ" of the growth and μ_m of *L. lactis*, under the conditions in which it was allowed to grow. This information will also help us to study the important primary metabolite nisin that is produced by *L. lactis* under exponential growth as related to its growth characteristics.

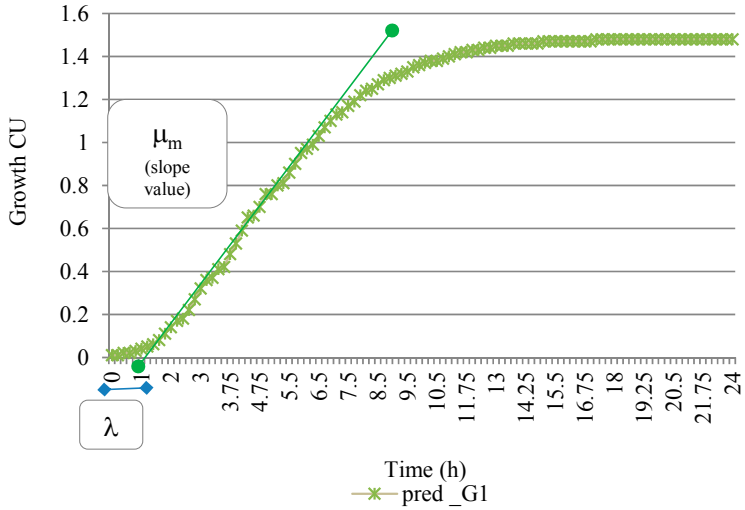

Figure 1. Growth curve of the data "CU" predicted from Gompertz sigmoidal function used to fit curve, showing the tangent on the exponential phase and lag time (λ), and data modeled (data of the curve at S No 1 of Table 2 has been used here).

Table 4. The derived maximal specific growth rate and lag time and associate information on the tangent line of exponential growth period of *L. lactis* under different factors of growth in different batch runs.

S. No	Growth Factors Used		Specific Growth Rate Maxima μ_{max} (h^{-1})	Inf pt: t (h)	Lag Time λ (h)	Time between Lag Time and Inflection Point T (h)
	Agitation (rpm)	Aeration (vvm)				
1	25	0.0	0.23	4.04	1.63	2.41
2	25	0.0	0.25	3.99	1.02	2.98
3	25	0.2	0.25	4.11	1.58	2.53
4	25	0.4	0.28	3.71	1.48	2.24
5	50	0.0	0.30	2.87	0.99	1.87
6	50	0.0	0.23	3.89	0.53	3.36
7	50	0.2	0.27	3.52	1.19	2.33
8	50	0.0	0.24	2.61	−0.08	2.68
9	50	0.4	0.23	3.28	0.06	3.23
10	100	0.0	0.24	4.09	1.03	3.06
11	100	0.2	0.26	2.99	0.37	2.62
12	100	0.4	0.25	3.80	1.09	2.71

The estimated specific growth rate maxima, correlated to the time between lag time and inflection point. The difference between the time of inflection point and the time of lag, correlated significantly (with a coefficient of −0.77) ($p \leq 0.01$). In other words, a decrease in time between lag time and inflection point showed an increase in the maximum maximal specific growth rate of the organism. On the other hand, if the lag time increased, the time at which the inflection point formed was also delayed correlated to as much as 0.69 ($p \leq 0.01$) coefficient of correlation (Table 5).

Table 5. Correlations between the different growth characteristics under batch runs.

One Tailed Correlations	Specific Growth Rate Maxima: $\mu_{max}(h^{-1})$	Inflection Point: t (h)	Lag Time: λ (h)	Time between Lag Time and Inflection Point: T (h)
Maximum specific growth rate μ_{max}	1			
Infpt: t	−0.39	1		
Lag time: λ(h)	0.23	0.69	1	
Time between lag time and inflection point: T(h)	−0.77	0.30	−0.49	1

The three functions used to model growth showed high R^2 values on the predicted data sets of growth modeled (Table 2) indicate high predictability. The Gompertz function to model growth of the *L. lactis* as observed (in CU). See the close/overlapping predicted values on (Figure 2) the growth data.

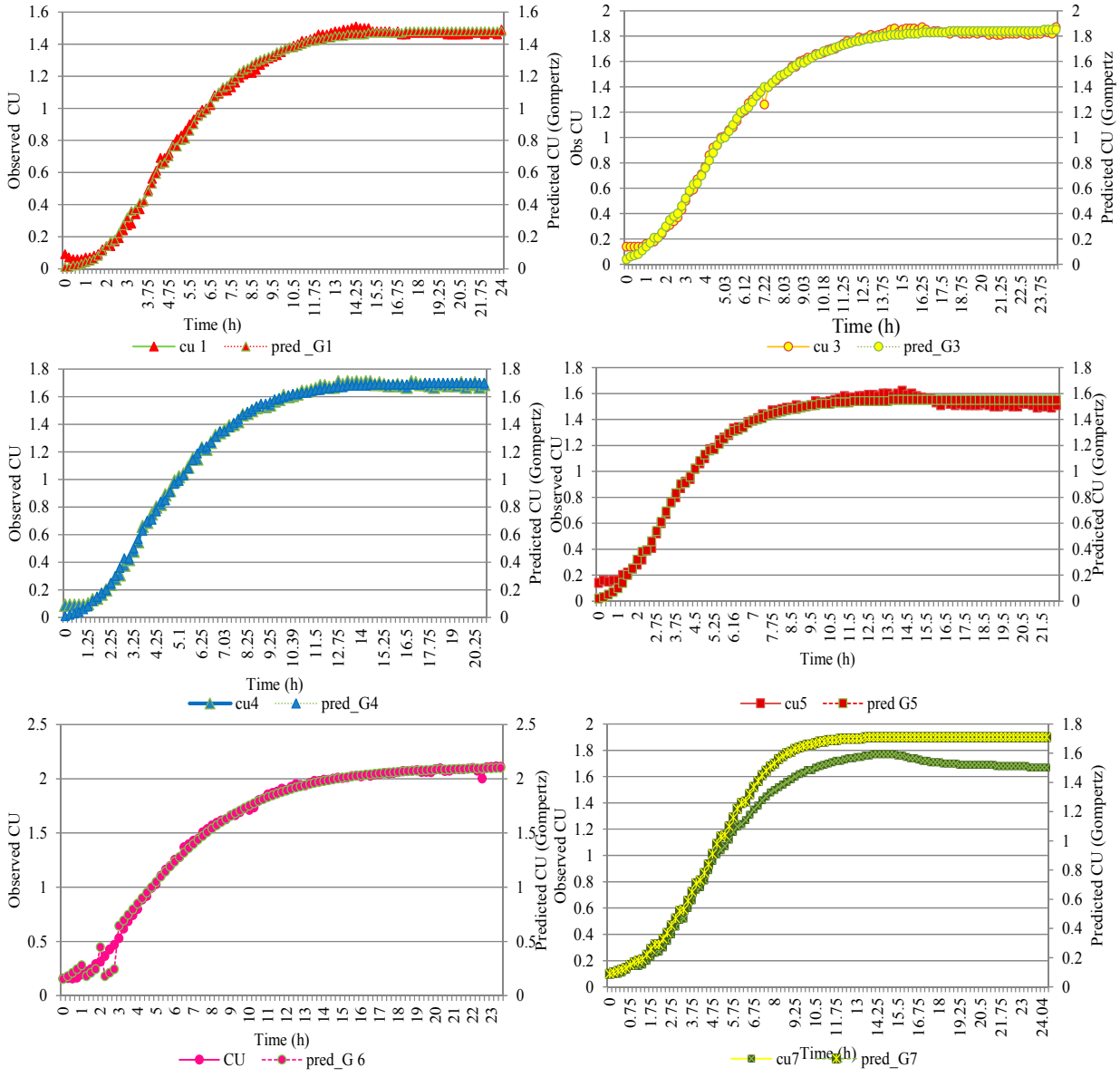

Figure 2. *Cont.*

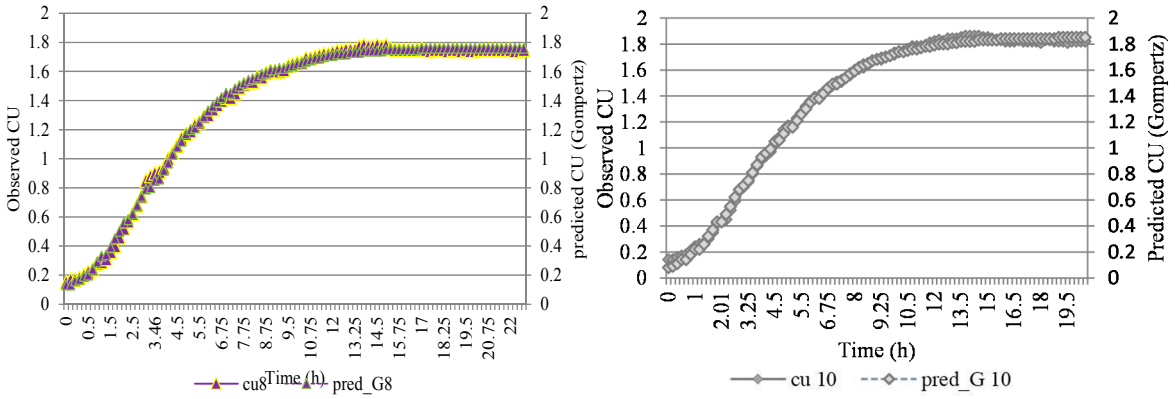

Figure 2. Growth curves of the data "CU" observed and predicted values on data fitted with Gompertz sigmoidal function to model growth (Data of the curves at S No 1, 3, 4, 5, 6, 7, 8, 10 of Tables 1 and 2 are shown).

This study is a new report on the use of concentration units "CU" as a measure of growth of *L. lactis*, modeled using sigmoidal growth functions on statistical software to derive its growth characteristics λ, μ and inflection point of *L. lactis*. The method involved measurements of "CU" at close intervals of time with a probe inserted directly into the bioreactor. The growth data was then downloaded and used to compute mathematical parameters a, b, c and d instantaneously on the three sigmoidal functions used to fit growth data, by Marquardt algorithm. The SPSS software package was used. This method calculated accurate, reliable values. Gompertz function yielded the most acceptable values.

4. Conclusions

The sigmoidal functions used to model the growth of *L. lactis*, showed their suitability to fit growth data of *L. lactis*. Since the Gompertz function had higher acceptability to model growth of *L. lactis*, it was used to derive the growth characteristics. Under the different growth factors (of aeration and agitation), the specific growth rates and lag time were derived from the mathematical parameters a, b and c on the Gompertz Sigmoidal function used. Thus, we have demonstrated that the use of a stochastic model along with the use of a statistical package software that can be a very precise and useful tool to study the exponential growth of *L. lactis*. Since the organism is an important lactic acid bacterium that produces a primary metabolite "nisin" early in the exponential phase, this study helps to understand lag and specific growth rate(s) which can be used to best grow the organism. This report offers a novel mechanistic approach to determine such growth characteristics.

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Author Contributions

Conceptualization and experimental work: Sunita Singh, Leonard Holmes and Siva Mandjiny; Manuscript preparation: Singh Sunita, Leonard Holmes; Statistical data analysis: Kamalesh N. Singh.

Conflicts of Interest

The authors declare no conflict of interest.

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