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Original Article

Effect of Temperature and pH on Formulating the Kinetic Growth Parameters and Lactic Acid Production of *Lactobacillus bulgaricus*

Marziyeh Aghababaie[1], Masood Beheshti[*2], Morteza Khanahmadi[3]

1. Biotechnology Department, Faculty of advanced Sciences and Technologies, University of Isfahan, Isfahan, Iran
2. Chemical Engineering Department, University of Isfahan, Isfahan, Iran
3. Isfahan Center for Research in Agricultural Science and Natural Resource, Isfahan, Iran

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ABSTRACT

Background and Objectives: *Lactobacillus delbrueckii* ssp *bulgaricus* is widely used in dairy industries as a starter for yogurt production. This study was designed using response surface methodology (RSM) in 12 batch pH-controlled cultures of *Lactobacillus delbrueckii* sub-ssp. *bulgaricus* for determining the effect of temperature and pH on the biomass production of a native strain of *L. bulgaricus*, and its main metabolite, lactic acid. The performance of Richards model for prediction of *L. bulgaricus* kinetic growth was verified using the data obtained from the experiments.

Materials and Methods: *L. bulgaricus* was isolated from a plain yogurt. The medium was composed of whey and yeast extracts. RSM was used to design the experiments and quantify the effects of temperature and pH on maximum cell concentration, maximum specific growth rate, total lactic acid concentration, relative growth rate (K) and exponent parameter (d) in Richards model, and product formation parameters of Luedeking-Piret equation (a & b). Matlab software (version 7.12.0) was used to estimate the parameters of Luedeking-Piret equation and Richards model for each experiment.

Results: Second order model for X_{max}, μ_{max}, P and K was significant but product formation parameters were almost constant. The optimum values of temperature and pH for attaining maximum biomass, maximum specific growth rate, and maximum acid production were obtained at 44 °C and 5.7, respectively.

Conclusions: The attained empirical mathematical correlations of RSM alongside the kinetic equations could be used to determine growth conditions under predefined temperature and pH in the fermentation process.

Keywords: *Lactobacillus bulgaricus*, Richards model, Response surface methodology, Lactic acid production, Luedeking-Piret model

Introduction

Lactobacillus delbrueckii ssp. *bulgaricus* (*L. bulgaricus*) is a thermophilic bacterium commonly used as a starter culture alongside *Streptococcus thermophilus* for yogurt production. Lactic acid is the main metabolite of homofermentative lactic acid bacteria, widely applied as an ingredient in food, cosmetic, chemical and pharmaceutic industries. It has become popular in the production of biodegradable poly (lactic acid) (1). Production of native *L. bulgaricus* strains develops starters, which can be easily used by industries in order to produce high quality dairy products, and pure (D-) lactic acid as the raw material for the related industries.

Whey, the byproduct of cheese manufacturing plants is a cheap nutritious material that contain lactose and milk peptides, making it suitable as a carbon source. For complete use of whey lactose, it is necessary to enrich the whey with an additional nitrogen source (2). Highly concentrated yeast extract, by itself gives higher lactic acid production than the mixture of yeast extract and peptone in low amounts (3). The effect of yeast extract peptone, milk powder, malt extract and whey concentrate has been investigated on the growth of *L. bulgaricus* in non-pH control batch condition. It has been reported that 10% (W/V) whey with yeast extract is suitable to obtain maximum biomass and specific growth rate (4).

Different influential parameters affect the lactic acid production through a fermentation process. Microbial strains, carbon and nitrogen sources, fermentation mode, pH content, and temperature have been discussed by (3). Mathematical modeling of growth phases is desirable for

***Address for correspondence:** Masood Beheshti, PhD of Chemical Engineering, Chemical Engineering Department, University of Isfahan, Isfahan, Iran. Tel: +98 3137934529; E-mail address: masbeh@yahoo.com

biotechnologists. Descriptive modeling for *Lactobacillus helveticus*, *Lactobacillus casei*, *Lactobacillus delbrueckii and Lactobacillus bulgaricus* growth and acid production has been reported by some researchers (5-10). As the Malthusian or exponential growth model is the simplest model for the growth of organisms (11). There are some other models like logistic, Von Bertalanffy, and Richards, which can be adopted in modeling of Sigmoid organisms' growth (11). A candidate regression model as a tool should be able to systematically assess and compare the growth-curve shapes in a precise manner. The Richards models class or family constitute a group of useful growth models that amongst a multitude of parameterizations, re-parameterizations and special cases, include familiar models such as the negative exponential, the logistic, the Bertalanffy and the Gompertz (11). The last three are Sigmoid, and have inflection points fixed at a given relative value (i.e., a percentage of the upper asymptote). These three models seem to be the most commonly fitted to the growth data of living organisms (11). The Richards' family models produce sigmoid shape curves with an upper asymptote. They are good choices for regression analysis of Sigmoid growth curves (11). Luedeking-Piret equation correlates the instantaneous acid formation rate to the instantaneous bacterial growth rate and to the bacterial concentration (7). Kinetics of lactic acid production by *Lactobacillus helveticus* (12), *L. delbrueckii* (7) *and Lactobacillus bulgaricus* (8, 9) have been investigated. Growth associated and non-growth associated parameters have been determined in different pH values (8) and medium compositions (12), Growth-associated parameter (*a*) was constant for *L. helveticus* (12); however, in some reports, it was a pH dependent term (7, 13).

Neither Richards model nor Luedeking-Piret equation dealt with the effects of temperature and pH, which are the dynamic operating factors that could be continuously monitored and easily adjusted. Therefore, they seem to be good means to control the trend of growth and product formation of the bacteria. An appropriate mathematical model, which correlates the rates of growth and lactic acid formation with the mentioned factors, could have a great value in this regard. It would be a good idea to add these effects to the models through making empirical correlation between their parameters with temperature and pH. RSM is a powerful tool for the design and analysis of experiments and estimating the effect of temperature and pH on the growth of *L. bulgaricus*. Schepers et al. (12) used RSM with three factors in order to optimize the growth conditions of *Lactobacillus helveticus* growth. Moreover, RSM was used by (14, 15) for the temperature and pH optimization in the growth of *L. bulgaricus* and *Streptococcus thermophilus* strains.

The objective of this study was to formulate the influence of two adjustable factors, temperature and pH, on the growth and lactic acid production of *L. bulgaricus*. To our knowledge, Richards model has not been applied in the modeling of the growth of lactic acid bacteria. Here, we used the specific re-parameterized Richards model and Leudking-Piert equation, respectively, for the growth kinetics and lactic acid production. RSM was used to design the experiments, evaluate the effect of temperature and pH on each parameter, and derive appropriate correlation for the parameters of the models. The parameters included the growth associated (*a*) and non-growth associated (*b*) parameters of Luedeking-Piret equation, as well as the maximum relative growth rate (K), exponent parameter (*d*) and growth rate constant (*k*) of Richards model. Furthermore, temperature and pH dependence of the important criteria of fermentation performance, namely maximum specific growth rate (μ_{max}), and maximum cell concentration (X_{max}), as well as the concentration of lactic acid produced at the onset of stationary phase (P) was determined.

Materials and Methods

Strain and media: *L. bulgaricus* was isolated from a plain yogurt. After several purifications, a single colony was examined by applying colony and cell morphology, gram staining, catalase reaction, and sugar fermentation pattern. The isolated bacterium was identified as a *L. bulgaricus* strain. Pure culture of the given strain was prepared in de Man, Rogosa and Sharpe (MRS) medium (Merck). The cultures were kept in deep-frozen stocks at -80°C. Working cultures were prepared from the frozen cultures 1% (v/v), incubated for 18h at 40°C.

Whey powder was obtained from Isfahan Pegah Dairy Company (Iran). Recombined whey solution of 10 % (W/V) was autoclaved at 110°C for 20 min and then filtered. According to the DNS method, this solution contained about 60g/l lactose. 800 ml of the filtrate with 1 mL Tween 80 (Merck) was sterilized in 1.0 L handmade bioreactor. 100 ml of the sterilized solution of 10 % (W/V) yeast extract (Merck) was added to the bioreactor mixture just before fermentation (15).

Also 1ml of $MgSO_4$ (Merck) (1M) and 1ml of $CaCO_3$ (Merck) (1M) were added to the bioreactor just before fermentation (16, 17).

Analysis: The twelve experiments were conducted in batches with different pH and temperature in 1.0 litere bioreactor. Temperature and pH were controlled at predefined values depending on the experiment. Constant pH during the fermentation was achieved by intermittent addition of 5N sodium hydroxide at appropriate time intervals using a Peristaltic pump. The volume of added base was used to compute the produced lactic acid (18).

The samples were taken at every half hour intervals and placed in the refrigerator (15). Cell population (X) was measured by a microscope (Eeverfocus YJ-2001T microscope) equipped with a Thoma lame with 0.02 mm

depth. The biomass specific dry weight (g/cell) was determined by measuring the weight of a dried sample containing a predetermined number of the bacterium cultivated in MRS medium

Experimental design: The Response Surface Methodology Central Composite, Uniform Precision with 12 runs including: a 4 factorial, 4 axial and 4 center point was selected for these experiments, as shown in Table 1. The values of each parameter assigned to the center points were chosen from reported optima (15); where, T=44°C and pH=5.8 for *L. bulgaricus* at the center points. Analysis was performed with the use of SAS 9.0 software. The above listed 8 parameters represent the responses of the 12 experiments conducted in this study.

The quadratic model for predicting the correlation between the responses and two adjustable factors (temperature and pH) can be expressed according to Eq. (1) (19):

$$y = B_0 + \sum_{i=1}^{2} B_i x_i + \sum_{i=1}^{2} B_{ii} x_i^2 + \sum \sum_{i>j=1}^{2} B_{ij} x_i x_j + e \qquad (1)$$

where, y could be any of the responses in each one of the 12 experiments (X_{max}, μ_{max}, p, k, K, d), x_1 is the temperature factor (°C), x_2 is the pH factor, and Bs are the coefficients of the predicting model.

Modeling: Richards model family has various forms, one of which was more useful in this study due to its specific curve characteristics (as starting value, placement of inflection, and maximum slope) (11). This unified-Richards model (11) was applied to the cell concentration of each experiment by the least square method on Matlab software (7.12.0. R2011a,The MathWorks Inc., Natick, MA, USA):

$$X(t) = A(1 + ((X_0 / A)^{(1-d)} - 1)\exp(-Kt / d^{d/(1-d)}))^{1/(1-d)} \qquad (2)$$

where, X(t)(cell/ml) is a cell concentration at time t (h), and X_0 (cell/ml) is the initial cell concentration. Each one of the four parameters controls a separate shape characteristic (11). This model constitutes a powerful tool for an interpretation of the important characteristics of the observed growth patterns, namely: i) maximum (relative) growth rate, i.e., slope at inflection (at the end of lag phase) (K (h^{-1}), ii) growth rate constant, i.e., relative value at an inflection ($k=K/d^{d/(1-d)}$), iii) value at age zero, i.e., starting value or initial biomass (X_0), and iv) asymptotic value, i.e., maximum value or upper bound (A (Cell/ml)) (11). These four parameters can characterize any Sigmoidal growth data in a unique manner. They were estimated for each experiment by applying the least square method on Matlab software.

Biomass concentrations (X_m (g/L)) along with the lactic acid concentrations data were applied to Luedeking-Piret equation (7) as below:

$$r_p = \frac{dP}{dt} = a\frac{dX_m}{dt} + bX_m \qquad (3)$$

The growth associated, a (g/h), and non-growth associated parameters, b (g), were estimated for each experiment by applying the least square method.

The four parameters of the Richards model and parameters of Luedeking-Piret equation, already estimated for each experiment, were analyzed with RSM in order to recognize their dependency on pH and temperature.

Results

Experiments were conducted according to Table 1. The results for some responses including maximum biomass concentration (X_{max}), maximum specific growth rate (μ_{max}), lactic acid produced at the end of fermentation (P), and Richards parameters (K and d) are presented in the same table. In the following section, details of fitting Eq. (1) to the data of Table 1 for each of the responses are discussed, and the resulting correlations and the optimum conditions for the growth and acid production of L. bulgaricus are presented.

Table 1. Experiments based on RSM; five basic responses are maximum biomass concentration (Xmax), maximum specific growth rate (μmax), lactic acid produced at the end of fermentation (P), and Richards parameters (K and d)

	Experimental conditions		Responses				
RUN	T	PH	X_{max} (cell/ml)	μ_{max} (h^{-1})	P(g/l)	K	d
1	40	4.9	1.5E+08	0.891	3.69	0.3253	1.9949
2	40	6.5	1.25E+08	0.609	2.385	0.4719	13.5524
3	48	4.9	1.3E+08	0.801	3.645	0.5719	6.7279
4	48	6.5	0.7E+08	0.5	2.295	0.2653	4.6353
5	38.34	5.7	1.8E+08	0.8	5.375	0.5978	8.7581
6	49.65	5.7	1.25E+08	0.486	2.7	0.4028	20.2673
7	44	4.56	2E+08	0.67	5.9	0.3962	4.6754
8	44	6.83	1.1E+08	0.586	2.97	0.3557	5.4907
9	44	5.7	2.0+08	0.92	5.94	0.588	6.0242
10	44	5.7	2.2E+08	0.919	5.265	0.52	3.868
11	44	5.7	2.16E+08	0.921	6.255	0.4301	3.275
12	44	5.7	1.8E+08	0.88	6.75	0.5239	3.3992

Maximum cell concentration (X_{max}): Table 2 shows the analysis of variance (AVOVA) for X_{max} and Figure 1 shows the response surface of X_{max} as a function of pH and temperature.

Table 2. ANOVA for second order response surface model for X_{max}

Source	Degree of freedom	Sum of squares	Mean of squares	F value	P value
T	1	2.73E+15	2.73E+15	4.13	0.0884
pH	1	5.37E+15	5.37E+15	8.13	0.0292
T*T	1	7.48E+15	7.48E+15	11.32	0.0152
T*pH	1	4.00E+14	4.00E+14	0.61	0.4662
pH*pH	1	6.94E+15	6.94E+15	10.50	0.0177
Model	5	2.05E+16	4.10E+15	6.21	0.0230
(Linear)	2	8.10E+15	4.05E+15	6.13	0.0355
(Quadratic)	2	1.20E+16	6.01E+15	9.09	0.0153
(Lack of fit)	3	2.92E+15	9.72E+14	2.77	0.2121
(Pure error)	3	1.05E+15	3.50E+14		
Total	11	2.45E+16			

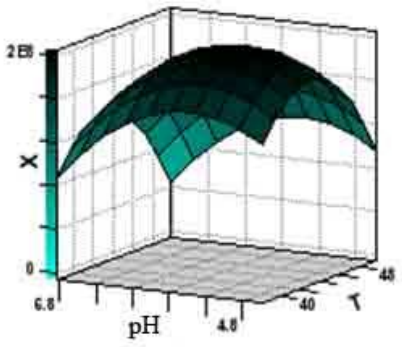

Figure 1. Response surface for X_{max}.

Maximum specific growth rate (μ_{max}): Maximum specific growth rate was calculated in accordance with the exponential (Malthusian) model $\left(\ln \frac{X}{X_0} = \mu t\right)$.

Second order model had statistical significance for μ_{max} rate ($P<0.05$) (Table3). However, a highly significant lack of fit ($P<0.01$) indicates that the response surface model could not adequately describe the observed data. Cubic and quadratic terms can be included in the model by using a model selection procedure in order to eliminate the lack of fit (12). Since F value for the interaction of temperature and pH (T*pH) was very low, it was excluded from the predicted model in order to overcome the lack of fit; as a result, the predicted model gained statistical significance. Table 4 shows the analysis of variance before the exclusion of T*pH factor.

Figure 2 illustrates the response surface of μ_{max} rate as a function of pH and temperature.

Table 3. ANOVA for second order response surface model for maximum specific growth rate (μ_{max})

Source	Degree of freedom	Sum of squares	Mean of squares	F value	P value
T	1	0.0517	0.0517	7.04	0.0378
pH	1	0.0616	0.0616	8.39	0.0275
T*T	1	0.0881	0.0881	12.00	0.0134
T*pH	1	0.0001	0.0001	0.01	0.9153
pH*pH	1	0.0997	0.0997	13.59	0.0103
Model	5	0.2700	0.0540	7.36	0.0153
(Lack of fit)	3	0.0428	0.0143	35.63	0.0076
(Pure error)	3	0.0012	0.0004		
Total	11	0.3140			

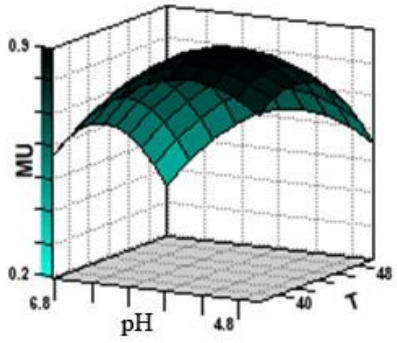

Figure 2. Response surface for μ_{max}.

Produced lactic acid: After the 5th hour of fermentation, all batches were in the stationary phase, and the lactic acid concentration at the onset of the stationary phase was analyzed with RSM.

ANOVA result for the produced lactic acid is presented in Table 4. Figure 3 shows the predicted second order model for the produced lactic acid.

Table 4. ANOVAfor produced lactic acid (P)

Source	Degree of freedom	Sum of squares	Mean of squares	F value	P value
T	1	1.9188	1.9188	1.88	0.2199
pH	1	5.7777	5.7777	5.65	0.0550
T*T	1	11.0776	11.0776	10.83	0.0166
T*pH	1	0.0005	0.0005	0.00	0.9830
pH*pH	1	7.9834	7.9834	7.80	0.0314
Model	5	23.6339	4.7268	4.62	0.0448
(Lack of fit)	3	4.9786	1.6595	4.29	0.1313
(Pure error)	3	1.1603	0.3868		
Total	11	29.7728			

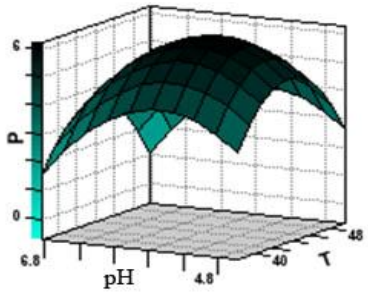

Figure 3. Response surface for the produced lactic acid

Richards' parameters: The experimental data and the fitted Richards model for the obtained optimum conditions (T=44°C and pH=5.7) are presented in Figure 4. Richards model was well fitted for all experiments. The upper bound of Richards model (A) was similar to X_{max}. Also initial cell concentration parameter was about $2*10^6$ (cell/ml) in all experiments; while other parameters (d, K and k) were different in each of the experiments. Thus, these parameters were analyzed with RSM in order to estimate their correlation as a function of temperature and pH.

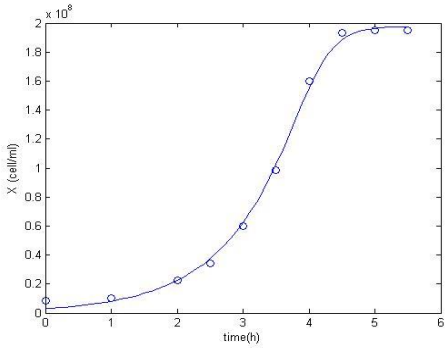

Figure 4. Experimental date (symbol) and fitted Richards model (line) for T=44°C and pH=5.7

Maximum relative growth rate (K): It was observed that the value of K from Richards model was different from maximum specific growth rate estimated from the exponential model. According to Table 5, the interaction of pH and temperature, and second order of pH had statistical significance (P<0.05). Fitted response surface model for K is presented in figure 5.

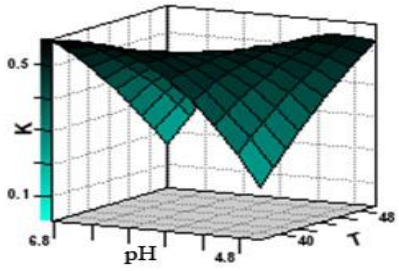

Figure 5. Response surface for maximum relative growth rate (K)

Table 5. ANOVA for maximum relative growth rate (K)

Source	Degree of freedom	Sum of squares	Mean of squares	F value	P value
T	1	0.0069	0.0069	1.4802	0.2694
pH	1	0.0059	0.0059	1.2570	0.3051
T*T	1	0.0014	0.0014	0.3060	0.6001
T*pH	1	0.0513	0.0513	10.9379	0.0163
pH*pH	1	0.0381	0.0381	8.1159	0.0292
Model	5	0.1023	0.0205	4.3583	0.0507
Error	6	0.0282	0.0047		
(Lack of fit)	3	0.0155	0.0052	1.2283	0.4349
(Pure error)	3	0.0126	0.0042		
Total	11	0.1305			

Exponent parameter (d): According to Table 6, lack of fit has statistical significance. After eliminating the non-significant factors, the predicted model gained statistical significance. Only T^2 had a significant effect on this parameter. Figure 6 shows the effect of temperature and pH on the exponent parameter of Richards model.

Table 6. ANOVA for exponent parameter (d)

Source	Degree of freedom	Sum of squares	Mean of squares	F value	P value
T	1	18.27794	18.27794	1.29418	0.298659
pH	1	14.09254	14.09254	0.99783	0.356402
T*T	1	124.9193	124.9193	8.844984	0.024827
T*pH	1	46.58131	46.58131	3.298217	0.119263
pH*pH	1	0.563946	0.563946	0.039931	0.848217
Model	5	213.1609	42.63218	3.018596	0.105579
Error	6	84.73908	14.12318		
(Lack of fit)	3	79.81789	26.60596	16.21922	0.023341
(Pure error)	3	4.921193	1.640398		
Total	11	297.9			

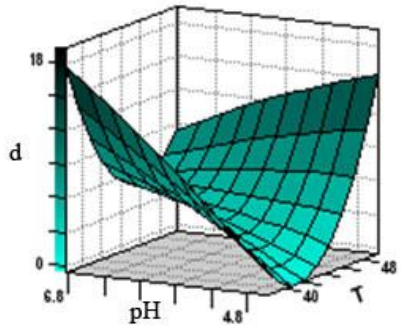

Figure 6. Response surface for exponent parameter (d)

Since $[k=K/d^{d/(1-d)}]$, then growth rate constant was a combination of d and K. The response surface analysis of growth rate constant was similar to that of the exponent parameter (d) (data not shown).

Growth associated and non-growth associated production parameters (a & b): None of the factors had significant effect on the growth associated and non-growth associated parameters. Parameter *a* was measured to be about 0.7 g lactic acid g.biomass^{-1} and parameter *b* was measured to be about 0.6 g lactic acid gbiomass^{-1}.h^{-1}.

The correlations: Coefficients of Eq. (1) are presented in Table 7 for X_{max}, μ_{max}, produced lactic acid and Richards parameters. Coefficients of each factor indicate the amount of the effect of that factor on each paramter.

Optimum conditions: The resulted correlations could be applied in estimating the optimum pH and temperature for X_{max}, μ_{max} and produced lactic acid (P). The results are presented in Table 8.

According to Table 8, optimum temperature for obtaining X_{max} and P is 44°C. Optimum pH for these factors is 5.7 and 5.13, respectively

Table 7. Coefficients of the response surface model for X_{max}, μ_{max} and P and Richards' parameters according to Equation 1

B_i Coefficients	X_{max}(cell/ml)	μ_{max}(h^{-1})	p	K	d	k
B_0+e	-6.00E+09	-14.565	-199.28	-13.58	252.1	17.1
B_1	2.01E+08	0.4259	7.133	0.2768	-18.17	-6.771
B_2	6.92E+08	2.338	18.98	2.898	48.58	46.54
B_{11}	-2.14E+06	-0.00496	-0.082	-0.0009	0.279	0.128
B_{22}	-5.15E+07	-0.2147	-1.745	-0.1205	n.s	-1.005
B_{12}	-3.13E+06	n.s	n.s	-0.0354	-1.066	-0.786
R^2 (R-square for RSM)	83.80%	87.92%	79.38%	78.41%	71.55%	82.24%

n.s: Non-significant
B_0: Model intercepts
B_1: Temperature factor coefficient
B_2: pH factor coefficient
B_{11}: T^2 coefficient
B_{12}: pH*T coefficient
B_{22}: pH^2 coefficient
e: Error in RSM model

Table 8. Optimization of temperature and pH for the growth and acid production of *L. bulgaricus*

T(°C)	pH	X_{max}	μ_{max}	P
44	5.13	204602328	0.90962	6.094
44	5.7	202749965	0.90999	6.052
41.17	5.7	198718452	0.90818	5.741
41.17	5.13	195570826	0.90543	5.777

Discussion

RSM results showed that temperature and pH were significant factors in the growth of *L.bulgaricus*. The fitted model for X_{max} had statistical significance ($P<0.05$), and lack of fit was not significant. Second order of temperature and pH had the maximum effect on maximum cell concentration with the statistical significance of $P<0.05$. This result is in agreement with of the one reported by Beal et al. (15).

Second order of pH and temperature had the maximum effect on the μ_{max} ($P<0.05$). Unlike the results of Beal et al. (15), the first order effect of these factors also had statistical significance ($P<0.05$).

The second order model was vital for prediction of the lactic acid produced at the end of fermentation ($P<0.05$). Regarding the order of efficacy, T^2, pH^2 and pH provided proper results, which are in agreement with the findings of Beal et al. (15). However, parameters of Luedeking-Piret equation were independent of pH and temperature. Growth associated parameter for acid production was lower than that reported by other researchers; however, the non-growth associated parameter was close to their findings (5, 8, 20).

Richards model is a good tool to predict and control the fermentation process. It was observed that pH and temperature had a significant effect on the parameters of this model.

The second order model was more efficient for predicting parameter K. Exponent parameter was the most important parameter in Richards model. It was repeated in the model four times.

Second order of temperature and interaction of pH and temperature had a significant effect on growth rate constant ($P<0.05$).

The optimum conditions didn't vary from that of reported by (15); while maximum cell concentration was lower in this study. It must be due to the different strains and media used in these two studies. It was obvious that the low and high levels of the factors were very important, so the predicted model highly depended on the considered range for pH and temperature.

X_{max}, μ_{max} and *P* at the end of the fermentation process can be obtained by applying the predicted model in different values of pH and temperature. Parameters of the Richards model can be obtained in the same way and use to predict the fermentation process in the desired pH and temperature.

Conclusion: In the present study, response surface analysis was used to study the effect of pH and temperature on the growth parameters and lactic acid production by *Lactobacillus bulgaricus* in the pH-controlled batch cultures. Richards model is a complicated model, which contains more parameters than simple exponential model. The results of the RSM analysis showed that exponent parameter (d) in Richards model highly depends on the second order of temperature; in contrast, maximum relative growth rate (K) highly depends on the second order of pH. RSM showed that the second order model was significant for $X_{max,}$ and produced acid lactic (P). In addition, lactic acid production was pH- and temperature-dependent; while Luedeking-Piret parameters were constant. Optimum pH and temperature for X_{max} and μ_{max} and P were 44 °C and 5.7, respectively. The attained empirical mathematical correlations of RSM alongside the kinetic equations could be used to determine the growth conditions under predefined values of pH and temperature of the fermentation. However, Richards model could be improved by formulating the pH- and temperature-dependent parameters to attain a unified model. The results of this study can be applied to the development of a kinetic model that describes the growth and lactic acid production of *L. bulgaricus* in pure pH-controlled batch cultures and in co-culture with *Streptococcus thermophilus* as a function of pH and temperature.

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