Non-linear modeling of kefir grains growth curve

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Abstract

The main objective of this research is non-linear modeling of growth curve during kefir grains batch propagation. For this purpose some laboratory experiments were performed in an RC1 reactor provided data for growth curve construction. Afterwards, we fitted several growth models (Logistic, Gompertz and Richards) and compared their biological parameters. Finally, we established the statistically most appropriate growth model for prediction of biomass increase during kefir grains batch propagation in milk under selected bioprocess conditions.

Keywords: kefir grains, batch propagation, growth models, statistical analysis

1. Introduction

Kefir is a self-carbonated refreshing dairy product that can be made with any kind of milk, such as cow, goat, sheep, camel and buffalo (Loretan *et al.*, 2003). Several methods for kefir production, which use (using) pure and isolated starter cultures, can be found in the literature (Assadi *et al.*, 2000; Beshkova *et al.*, 2002). However, original kefir can only be produced using the traditional method of adding kefir grains to a quantity of milk (Tamime *et al.*, 1999). Kefir grains are complex symbiotic colony containing more then 35 probiotic bacteria proven to be highly beneficial for humans (http://kefir.org/kefir_manual.htm, 2006). The variegated microbial composition of kefir grains enable their application not only in large scale kefir fermentation but also, for instance, in bread production as baker's yeast (Plessas *et al.*, 2005), volatile aroma compounds' production (Beshkova *et al.*, 2003) and ethanol production using immobilized kefir yeast cells (Athanasiadis *et al.*, 1999). Therefore, when kefir grains are used in commercial applications, then their production, using traditional cultivation in milk with very low biomass increase (only 5–7 %/d) (Libudzisz and Piatkiewicz, 1990), has to be improved. Some studies report considerable improvements in kefir grain biomass increase during its production. For example, Schoevers and Britz (2003) investigated the influence of different culturing conditions on kefir grain biomass increase. They established process conditions, which result in a biomass increase of more then 580 % over a 22 day period (26.3 %/d). Furthermore, Harta *et al*. (2004) studied the propagation of kefir grains in a vigorously aerated batch system, using various pure and mixed water solutions of carbohydrates. Their results indicate, in the most effective case, an admirable daily increase of 545 %. Kefir grain biomass propagation is inherently a very complex process, therefore for its optimization and control it is extremely important to develop models that are able to provide an accurate description of kefir grain biomass growth curve.

The time-dependent increase in the microbial population in a closed bioreaction system is referred to as a growth curve (Perni *et al.*, 2005). A typical microbial growth curve can be divided into different regions: lag, logarithmic exponential growth, inhibition, stationary and decay phase (Vadasz and Vadasz, 2005). Moreover, it is generally sigmoid on a semi-logarithmic plot. Several different growth models can be found in literature for describing such a sigmoidal-shaped curve (Lopez *et al.*, 2004). Most of them are based on mathematical equations such as Logisitc (LOG), Gompertz (GOM) and Richards (RIC) (Zwietering *et al.*, 1990). Using these mathematical models, we can estimate the important growth kinetic parameters (lag time duration and maximum specific growth rate) under different process conditions. Finally, in the predictive microbiology field, sigmoidal shaped growth models have been used to forecast cell growth rates (Speers *et al.*, 2003). Irrespective of the importance of predictive modelling, there is still a lack of published information concerning a mathematical description of growth curve during traditional kefir grains production.

The objective of the present study was to analyze the different growth models for describing the kefir grain biomass propagation using experimental measurements. The propagation was performed in RC1 batch reactor under selected bioprocess conditions using traditional cultivation in milk. We compared the values of biological parameters describing kefir grain biomass growth, obtained by using different models. Moreover, we determined the most statistically appropriate biomass growth model.

2. Materials and methods

2.1. Equipment, kefir grains culture, propagation medium and other chemicals

Kefir grain biomass propagation using traditional cultivation in milk was performed in a computer-controlled batch laboratory RC1 reactor (Mettler Toledo). It consists of a double walled glass reactor (V = 2 L), RD10 universal controller and PC with the ‘WinRC1 for RC1’ software. RC1 allows the measurement and control of important process parameters and determination of the complete mass and heat balance of the entire chemical process. Furthermore, using specific modifications it could also be used for investigating thermal effects during bioprocess.

Fresh initial kefir grain culture, originating from the Caucasian Mountains, was kindly supplied by an existing Slovenian local dairy. Grain biomass growth was studied using traditional propagation in fresh HTP whole fat cows’ milk, produced by Ljubljanske mlekarne d.d. (Ljubljana), as a propagation medium. Glucose anhydrous

(Fluka), purchased from Sigma-Aldrich Co (St. Lous, MO), was used as an addition to the propagation medium.

2.2. Kefir grain biomass growth model

Kefir grain biomass growth models can be used as a tool in all kefir grain biomass batch propagation studies to describe mass increases over a particular time, until reaching maximum value. The growth curve in a semi-logarithmic plot (logarithm of the relative kefir grain mass, versus batch propagation time, t_P) has the characteristic elongated S-shape, also known as a sigmoidal curve. There are several mathematical well known relationships, such as LOG, GOM and RIC, that can describe this type of curve (Zwietering *et al.* 1990). These equations contain some mathematical parameters, a, b, c, τ, and v, without biological meaning. Therefore, Zwietering *et al.* (1990) reparameterized these equations and substitute the mathematical parameters with maximum specific growth rate, μ_{max}, lag time duration, t_L, and asymptotic value, A. The modified forms of LOG, GOM and RIC equations (growth models) are collected in Table 1.

Model	**Expression**
LOG	$\ln\left(m_{KG}/m_{KG,0}\right) = A\left\{1+\exp\left[4\mu_{max}A^{-1}\left(t_L - t_P\right) + 2\right]\right\}^{-1}$
GOM	$\ln\left(m_{KG}/m_{KG,0}\right) = A\exp\left\{-\exp\left[\mu_{max}\exp(1)A^{-1}\left(t_L - t_P\right) + 1\right]\right\}$
RIC	$\ln\left(m_{KG}/m_{KG,0}\right) = A\left\{1 + v\exp(1+v)\exp\left[\mu_{max}A^{-1}(1+v)^{(1+1/v)}\left(t_L - t_P\right)\right]\right\}^{(-1/v)}$

Table 1: LOG, GOM and RIC growth models.

Furthermore, at the inflection point of the growth curve, the second derivative of the growth equation with respect to t, is equal to zero. By considering that definition and proposed models in Table 1, the batch propagation time at μ_{max}, t_μ, can be expressed by equations, as presented in Table 2.

Model	**Expression**
LOG	$t_\mu = t_L + A\left(2\mu_{max}\right)^{-1}$
GOM	$t_\mu = t_L + A\left[\exp(1)\mu_{max}\right]^{-1}$
RIC	$t_\mu = t_L + A(1+v)^{(-1/v)}\mu_{max}{}^{-1}$

Table 2: LOG, GOM and RIC model – estimation of t_μ.

From the experimental data of kefir grain mass at different t_P under selected process conditions, it is possible to determine the shape (v) and biological parameters (μ_{max}, t_L, A and t_μ) of the proposed LOG, GOM and RIC growth models.

2.3. Analytical methods and non-linear regression of experimental data.

A growth curve was constructed using the experimental data of time-dependent kefir grain mass during batch propagation in a selected medium. Therefore, gravimetric kefir grain wet-weight determination was used. The propagations were performed at different batch times. When the separate experiment was completed, the kefir grains were harvested after separation using a household sieve. Then, the grains were washed with cold water and dried carefully on paper towelling. Finally, the kefir grain mass was determined gravimetrically, using a Mettler-Toledo analytical balance (PG5002-S).

Non-linear regression of experimental data was used to evaluate the parameters of the proposed growth models with the Marquardt-Levenberg algorithm using the commercial available SigmaPlot®9.0 software. The goodness of fit and the most statistically appropriate kefir grain biomass growth model were determined using the six well known statistical indicators, i.e. standard error, *SE*, coefficient of the variation, *CV*, adjusted coefficient of the determination, R_{adj}^2, root mean squared error, *RMSE*, variance ratio (statistical F-criterion), *F*, predicted residual error sum of squares, *PRESS*, and t-statistic value, *t-st*.

2.4. Kefir grain biomass activation

Kefir grain biomass adaptation to the propagation medium and, simultaneously, constant initial kefir grain biomass viability were attained during activation. We applied a similar activation procedure as Schoevers and Britz (2003), using a glass lab beaker (V = 1 L). Inactive kefir grains (γ_{KG} = 75 g/L) were added to fresh milk and incubated at room temperature (ϑ = (22 ± 2) °C). After 24 hours incubation, the kefir grains were retrieved using a household sieve, washed with cold water, and reinoculated into a new batch of fresh milk. The same procedure was repeated for the next six subsequent days until grains were considered active.

2.5. Kefir grain biomass propagation

In order to obtain experimental data of time-dependent kefir grain mass during batch propagation, for growth curve construction, a series of experiments were performed with different t_P. It is well known, that a combination of different bioprocess conditions, has an influence on kefir grain biomass increase (Schoevers and Britz, 2003; Harta *et al.*, 2004). However, we proposed the following values of bioprocess conditions: temperature, ϑ = 24 °C, rotational frequency of the stirrer, f = 90 (1/min), initial kefir grain mass concentration, $\gamma_{KG,0}$ = 75 g/L, and glucose mass concentration, γ_G = 20 g/L. The individual experiment was started by charging the RC1 reactor using 1 L of milk (V = 1L) and glucose (m_G = 20 g). This propagation medium was heated up and incubated at ϑ = 24 °C and f = 90 (1/min), for t = 20 min. Then, the propagation with previously defined time was triggered by inoculating 75 g of active grains as initial kefir grain culture. When the propagation was completed, the final kefir grain mass was determined.

3. Results and discussion

The final kefir grain mass, m_{KG}, kefir grain mass increase, $m_{KG,i}$, and kefir grain increase mass fraction, $w_{KG,i}$, determined under different t_P, are presented in Table 3.

Exp. No.	t_P/h	m_{KG}/g	$m_{KG,i}$/g	$w_{KG,i}$/%
1	3	75.51	0.51	0.7
2	4	76.19	1.19	1.6
3	6	77.02	2.02	2.7
4	9	81.50	6.50	8.7
5	12	85.36	10.36	13.8
6	16	88,92	13.92	18.6
7	22	91.71	16.71	22.3
8	24	94.37	19.37	25.8
9	26	93.20	18.20	24.3
10	28	93.46	18.46	24.6
11	30	93.50	18.50	24.7

Table 3: Experimental (m_{KG}) and calculated data ($m_{KG,i}$, $w_{KG,i}$) at different batch propagation times.

In Table 3, $m_{KG,i}$ is the difference between m_{KG} and the initial kefir grain mass, $m_{KG,0}$, meanwhile, $w_{KG,i}$ is the quotient between $m_{KG,i}$ and $m_{KG,0}$. The final and highest kefir grain increase mass fraction (average value of experiments 8, 9, 10 and 11), $w_{KG,i}$ = (24.85 ± 0.95) %, was achieved after a t_P =24 h. Further increase on t_P did not significantly effect on $w_{KG,i}$. Therefore, we can establish that after t_P = 24 h the batch fermentation under selected bioprocess conditions was completed. Moreover, a comparison with the data reported by Schoevers and Britz (2003), indicates a similar daily $w_{KG,i}$. Changes in the logarithm of relative kefir grain mass versus t_P, obtained from experimental data, are presented in Fig. 1. Moreover, growth curves fitted with LOG, GOM and RIC model are also shown.

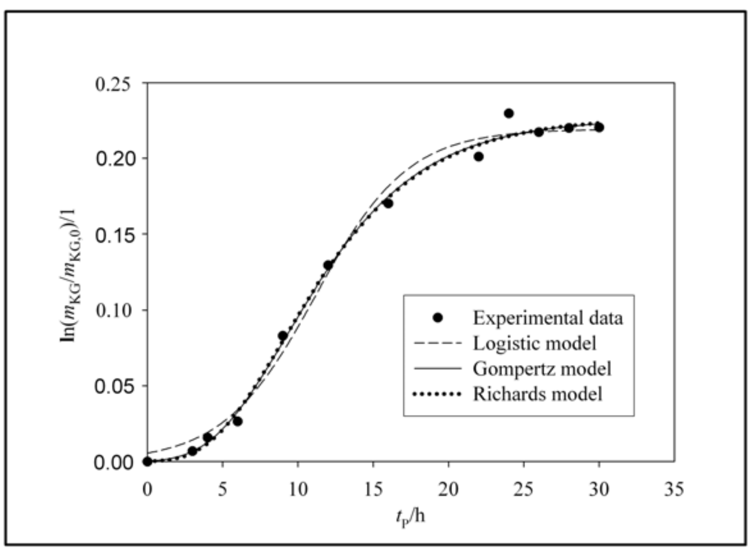

Figure 1: Kefir grain biomass growth curves.

It is evident from Fig. 1, that all models satisfactorily fitted the experimental values and, therefore, can be used for describing kefir grain biomass growth during batch propagation. However, the most statistically appropriate model can not be determined from graphic results. Parametric analysis was used instead, enabling us to define the important kinetic parameters of the proposed models and to establish the most adequate growth model. The results are collated in Table 4. A very significant feature of the parametric results from fitting the LOG, GOM and RIC models is that the values of A and μ_{max}, proposed by all models, are very similar to each other, whilst, t_L values are markedly different. The variations of estimated values A, μ_{max} and t_L are within the ranges ±3.8, ±6.6 and ±19.0 %, respectively

Model	**Parameter**	**Estimate**	***SE***	***CV*/%**	R_{adj}^2	***RMSE***	***F***	***PRESS***	***t-st***
LOG	A/1	0.2193	0.0046	2.1	0.991	0.009	573	0.0016	47.5
	μ_{max}/(1/h)	0.0178	0.0017	9.6					10.4
	t_L/h	5.0319	0.6019	12.0					8.4
GOM	A/1	0.2263	0.0044	1.9	0.995	0.007	1125	0.0007	51.6
	μ_{max}/(1/h)	0.0167	0.0011	6.6					14.7
	t_L/h	4.2271	0.4103	9.7					10.4
RIC	A/1	0.2275	0.0067	2.9	0.995	0.007	673	0.0008	33.8
	μ_{max}/(1/h)	0.0167	0.0012	7.2					13.7
	t_L/h	4.2163	0.4977	11.8					8.5
	ν/1	–0.0870	0.3294	378.6					–0.3

Table 4: Parametric analysis results and statistical indicators of different growth models.

The statistically most appropriate growth model was determined using the six statistical indicators. Firstly, R_{adj}^2 criterion was applied. The R_{adj}^2 values were, in all cases, almost equal with variation ±0.4 %. Therefore, this statistic indicator can not be used as a single basis for a statistical growth model ranking. Secondly, the *RMSE* and *PRESS* criteria were considered simultaneously in order to choose the statistically best kefir grain biomass growth model. GOM and RIC models had equal *RMSE* (0.007) and similar *PRESS* values (0.0007 and 0.0008, respectively). The LOG model had smaller *RMSE* and *PRESS* values, compared to the GOM and RIC models, and therefore, it is less statistically suitable for describing a kefir grain biomass growth curve. However, using these criteria, we could still not clearly distinguish statistically between GOM and RIC models. Therefore, we finally applied *F* and *t-st* statistical indicators. The results in Table 4 show that the GOM, compared to RIC model had greater *F* value. The relative difference is more than 63 %. Moreover, it also had greater *t-st* values for all biomass growth parameters. This result could lead to the conclusion that the GOM model is statistically more suitable than the RIC one.

On the basis of the applied parametric analysis and the statistical indicators, we can summarise that the kefir grain biomass growth curve during traditional batch propagation in fresh HTP whole fat cows' milk under selected bioprocess conditions can be most successfully described statistically by the GOM growth model as follows:

$$\ln\left(m_{KG}/75\right) = 0.2263\exp\left\{-\exp\left[0.2006\left(4.2271 - t_P\right) + 1\right]\right\} \quad (1)$$

Finally, in order to represent the considerable difference between all the proposed growth models, we also estimated their t_μ values. The results are presented in Table 5.

Model	t_μ**/h**		***CV*/%**	k_G**/1**		***CV*/%**
	Estimate	***SE***		**Estimate**	***SE***	
LOG	11.2	1.3	12	1.22	0.26	22
GOM	9.2	0.8	9	1.00	0.00	0
RIC	9.0	2.0	23	0.98	0.31	32

Table 5: t_μ estimations with LOG, GOM and RIC model.

The t_μ estimations and appurtenant *SE* and *CV* values were calculated using the equations and parametric results collected in Table 2 and 4, respectively. Moreover, the deviation coefficient according to GOM model, k_G and their *SE* and *CV* values are also presented in Table 5. The k_G (quotient between t_μ and $t_{\mu,GOM}$) nearer to 1 indicates that the growth model predict a similar t_μ value to the GOM model. The t_μ times, estimated by the GOM and RIC models, are similar (9.2 and 9.0 h, respectively) meanwhile the t_μ obtained by the LOG model is perceivably higher (t_μ = 11.2 h). The considerable difference and, consequently, the smaller statistical suitability of the LOG model compared to others can also be explained by the highest deviation of their k_G value from unity. In addition, the RIC model has the highest *SE* and *CV* values for estimates, t_μ and k_G. Consequently, these values indicate their high multicolinarity level, which is an indirect outcome of high *SE* value for v. Finally, a comparison of *CV* values for t_μ once again confirms that the GOM model is statistically the most suitable for describing a kefir grain biomass growth curve.

4. Conclusion

In this study LOG, GOM and RIC models were compared to describe kefir grain biomass growth during traditional batch propagation in milk under selected bioprocess conditions (ϑ = 24 °C, f = 90 1/min, $\gamma_{KG,0}$ = 75 g/L and γ_G = 20 g/L). The results showed that the highest statistical deviation between the predicted and experimental data belongs to the LOG model and, therefore, it is less statistically suitable for describing growth curve. On the other hand, the statistical indicators proved that the GOM model was the best for describing the kefir grain biomass growth curve during traditional batch propagation in milk. The presented results are specific for the selected bioprocess conditions, for the used propagation medium and for the initial kefir grain culture. It is well known that microbial composition in milk and kefir grains considerable vary with time and are dependent on age and storage conditions. Therefore, the results cannot be presented as general for all kinds of batch kefir grain biomass propagations. In spite of all that, the experimental and statistical procedure given in this paper can be used to find the statistically best growth model for describing batch kefir grain biomass growth curve under different experimental and propagation setups.

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