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# A Novel Approach for Assuring and Following Inoculum Activity during Kefir Grains Growth Studies – Application of Dynamic pH Profiles

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*Dedicated to the memory of professor Vojko Ozim*

## Abstract

The profiles of kefir grains growth curves strongly depend on bioprocess conditions and inoculum viability. Therefore, accurate growth curve studies require the use of optimally active kefir grains as inoculum. Accordingly, the main objective of our study was experimental optimization of classic kefir grains activation procedure and afterwards, comparison among growth curves of differently activated kefir grains. For this purpose some experiments were initially performed in computer-controlled RC1 reactor provided data of dynamic pH profiles during batch propagation of differently activated grains. Experimental data were mathematically analyzed and using the special methodology of minimizing the absolute deviation of pH($t$) profiles the minimal time for grains activation was determined. We established that optimal inoculum activity could be ensured only by the kefir grains, which were previously activated at least over eleven successive days. In the second part of research the growth curve of optimally activated kefir grains was constructed using experimental measurements and fitted with Gompertz model. The present results were compared with the results obtained by our previous study[14], where classically activated grains were used. We established that, in spite of equal daily kefir grain mass increase, considerable difference in growth curves of differently activated kefir grains exist.

**Keywords:** Kefir grains, minimal activation time, growth curve, dynamic pH profiles, Gompertz model

## 1. Introduction

Kefir grains are complex symbiotic microbial community entrapped into matrix of protein and polysaccharide and is believed to have its origins in the Caucasian mountains.[1,2] During the last few years, many researches have been focused on analysis of their microbial composition. Summarily, they contain gram-positive homofermentative and heterofermentative lactic acid bacteria, gram-negative acetic acid bacteria and both lactose fermenting and non-fermenting yeasts.[3–5] Traditionally, kefir grains are the key to original kefir (viscous, fermented dairy beverage that contains small quantities of alcohol) production.[6] However, the unique microbial composition of kefir grains enable their application also, for instance, in bread production as baker's yeast[7], polysaccharide production[8], ethanol production using immobilized kefir yeast cells[9] and novel low-alcoholics drink production from suspension of whey and raisin extracts.[10] If kefir grains are commercially included in above-quoted applications, their traditional production using classical propagation in milk with relatively low daily mass increase fraction ($w_{KG,24}$ = (5–7) %/d)[11] has to be optimized and eventually improved. Therefore, it is very important for optimization, control and monitoring purposes to develop predictive kefir grains growth models.

The increase of the microbial population versus propagation time in a closed bioreaction system is referred to as a growth curve, which can be divided into different well known regions: lag, logarithmic exponential growth, inhibition, stationary and decay phase.[12] The shape of the curve strongly depends on process conditions and inoculum viability. It was established by our preliminary experiments that irrespective of equal daily kefir grain mass increase, the lag phase of less active grains is longer compa-

red to the optimal active ones. Classic, six daily, kefir grains activation is the only one found in the literature.[13] We used the same procedure during our previous study,[14] where some different well-known predictive growth models (logistic, Gompertz and Richards ones) for describing kefir grains increase using experimental measurements were compared. The statistical indicators proved that the Gompertz model was the best for describing the biomass growth curve during traditional propagation in milk under optimal batch bioprocess conditions. However, we hypothesize that more efficient and repeatable kefir grains growth modeling probably requires some experimental measurements using long-continued and constant active and viable kefir grains. The important fact is that, observed daily kefir grain mass increases in batch reactor at optimal process conditions confirmed the adequacy of the classic activation procedure if grains increase maximization is discussed. Nevertheless, for the more accurate modeling purposes not only the repeatable daily grains increase is important but also the total concentration profile (followed by pH measurements in our case). Therefore, the needfulness existed for the improved activation procedure. It was expected that kefir grains activated according to improved (optimal) procedure will assure also the repeatability of time dependent medium pH values during the fermentation.

The aim of the present study was experimental optimization of the classic kefir grains activation procedure and afterwards comparison between growth curves of classically and optimally activated grains. For this purpose our study was carried out in two parts. Firstly, we determined the minimal time needed for kefir grains activation where absolute deviation of dynamic pH profile followed the selected tolerance criterion. Based on the mathematical analysis of experimentally measured pH values, special method for minimisation of absolute deviation in pH profiles was developed. In the second part of the study, on the basis of experimental data we constructed the growth curve of optimally activated grains and their biological parameters (lag time duration, maximum specific growth rate and asymptotic value) using Gompertz predictive growth model were estimated. Finally, we compared these values with the previously obtained results, where growth curve was based on classically activated kefir grains.

# 2. Experimental

## 2. 1. Materials and Methods

### 2. 1. 1. Equipment

Kefir grains propagation was performed in an RC1 reaction calorimeter (Mettler–Toledo, model: RC1 classic). RC1 is a computer-controlled laboratory reaction calorimeter containing a double walled glass reactor with a 2 L working capacity and optional equipment such as pH electrode (Mettler-Toledo, model: pH01). It is an excellent tool for investigating the thermal characteristics of desired chemical reactions, and for assuring safe process performance. RC1 data provide information such as the energy of chemical reaction, specific heat, adiabatic temperature rise, heat transfer coefficient, and media pH value. By using specific modifications in hardware and software, it could also be used for investigating during bioprocesses.[15] A detailed description of RC1 can be found in our previous paper.[16]

### 2. 1. 2. Chemicals, Kefir Grains Culture and Culture Medium

Kefir grains activation and afterwards growth curve were studied using fresh HTP whole fat cows milk (Ljubljanske mlekarne d.d.) as a culture medium. Its chemical composition is 3.2% proteins, 4.6% carbohydrates, 3.5% fat and 0.13% calcium. 3D-(+) Glucose anhydrous (Fluka) was obtained from commercial sources. Kefir grains originate from the Caucasian Mountain and were acquired from an existing local dairy (Kele&Kele d.o.o.). The microbial population (bacteria and yeasts) of kefir grains depends on many different factors (age, storage conditions and fermentation medium) and varies with the season. It is almost impossible to assure equal microbial composition during long term period, therefore for sets of experiments within one research, kefir grains with the same viability should be used.

### 2. 1. 3. Kefir Grains Activation

Kefir grains activation was performed in a glass lab beaker. The collected inactive kefir grains ($\gamma_{KG}$ = 75 g/L) were inoculated in 1 L of fresh HTP whole fat cows milk. After incubation at room temperature ($\vartheta$ = (22 ± 2) °C) for 24 h, the grains were separated from the kefir beverages using a household sieve. After washing, they were reinoculated ($\gamma_{KG}$ = 75 g/L) into the fresh milk. The same procedure was repeated over subsequent days.

### 2. 1. 4. Methodology for Determination of Optimal Activation Time

Optimal constant initial activity and viability of inoculum, which simultaneously enables the repeatability of daily increase and concentration profile of kefir grains in batch reactor, can be achieved using adequate activation strategy. For the experimental determination of minimal time for kefir grains activation, $t_{act,min}$, we developed the special methodology of minimizing the absolute deviation of pH ($t$) profiles. It is based on mathematical analysis of experimentally determined pH profiles during traditional propagation of differently activated kefir grains in milk under previously defined[17] optimal process conditions.

The fundamental principle of mathematical analysis is based on the assumption that $n$ experiments are performed with different activated kefir grains (e.i. for $i^{th}$ experiment the grains are activated for $t_{act,i}$ days, where: $t_{act,i} - t_{act,i-1} \geq 1$ d; $i \in N$; $t_{act,0} = 0$ d). During the experiments pH values versus fermentation time are monitored, therefore $pH_i(t)$ profile is assigned to $i^{th}$ experiment. Absolute deviation of pH ($t$) profile at $n$ experiments is calculated by the equation:

$$\Delta pH(t) = \max(A(t)) - \min(A(t)) \quad (1)$$

where the group of pH($t$) profiles is $A(t) = \{pH_1(t), pH_2(t), \ldots pH_n(t)\}$.

If absolute deviation at $n$ experiments meets the criterion of optimal activation time (tolerance criterion):

$$\Delta pH(t) \leq d_{max} \quad (2)$$

where $d_{max}$ is maximal allowed absolute deviation of pH($t$) profile, then minimal activation time for kefir grains is defined as $t_{act,min} = t_{act,1}$. In the opposite case, we eliminate $pH_1(t)$ from the group of pH($t$) profiles, $A(t)$, and recalculate the value of $\Delta pH(t)$ for $n - 1$ experiments. The procedure of successive elimination of pH($t$) profiles is repeated until absolute deviation reaches tolerance criterion. The group $A(t)$ have to include at least three elements. In the case of $j$ removed pH($t$) profiles, minimal time for kefir grain activation is $t_{act,min} = t_{act,j+1}$.

### 2. 1. 5. Predictive Kefir Grains Growth Model

Biomass growth during batch conditions yields well-known sigmoidal curve from which important biological parameters such as lag time duration, $t_L$, maximum specific growth rate, $\mu_{max}$, and asymptotic value, $A$, may be estimated.[18,19] There are several well-known growth models, such as logistic, Gompertz, Richards, that can describe this type of curve. Nevertheless, our previous study[14] shoved that the biomass growth curve during traditional batch propagation of classically activated kefir grains in fresh HTP whole fat cow's milk under optimal bioprocess conditions ($\vartheta$ = 24 °C, $\gamma_G$ = 20 g/L, $f_s$ = 90 $min^{-1}$) and $\gamma_{KG,0}$ = 75 g/L can be statistically most successfully described by the Gompertz predictive growth model as follows:

$$\ln\left(\frac{\gamma_{KG}}{\gamma_{KG,0}}\right) = A \exp\left(-\exp\left(\frac{\mu_{max}\exp(1)}{A}(t_L - t) + 1\right)\right) \quad (3)$$

where: $\gamma_{KG}$ – kefir grain mass concentration at $t$, g
$\gamma_{KG,0}$ – initial kefir grain mass concentration (inoculum), g, and
$t$ – fermentation (propagation) time, h

### 2. 1. 6. Analytical Methods and Biological Growth Parameters' Estimation

Experimental data of kefir grain mass versus time during batch propagation at previously defined optimal bioprocess conditions were used for optimally activated kefir grains growth curve construction. Kefir grain mass was determined using gravimetric method. Therefore, kefir grains were separated first from the culture media with a plastic household sieve. Then, the grains were carefully washed with cold water and dried on filter paper. Finally, kefir grain mass was determined by weighting on Mettler-Toledo analytical balance (PG5002-S).

Non-linear regression of experimental data using commercial available SigmaPlot 9.0 was used to estimate the biological parameters of Gompertz predictive growth model. The goodness of model's fit was assessed using the seven 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*.[14]

## 2. 2. Experimental Work

### 2. 2. 1. Experimental Optimization of Kefir Grains Activation Procedure

For experimental determination of minimal time for kefir grains activation the set of eight experiments at optimal process conditions has been performed – Tab. 1. Grains were activated according to the procedure described above. Activation time, $t_{act}$, was different for each experiment. For example, grains for experiment A-11-28 were activated over 11 subsequent days. Fermentation times were also different. Our preliminary experiments (using differently active kefir grains of unknown origin) indicated that fermentation could be completed after (12–14) h in the first place and after 28 h in the worst case. Therefore, the fermentation times were equally distributed wit-

**Table 1.** Set of experiments performed for $t_{act,min}$ determination.

| Experiment | | $t$ (h)[a] | $t_{act}$ (d) |
|---|---|---|---|
| Exp. No. | Designation | | |
| 1 | A–6–20 | 20 | 6 |
| 2 | A–7–16 | 16 | 7 |
| 3 | A–8–22 | 22 | 8 |
| 4 | A–9–26 | 26 | 9 |
| 5 | A–11–28 | 28 | 11 |
| 6 | A–13–18 | 18 | 13 |
| 7 | A–14–12 | 12 | 14 |
| 8 | A–15–24 | 24 | 15 |

[a] $t$ – fermentation time in RC1.

hin the interval, $t$ = (12–28) h and randomly assigned to individual experiments.

The incubation procedure was the same for all experiments. Individual experiment was performed by means of first charging the reactor by 1 L of fresh milk and adding 20 g of glucose ($\gamma_G$ = 20 g/L). This medium was heated up to working temperature ($\vartheta$ = 24 °C) at the optimal rotational frequency of the stirrer ($f_s$ = 90 min$^{-1}$).

After establishing the temperature steady state and dissolved glucose, we inoculated the medium with the mass, $m_{KG}$ = 75 g, of active kefir grains, which corresponds to initial kefir grain mass concentration, $\gamma_{KG,0}$ = 75 g/L. The addition of grains caused the fermentation of milk. Fermentation medium pH values were monitored during the process.

### 2. 2. 2. Experimental Data for Kefir Grains Growth Curve Construction

In order to obtain data for biomass growth curve construction during batch propagation of optimally activated kefir grains at previously defined optimal bioprocess conditions, a series of seven experiments were performed at different propagation times (4, 8, 16, 20, 24, 28 and 36 h). The experimental procedure was identical as the one described in section 2. 2. 1. The inoculation of optimally activated kefir grains caused the acidification (lactic, acetic and other organic acid formation) of culture medium and therefore, fermentation medium pH value was dynamically monitored during the kefir grains propagation. When the propagation was completed, the final kefir grain mass was gravimetrically determined.

## 3. Results and Discussion

### 3. 1. Graphic Analysis of Dynamic pH Profiles

Graphic comparison between experimentally measured pH($t$) profiles during traditional propagation of differently activated kefir grains in fresh milk at $\vartheta$ = 24 °C, $f_s$ = 90 min$^{-1}$, $\gamma_G$ = 20 g/L and $\gamma_{KG,0}$ = 75 g/L is presented in Fig. 1. Initial pH of fermentation medium was the same for all experiments, $pH_{FM,0}$ = 6.7 ± 0.02. After adding the kefir grains, fermentation started, where, among others, lactic, acetic and other organic acids synthesized. Therefore, pH started to decrease. Nevertheless, the formation of acids is not the only reason for pH drop. There is also some pH gradient between grains and fermentation medium. It has to be taken into account that grains are sourly and, therefore, their inoculation into the medium additionally reduces pH value. Within first 10 minutes after the inoculation, medium pH value dropped to, pH = 6.35 ± 0.05. It is evident from Fig. 1 that during this period the deviation in pH($t$) profiles of differently activated grains is insignificant, thereupon starts increasing. This behavior is result of different kefir grains activity. Maximum deviation appears in period between the sixth and seventh hour after the inoculation. Afterwards, the difference starts to decrease and after $t$ > 24 h reaches its minimum again.

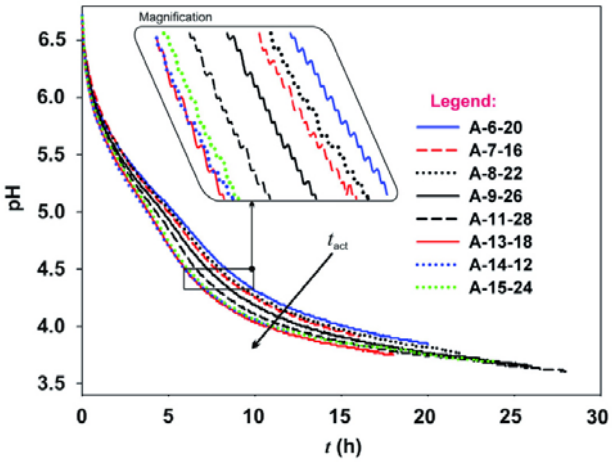


**Figure 1.** pH($t$) profiles of traditionally propagated kefir grains with different activation times in milk.

Within the region of maximum deviation in pH($t$) profiles experiments with less active grains (i.e. A-6-20) result in higher pH of fermentation medium compared to the more active ones (i.e. A-15-24). Furthermore, minimal time for kefir grains activation, where validity and repeatability of concentration profiles is assured, was defined using mathematical analysis of absolute pH($t$) deviation.

### 3. 2. Determination of Optimal Time for Kefir Grains Activation

Optimal time for kefir grains activation was determined using the methodology of minimizing the absolute de-

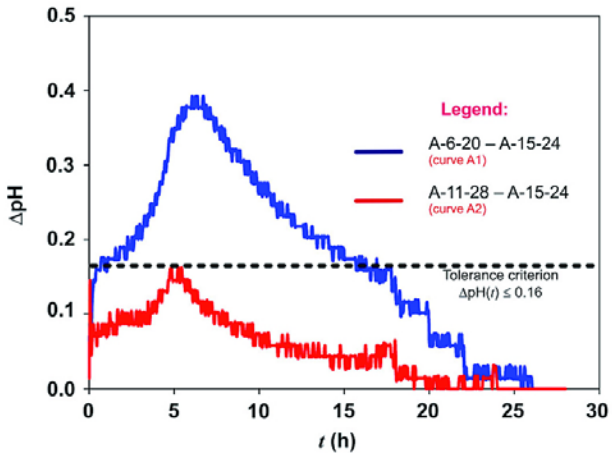


**Figure 2.** Mathematical analysis of pH($t$) profiles absolute deviation.

viation of pH($t$) profiles, ΔpH($t$), at proposed value $d_{max}$ = 0.16. Mathematical analysis of ΔpH($t$) for all eight experiments (Fig. 2, curve A1) showed that tolerance criterion (Eq. (2)) is not satisfied. According to the algorithm of minimum absolute deviation we removed from the group of pH($t$) profiles the ones for experiments A-6-20, A-7-16, A-8-22 and A-9-26. Afterwards, ΔpH($t$) for remained four experiments (A-11-28, A-13-18, A-14-12 and A-15-24) was calculated again.

Curve A2 in Fig. 2 confirms minimal absolute deviation of the rest of the pH($t$) profiles in group A($t$), which corresponds to the tolerance criterion over complete fermentation time interval. For this reason minimal time for kefir grains activation, $t_{act,min}$, was established as 11 d.

## 3. 3. Growth Curve of Optimally Activated Kefir Grains

Measured ($m_{KG}$) and calculated ($m_{KG,i}$, $w_{KG,i}$) values obtained for different (regarding the fermentation time) batch propagations of optimally activated kefir grains at previously defined optimal bioprocess conditions are presented in Tab. 2. Kefir grain mass increase, $m_{KG,i}$ is the difference between final kefir grain mass, $m_{KG}$ and initial kefir grain mass, $m_{KG,0}$, meanwhile, kefir grain increase mass fraction $w_{KG,i}$is defined as quotient between $m_{KG,i}$ and $m_{KG,0}$ multiplied with 100.

According to the results presented in Tab 2, we inferred that the highest kefir grain mass increase fraction (average value of experiments 5, 6 and 7), $w_{KG,i}$ = (24.0 ± 0.5) % was achieved already after 24 h. Moreover, the daily $w_{KG,i}$ during propagation of optimally activated kefir grains is almost the same as the one obtained in our previous study ($w_{KG,i}$ = (24.9 ± 1.0) %)[14], where classically ($t_{act}$ = 6 d) activated kefir grains were used. Generally, we can establish that daily $w_{KG,i}$ is independent of kefir grains activity, although at the same time, it should be emphasized that weakly or even non-active kefir grains yield much smaller daily $w_{KG,i}$.

Growth curve, i.e. logarithm of relative kefir grain mass versus time, during batch propagation of optimally activated ($t_{act,min}$ = 11 d) kefir grains at optimal process conditions is presented in Fig. 3. Moreover, the present data are also graphically compared with the data obtained by our previous study,[14] where classically activated kefir grains were used.

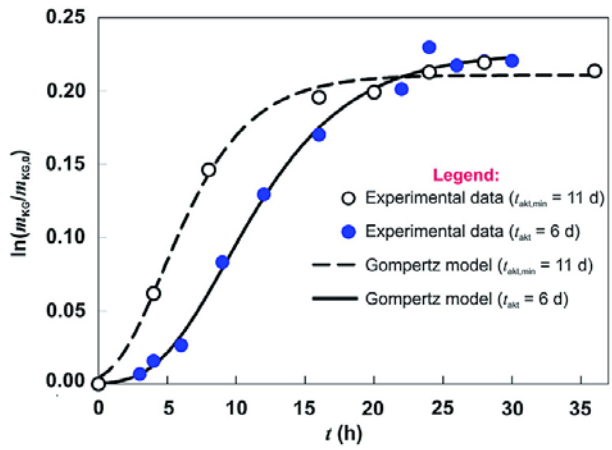


**Figure 3.** Growth curves of optimally ($t_{act,min}$ = 11 d) and classically ($t_{act}$ = 6 d) activated kefir grains fitted with Gompertz model.

The experimental data (Fig. 3) were fitted to Gompertz model (Eq. 3) by non-linear regression. Estimated biological growth parameters ($\mu_{max}$, $t_L$, $A$) and statistical indicators (*SE*, *CV*, $R_{adj}^2$, *RMSE*, *F*, *PRESS* and *t-st*) are collected in Tab. 3. The values of statistical indicators prove that Gompertz model is statistically appropriate not only for describing the classically, as we established in our previous paper,[14] but also for optimally activated kefir grains growth curve.

Results presented in Tab. 3 shows a significant feature. The values of $A$ are very similar to each other in both cases, i.e. growth curves of classically and optimally activated kefir grains, whilst $t_L$ and $\mu_{max}$ values are observably different. The growth curve of optimally compared to classically activated kefir grains has much shorter lag phase which has been confirmed with smaller $t_L$ value. Higher $\mu_{max}$ value of optimally activated grains indicates their greater activity. On the basis of graphical (Fig. 3) and biological parameters' analysis (Tab. 3), we can establish that, in spite of equal daily kefir grain mass increase, considerable difference in growth curves (and consequently in concentration profiles) during propagation of classi-

**Table 2.** Experimental and calculated data at different batch propagation times.

| Experiment | | $t$ (h) | $m_{KG}$ (g) | $m_{KG,i}$ (g) | $w_{KG,i}$ (%) |
|---|---|---|---|---|---|
| Exp. No. | Designation | | | | |
| 1 | GC-O-4 | 4 | 79.78 | 4.78 | 6.4 |
| 2 | GC-O-8 | 8 | 86.79 | 11.79 | 15.7 |
| 3 | GC-O-16 | 16 | 91.20 | 16.20 | 21.6 |
| 4 | GC-O-20 | 20 | 91.52 | 16.52 | 22.0 |
| 5 | GC-O-24 | 24 | 92.78 | 17.78 | 23.7 |
| 6 | GC-O-28 | 28 | 93.39 | 18.39 | 24.5 |
| 7 | GC-O-36 | 36 | 92.85 | 17.85 | 23.8 |

**Table 3.** Comparison of estimated biological parameters ($\mu_{max}$, $t_L$, $A$) and statistical indicators (*SE, CV,* $R_{adj}^2$, *RMSE, F, PRESS* and *t-st*) derived from fitting growth curves of differentlly activated kefir grains with Gomperz model.

| Curve | Parameter | Estimate | *SE* | *CV* (%) | $R_{adj}^2$ | *RMSE* | *F* | *PRESS* | *t–st* |
|---|---|---|---|---|---|---|---|---|---|
| C | $\mu_{max}$ (1/h) | 0.0167 | 0.0011 | 6.6 | 0.995 | 0.007 | 1125 | 0.0007 | 14.7 |
| | $t_L$ (h) | 4.2271 | 0.4103 | 9.7 | | | | | 10.4 |
| | $A$ (1) | 0.2263 | 0.0044 | 1.9 | | | | | 51.6 |
| O | $\mu_{max}$ (1/h) | 0.0223 | 0.0026 | 11.7 | 0.992 | 0.007 | 460 | 0.0007 | 8.5 |
| | $t_L$ (h) | 1.2256 | 0.5398 | 44.0 | | | | | 2.3 |
| | $A$ (1) | 0.2108 | 0.0036 | 1.7 | | | | | 58.8 |

C – Growth curve of classically activated kefir grains, O – Growth curve of optimally activated kefir grains

cally and optimally activated kefir grains exists. Therefore, repeatability of kefir grain propagations in milk under optimal process conditions, which means approaching growth curve to the optimal one, can be achieved only if optimally activated kefir grains are used as inoculum.

Finally, in order to confirm the repeatability of concentration profiles (using pH measurements) during propagations of optimally activated kefir grains we dynamically measured the fermentation pH values and afterwards mathematically analyzed absolute deviation of pH($t$) profile, ΔpH($t$), (Eq. 1) for all seven experiments (GC-O-4 – GC-O-36). The graphic results, i.e. pH and ΔpH (curve C) versus time, are presented in Fig. 4.

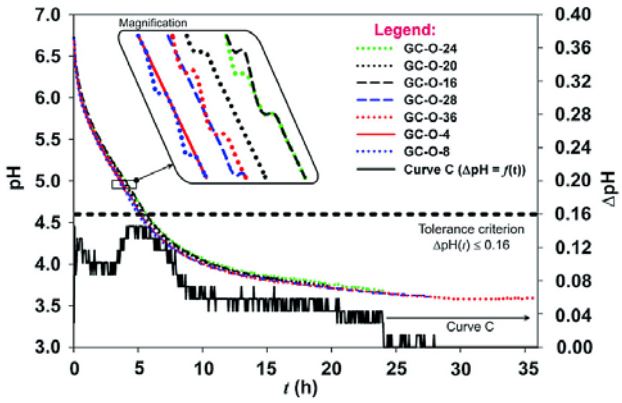


**Figure 4.** pH analysis of growth curve – optimally activated kefir grains.

Curve C in Fig. 4 confirms that absolute deviation of all pH($t$) profiles corresponds to the tolerance criterion over complete batch propagation time interval. Therefore, we can affirm that repeatability of concentration profiles during experiments was assured and consequently the results of growth curve modeling of optimally activated kefir grains are valid.

## 4. Conclusion

The novel approach of minimizing the absolute deviation of pH($t$) profiles was used as a basis for improving the activation procedure. It was established that validity and repeatability of fermentation medium concentration profiles (followed by pH($t$) measurements) in traditional kefir grains propagation could be ensured only by the kefir grains, previously activated at least eleven successive days. This statement is valid for grains which were previously activated according to procedure described above and under optimal propagation conditions ($\vartheta$ = 24 °C, $\gamma_G$ = 20 g/L, $f_s$ = 90 min$^{-1}$). Furthermore, regression analysis of experimental data using Gompertz predictive growth model confirmed our previsions that growth curves of optimally and classically activated kefir grains both have equal asymptotic value, but their profiles are markedly different. Also optimally activated kefir grains have much shorter lag phase and attain higher maximum specific growth rate.

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## Povzetek

Profili krivulj rasti kefirnih zrn so v veliki meri odvisni od bioprocesnih pogojev in aktivnosti inokuluma, zato so za njihove natančne študije potrebna optimalno aktivna zrna. Namen naše raziskave je bil v prvi fazi eksperimentalno optimiranje klasičnega postopka aktivacije kefirnih zrn in nadalje primerjava krivulj rasti različno aktiviranih zrn. V ta namen smo v računalniško vodenem reakcijskem kalorimetru RC1 izvedli serijo eksperimentov, pri katerih smo zasledovali spreminjanje pH vrednosti med šaržnim razmnoževanjem različno aktiviranih kefirnih zrn. Eksperimentalne podatke smo matematično analizirali in hkrati s posebno metodologijo minimiziranja absolutnega odstopanja pH($t$) profilov določili minimalni čas aktivacije zrn. Dokazali smo, da optimalno aktivnost inokuluma lahko zagotovimo le z zrni, ki so bila predhodno aktivirana najmanj 11 zaporednih dni. V drugem delu raziskave smo na osnovi eksperimentalnih meritev konstruirali krivuljo rasti optimalno aktiviranih zrn in jo prilegali k Gompertz-ovemu modelu. Dobljene rezultate smo primerjali s podatki iz predhodne raziskave[14], kjer so bila uporabljena klasično aktivirana zrna. Ugotovili smo, da kljub enakemu dnevnemu prirastu obstajajo znatne razlike v krivuljah rasti različno aktiviranih kefirnih zrn.