Influence of temperature variations on ethanol production by kefir grains – mathematical model development

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Mathematical models are useful for describing microbial growth, product formation and substrate utilization, both in natural ecosystems and under research conditions. To this end, we developed a mathematical model for the kinetics of ethanol production that incorporates the influence of the temperature variations and fermentation time. Modified Gompertz model could be regarded as sufficient to describe the ethanol production by mixed kefir grains yeast population with high determination coefficients indicated that. Biological kinetic parameters of the model were estimated using the least-square method on the basis of experimental data obtained in batch fermentations. The influence of temperature on kefir grains biomass increase and microbiological composition of kefir product and kefir grains was also investigated and quantified. The temperature dependence of the maximum ethanol production rate constant was analyzed based on the Arrhenius model. The activation energy value of ethanol production was 64.3 kJ/mol. The adopted mathematical model could describe very well the dynamics of ethanol production from the beginning up to the stationary phase during the kefir fermentation.

1. Introduction

Kefir is a probiotic mixture which has proven beneficial properties for illnesses (Urdaneta et al., 2007). It is an acid-alcoholic dairy beverage, traditionally produced with kefir grains which have a complex microbiological composition. Lactobacilli, lactic streptococci, yeast, and acetic acid bacteria have been shown to be present in them (Bosch et al., 2006). The kefir-specific yeasts, which during the fermentation cause the generation of ethanol (0.7–2.5 %) and CO_2, play a key role in the creation of kefir characteristics (Simova et al., 2002). Ethanol, together with other flavour compounds, creates the desired aroma and flavour of kefir (Beshkova et al., 2003). Factors effecting the ethanol production include pH of the culture medium, concentrations of inoculums and substrates, substrate composition, washing of grains, the type of milk. One of the most important operating variables is temperature (Sánchez et al., 2004).

Therefore, in the present paper, insight into the influence of fermentation temperature on kefir fermentation was investigated. In order to describe temperature effect, mathematical model was developed to illustrate the effect of temperature on kefir grains biomass increase and ethanol production during the 24 h kefir fermentation. Likewise, the objective of the research reported in this paper was also to study the influence of temperature on the microbiological composition of kefir grains and kefir product in the manufacture of kefir made from cows' milk. Furthermore, the dependency of the maximum ethanol production rate on the temperature was explained by the superposition of activation energy for ethanol production. The dependence of the kinetic coefficients of ethanol production on temperature using Arrhenius-type equations for kefir fermentation was proposed. The results consequently provide a better understanding of temperature effects on the cell activities for further development of the fermentation process.

2. Materials and methods

2.1 Fermentations

One liter of fresh HTP milk, was added to the batch reactor and heated up to the reaction temperature, ϑ = (15–31) °C. After reaching the temperature steady state the kefir grains were inoculated (γ_{KG} = 42 g/L). During the 24 h experiment, the milk-grains mixture was agitated periodically each hour for 1 min at f_{m} = 60 min^{-1}. Furthermore, the additional four experiments at the selected temperatures (15, 20, 25, and 30 °C) were necessary for determination of the activation energy for ethanol production.

2.2 Assays

Kefir grains dry mass was determined by weighting on analytical balance. After determining the grains mass, the grains were returned to the fresh milk.

Ethanol concentration was determined by ReactIR$^{\mathrm{TM}}$iC10 system from Mettler Toledo.

2.3 Microbiological analysis

Kefir grains (m_{KG} = 10 g) and 90 mL of a sterile solution of sodium citrate were homogenized for 5 min prior to plating. Samples of kefir and grains homogenates were serially diluted in sterile ¼ strength Ringer solution (IDF, 1992). Subsequent serial dilutions were plated on Rogosa, M-17, and on YGC agar for enumeration of respectively the lactobacilli, the lactic streptococci and the yeasts. Plates were incubated during 3–4 days at 37 °C for the lactobacilli, 2–3 and 3–5 days at 28 °C for the lactic streptococci and the yeasts, respectively.

3. Results and discussion

3.1 Influence of temperature

3.1.1 Kefir grains biomass increase

The experimentally determined values of kefir grains biomass increase are plotted against the temperature in Fig. 1. It is evident, that throughout the fermentations, temperature has a high positive linear effect on the kefir grains biomass increase. Therefore, the relationship between the kefir grains biomass increase and temperature can be mathematically described with the linear equation:

$$\Delta\gamma_{\mathrm{KG}}(\vartheta) = k\vartheta + \Delta\gamma_{\mathrm{KG,0°C}} \tag{1}$$

where ϑ is the temperature (°C), $\Delta\gamma_{\mathrm{KG}}$ is the kefir grains biomass increase after 24 h fermentation at selected temperature (g/L), $\Delta\gamma_{\mathrm{KG,0°C}}$ is the kefir grains biomass increase after 24 h at 0 °C (g/L) and k is the constant, which represents the change of kefir grains biomass increase per temperature unit (g/L °C). Using the last-squares curve-fitting method (SigmaPlot$^{®}$10.0 software), values of the $\Delta\gamma_{\mathrm{KG,0°C}}$ and k estimated from the experimental data in the range of (15–31) °C were obtained.

The estimated values of $\Delta\gamma_{\mathrm{KG,0°C}}$ and k were (–3.866 ± 1.056) g/L and (0.545 ± 0.045) g/L°C, respectively. The negative value of $\Delta\gamma_{\mathrm{KG,0°C}}$ indicates that at 0 °C the initial value of kefir grains biomass ($\gamma_{\mathrm{KG,0}}$ = 42 g/L) is going to be decrease for about (3.866 ± 1.056) g/L. The linear equation was found to be the appropriate (R^2 = 0.955) to successfully describe the temperature dependence of kefir grains biomass increase after 24 h fermentation in the range of 15–31 °C.

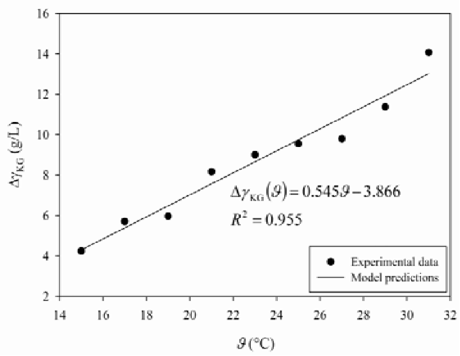

Fig. 1 Influence of fermentation temperature on the kefir grains biomass increase.

3.1.1 Ethanol mass concentration

It was found out that non-linear Gompertz model successfully describes the ethanol production during kefir fermentation (Zajšek and Goršek, 2009):

$$\gamma_{\mathrm{EtOH}} = \gamma_{\mathrm{EtOH,m}} \exp\left\{ -\exp\left[\frac{r_{\mathrm{EtOH,m}} \exp(1)}{\gamma_{\mathrm{EtOH,m}}} \left(t_{\mathrm{L}} - t\right) + 1 \right] \right\} \tag{2}$$

where γ_{EtOH} is the ethanol mass concentration (g/L), $\gamma_{\mathrm{EtOH,m}}$ is the potential maximum ethanol mass concentration (g/L), $r_{\mathrm{EtOH,m}}$ is the maximum ethanol production rate (g/L h) and t_{L} is the lag phase or the time to exponential ethanol production (h). Our studies have shown that temperature does not affect $\gamma_{\mathrm{EtOH,m}}$. Therefore, $\gamma_{\mathrm{EtOH,m}}$ was assumed to be a constant value. It has been reported that throughout the fermentation, temperature is the factor which has an important influence on the ethanol production (Dragone et al., 2004). Therefore we expressed the model parameters ($r_{\mathrm{EtOH,m}}$ and t_{L}) as the linear functions of temperature:

$$r_{\mathrm{EtOH,m}} = a + b\vartheta \quad (3) \qquad \text{and} \qquad t_{\mathrm{L}} = c + d\vartheta \quad (4)$$

where a, b, c, and d are the constants.

If γ_{EtOH} is expressed as a function of fermentation time and temperature, using Eqs. (3, 4), Eq. 2 becomes:

$$\gamma_{\mathrm{EtOH}} = \gamma_{\mathrm{EtOH,m}} \exp\left\{ -\exp\left[\frac{\left(a + b\vartheta\right)\exp(1)}{\gamma_{\mathrm{EtOH,m}}} \left(\left(c + d\vartheta\right) - t\right) + 1 \right] \right\} \tag{5}$$

Ethanol production is shown in Fig. 2. Lag time for ethanol production varied from (5–19) h. Furthermore, with regard to fermentation temperature influence, the experimental data of ethanol concentration show that at the end of lag phase, yeasts produced similar ethanol values at 15 °C and 17 °C, while ethanol level was found to be higher in the range of (19–31) °C.

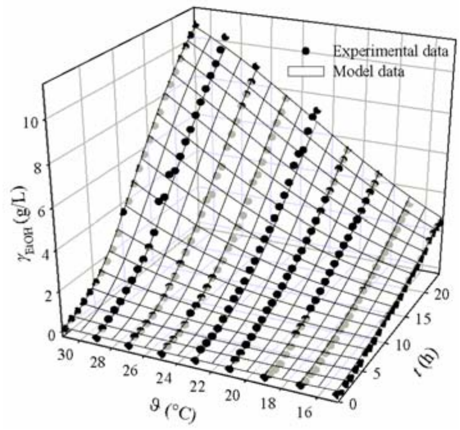

Fig. 2 Ethanol concentrations at different controlled temperatures.

The highest ethanol concentration was observed at 31 °C (γ_{EtOH} = 10.7 g/L). Variation in ethanol concentration produced during kefir fermentation could be due to changes in the availability of nutrients and other environmental factors which influence the population of kefir grains yeasts. The determination coefficient (R^2) and constants, estimated by using the Eq. (5), are given in Table 1.

Table 1 Predicted parameters obtained from modified Gompertz model.

$\gamma_{\mathrm{EtOH,m}}$ (g/L)	a (g/L h)	b (g/L h °C)	c (h)	d (h/°C)	R^2
13.425 ± 0.379	− 0.182 ± 0.025	0.025 ± 0.001	17.044 ± 0.718	− 0.416 ± 0.026	0.991

High value of determination coefficient (R^2 = 0.991) shows that Eq. (5) can be used to describe the effects of fermentation temperature and time on the ethanol production by kefir grains within the limits of this study (15–31 °C, 0–24 h).

3.1.2 Effect of temperature on microbiological characteristics

Fig. 3 shows the temperature effect on the mean changes in the counts of the different microbial groups. Counts of presumptive lactobacilli and yeasts in kefir increased slowly within the range of (15–31) °C by increasing the temperature. This indicates that all the yeasts and lactobacilli cultures, have a potential to proliferate in the milk during the fermentation inside the selected temperature interval. In kefir grains the number of counts of lactobacilli was relatively constant, showing only a slight increase in the range of 25–29 °C. The counts of yeasts in kefir grains decrease slightly in the range from 17 to 19 °C and thereafter increased progressively between (19–25) °C. In the range of temperature higher than 25 °C, there was a progressive decrease in the counts of yeasts, presented in kefir grains. This could indicate that at higher temperatures yeasts started to die quickly. The counts of presumptive lactic streptococci in kefir and kefir grains increased considerably in the range of temperature, ϑ = (15–19) °C. Lactic streptococci grow and poliferate rapidly at temperatures lower than 20 °C, when they are in the symbiosis with yeasts. From then onwards, there was a progressive and marked decrease until the 23 °C. At temperatures higher than 23 °C the counts of lactic streptococci were approximately constant. Lower counts suggest possible inhibition of lactic streptococci in the presence of yeasts due to high levels of gas and alcohol produced as it grows in milk. It was previously reported that high levels of alcohol in naturally fermented milk retarded the growth of LAB, which include streptococci (Kebede et al., 2007). The counts of presumptive lactobacilli obtained at 24 h fermentation and 23 °C are of the order of 1 and 0.5 log units lower for kefir and kefir grains respectively than those observed by Farnworth (2005). Moreover, in the current study the counts of yeasts after 24 h of fermentation at 23 °C are higher (1.5 and 0.3 log units for kefir and kefir grains) compared to those observed by Farnworth (2005), even though he did not specify the fermentation conditions.

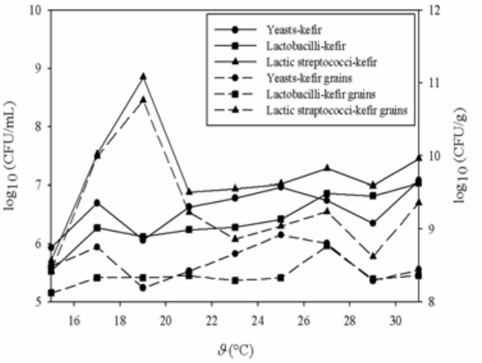

Fig. 3 Influence of fermentation temperature on the changes in the microbial counts in kefir and kefir grains.

3.2 Activation energy

Fig. 4 demonstrated the experimental results, as well as the modelled values of the ethanol concentrations at different temperatures.

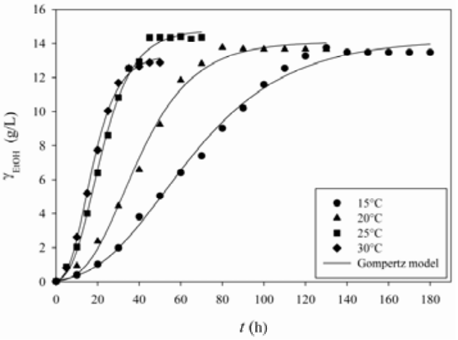

Fig. 4 Experimental results and simulations of ethanol concentrations at different operating temperatures.

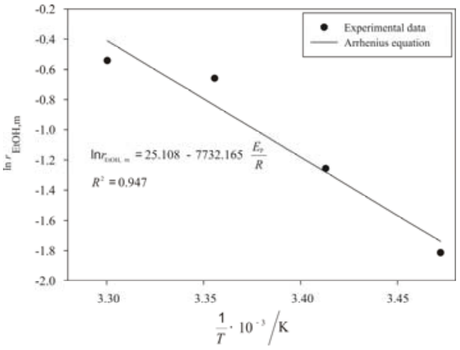

Fig. 5 Arrhenius plot for ethanol production by kefir grains yeast.

Experimental data show that the maximum ethanol production rates ($r_{\mathrm{EtOH,m}}$) enhance by the increase of the isothermal control from (15–30) °C. At low temperature the duration of lag phase was around 20 h, whilst at temperature 30 °C it decreased to less than 6 h. Following this there was a relatively short exponential production phase between (2–10) h for temperatures within the range of (30–15) °C. Subsequently there was a considerably longer period of between (15–80) h during which ethanol production remained fairly linear. The maximum ethanol concentration was obtained after 45 h of the fermentation at 25 °C. The effects of the temperature on the biological parameters *i.e.*, $\gamma_{\mathrm{EtOH,m}}$, $r_{\mathrm{EtOH,m}}$, and t_{L} were calculated using the Gompertz model from Eq. (2), are given in Table 2.

Table 2 Predicted values of kinetics parameters obtained from the Eq. (2).

ϑ (°C)	$\gamma_{\mathrm{EtOH,m}}$ (g/L)	$r_{\mathrm{EtOH,m}}$ (g/L h)	t_{L} (h)	R^2
15	14.215 ± 0.246	0.163 ± 0.008	19.778 ± 2.069	0.995
20	14.092 ± 0.239	0.284 ± 0.019	14.233 ± 1.710	0.994
25	14.757 ± 0.171	0.518 ± 0.022	7.245 ± 0 615	0.997
30	13.312 ± 0.161	0.582 ± 0.022	5.918 ± 0.429	0.998

The validation of the model was checked by using R^2 obtained from the Gompertz model. The $\gamma_{\mathrm{EtOH,m}}$ values for the ethanol production varied from (14.215 ± 0.246) g/L to (13.312 ± 0.161) g/L at 15 °C and 30 °C, respectively. Statistical analysis showed that temperature had no important impact on the $\gamma_{\mathrm{EtOH,m}}$ (Table 2). Through the fermentation, the maximum production rate for ethanol formation by kefir grains yeast ranged from, (0.163 ± 0.008) g/L h to (0.582 ± 0.022) g/L h. The highest $r_{\mathrm{EtOH,m}}$ was found at 30 °C.

Lag time varied between (5.918 ± 0.429) h and (19.778 ± 2.069) h. Statistical analysis indicated that fermentation temperature altered the length of t_{L} of ethanol production (Table 2). It could be consequence of acclimated kefir grains transferred to the milk medium, which caused different adaptation to change in temperature condition.

On the basis of the estimated $r_{\mathrm{EtOH,m}}$ values, shown in Table 2, it can be seen that the maximum ethanol production rate was achieved at 30 °C. Expressed by the Arrhenius relationship:

$$r_{\mathrm{EtOH,\,m}} = r_{\mathrm{EtOH,0}}\ e^{-\frac{E_{\mathrm{p}}}{RT}} \tag{6}$$

where $r_{EtOH,m}$ represents the maximum ethanol production rate (g/L h), E_p represents the apparent energy of ethanol production (kJ/mol), T is temperature (K), R is ideal gas constant (8.314 J/mol K), and $r_{EtOH,0}$ the corresponding pre-exponential factor (g/L h). The temperature dependency of the maximum ethanol production rate was fitted very well with the experimental results (Fig. 5). The results obtained via non-linear regression are set out in Table 3, where it can be seen that value for E_p is in the range of activation energies for enzyme reactions, E_a = (40–80) kJ/mol, (Doran, 2004).

Table 3 Apparent activation energy and pre-exponential factor.

E_p (kJ/mol)	$r_{EtOH,0}$ (g/L h)	R^2
64.3	8.0×10^{10}	0.946

During the long period after exponential production phase, ethanol concentration increased linearly. The linear ethanol production begins earlier as the temperature rises. This behaviour may be explained by the fact that solubility of oxygen in the culture diminishes concomitantly with a rise in temperature. The duration of the linear ethanol production phase also decreases in regard to different temperatures: at 15 °C it lasts up to 80 h whilst at 30 °C it lasts no more than 15 h, which might be expected if we bear in mind that the coefficient of oxygen diffusion within the medium, and thus the coefficient of oxygen transport, increases exponentially with temperature.

4. Conclusion

A linear mathematical model was developed in order to follow the kefir grains biomass increase in dependence of fermentation temperature after 24 h fermentation. Modified Gompertz model was well fitted to the experiment data and it could be regarded as sufficient to describe the ethanol production by mixed kefir grains yeast population in the milk medium as a function of fermentation time and temperature regimes. The estimated activation energy for ethanol production was 64.3 kJ/mol.

Utilization of mathematical models will contribute to a better understanding of the environmental effect on the biomass activities and therefore on the bio-products production and could be applied as a tool for further process development.

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