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## KINETICS OF SACCHAROSE FERMENTATION BY KOMBUCHA

### Article Highlights

- Kinetics of saccharose fermentation by Kombucha was analysed
- A saccharose concentration model was defined as a sigmoidal function
- Reaction rates were calculated as first derivatives of Boltzmann's functions
- Saccharose fermentation by Kombucha occurred according to complex kinetics

### Abstract

*The kinetics of saccharose fermentation by Kombucha is not yet well defined due to lack of knowledge of reaction mechanisms taking place during this process. In this study, the kinetics of saccharose fermentation by Kombucha was analysed using the suggested empirical model. The data were obtained on 1.5 g L<sup>-1</sup> of black tea, with 66.47 g L<sup>-1</sup> of saccharose and using 10 or 15% (V/V) of Kombucha. The total number of viable cells was as follows: approximately 5×10<sup>6</sup> of yeast cells per mL of the inoculum and approximately 2×10<sup>6</sup> of bacteria cells per mL of the inoculum. The samples were analysed after 0, 3, 4, 5, 6, 7 and 10 days. Their pH values and contents of saccharose, glucose, fructose, total acids and ethanol were determined. A saccharose concentration model was defined as a sigmoidal function at 22 and 30 °C, and with 10 and 15% (V/V) of inoculum quantity. The determination coefficients of the functions were very high (R<sup>2</sup> > 0.99). Reaction rates were calculated as first derivatives of Boltzmann's functions. No simple correlation between the rate of reaction and independent variables (temperature and inoculum concentration) was found. Analysis of the empirical model indicated that saccharose fermentation by Kombucha occurred according to very complex kinetics.*

*Keywords:* Kombucha, saccharose fermentation, kinetics, empirical model.

The consortium of yeasts and acetic acid bacteria known as the Kombucha culture exhibits a metabolic activity on sweetened tea, under batch conditions, giving a pleasant sour beverage containing useful compounds such as some organic acids and certain vitamins. The activity of Kombucha on the traditional carbon source saccharose was investigated by several authors [1-6] and the main pathways of saccharose transformation were determined. It has been proven that the yeast cells are responsible for the extracellular enzymatic hydrolysis of saccharose into glucose and fructose, and transformation of glucose and fructose into ethanol and CO<sub>2</sub>, while acetic acid

bacteria cause the conversion of glucose into gluconic and ketogluconic acid and fructose into acetic acid as a consequence of the metabolic chain reactions [1]. The association between the two types of microorganisms is unique. Yeasts produce ethanol, which stimulates the growth of acetic acid bacteria and production acetic acid [7]. Thanks to the bacteria's ability to synthesize cellulosic network during fermentation, parts of the yeast cells are entrapped within this network.

Apart from the monosaccharides and the mentioned main metabolites, reaction mixture contains a great number of organic acids, such as glucuronic, succinic, mannonic, propionic, ascorbic, saccharic, etc. [8-13]. Also present are proteins (enzymes) produced during fermentation, tannins and their derivatives introduced by the tea broth [14,15], terpenoids, carotenoids, lipids and linoleic acid, due to

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degradation of amino acids [16], as well as a certain amount of useful compounds [14,17-22] such as vitamins B<sub>1</sub>, B<sub>2</sub>, B<sub>3</sub>, B<sub>6</sub> and B<sub>12</sub>, folic acid [23,24] and vitamin C [10,25].

Kombucha beverages have been intensively consumed during a long time worldwide for its prophylactic and therapeutic properties. Novel studies show antimicrobial activity [26], hepatoprotective properties [27] and beneficial effects on different diseases including diabetes [28].

According to the available published literature, an exact mechanism of saccharose fermentation by Kombucha is not known. Accordingly, very little knowledge on the kinetics of such fermentation is available. Also, no attempts to suggest an empirical model has been reported to date. Although hydrolysis of saccharose is a single-substrate step, subsequent reactions are multi-substrate with extremely complex kinetics [2]. The best understood reaction parameters are temperature, composition of Kombucha and inoculum concentration. Several authors reported 28 °C as an optimal temperature for Kombucha fermentation [25,29]. It has been proven that the composition of fermented tea greatly depend on the individual Kombucha association [1,23] as well as on the concentration of inoculum solution. As far as saccharose (substrate) is concerned, several concentrations were studied: 30, 50, 70 and 100 g L<sup>-1</sup> [1,2,6,23].

This paper presents the kinetics of saccharose fermentation by Kombucha, as a function of temperature and inoculum concentration. Experimental data were fitted to an empirically-derived model.

## EXPERIMENTAL

### Kombucha culture

Local domestic Kombucha, used for the fermentation, contains at least five yeast strains (*Saccharomyces ludwigii*, *Saccharomyces cerevisiae*, *Saccharomyces bisporus*, *Torulopsis* sp. and *Zygosaccharomyces* sp.) as determined by Markov *et al.* [30]. It is well known that Kombucha bacteria belong to the strains of the genus *Acetobacter* [1,2,5]. The total number of viable cells was as follows: approximately 5×10<sup>5</sup> of yeast cells per mL of the inoculum and approximately 2×10<sup>6</sup> of bacteria cells per mL of the inoculum.

### Kombucha fermentation

Tap water was boiled, sweetened with 66.47 g L<sup>-1</sup> of saccharose and mass of 1.5 g L<sup>-1</sup> of black tea (Indian tea, "Vitamin", Horgoš, Serbia) was added and removed by filtration after 15 min. Tea was

cooled to room temperature and afterwards inoculated with 10 or 15% (V/V) of fermentation broth obtained from previous Kombucha fermentation. The beakers (beaker volume 2 L, diameter 13 cm, liquid volume 1.1 or 1.15 L) were covered with cheesecloth and incubated in a temperature-controlled bath at 22 and 30 °C for 10 days. The samples from Kombucha fermentation were taken after 0, 3, 4, 5, 6, 7 and 10 days and following quantities were measured: pH value, total acids, saccharose content, glucose content, fructose content and ethanol content.

### Methods of analysis

The pH values were determined using a pH-meter (pH Spear, Eutech Instruments Oakton, England).

Total acids content was determined as titratable acidity. After removing CO<sub>2</sub> from the fermentation broth, a sample of 20 mL was taken and titrated with 0.1 mol L<sup>-1</sup> of NaOH, while phenolphthalein was used as an indicator and calculated in g L<sup>-1</sup> acetic acid according as:  $m_{\text{acetic acid}} = 50(V_{\text{NaOH}} C_{\text{NaOH}} M_{\text{acetic acid}} / 1000)$ .

Saccharose, glucose and fructose contents were determined by using the Boehringer Mannheim Kit (Cat. No. 716260) and ethanol content by Boehringer Mannheim Kit (Cat. No. 176290).

Total counts of cells of yeasts and acetic acid bacteria in the fermentation mixture were determined by plate counting method. The medium for determining yeasts was Sabouraud-4% maltose Agar (Merck, Darmstadt, Germany) with addition of 50 mg L<sup>-1</sup> of chloramfenicol (Sigma-Aldrich, St. Louis, MO, USA). The plates were incubated for 72 h at 28 °C. The medium for determining acetic acid bacteria was yeast peptone mannitol Agar (Difco, Detroit, MI, USA), containing 500 mg L<sup>-1</sup> cycloheximide (actidione; Sigma-Aldrich, St. Louis, MO, USA) to inhibit yeasts growth. The incubation at 28 °C lasted for 5-7 days.

All experiments were performed in duplicate, under the same conditions. Each quantity was measured three times and the arithmetic mean values were calculated. The means were used as relevant data basis for further processing.

## RESULTS AND DISCUSSION

### Results of measurements

Kinetics of saccharose fermentation by Kombucha, at two temperatures (22 and 30 °C) and in the samples inoculated with two inoculum quantities (10 or 15% (V/V)), was examined through measuring pH change during fermentation (Figure 1) as well as

through determining concentration change of substrate (saccharose) and intermediates/products - glucose, fructose, total acids and ethanol (Figure 2). Such a plan of experiments intended to approve the assumed chemical model written in a general form:

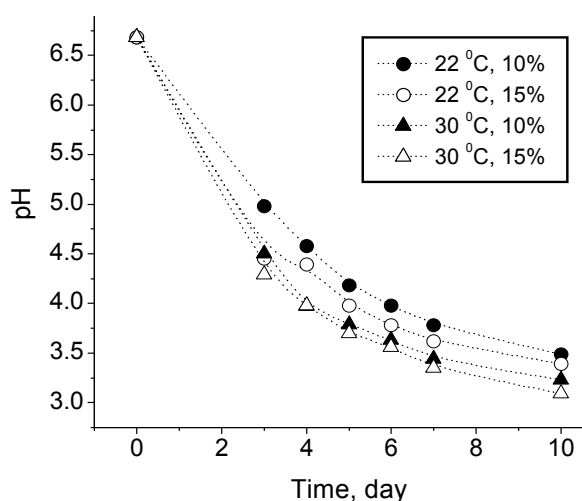
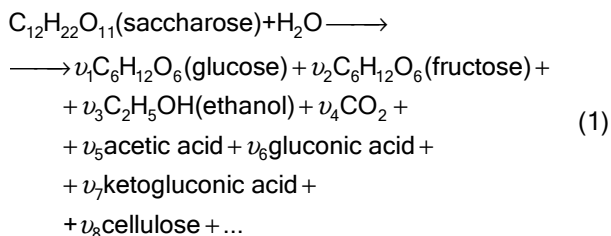


Figure 1. Change of pH as a function of temperature and inoculum concentration; lines (B-spline) used only as guide to the eye.

As it is known, reaction (1) is catalysed by enzymes produced by the cultures from Kombucha inoculum. Saccharose molecules bind to the enzyme's active sites where they are transformed into glucose and fructose, which continued to convert to other products through a number of consecutive and parallel reactions. Therefore, the reaction mixture changes its composition (qualitative and quantitative) during fermentation. This change is described by the stoichiometric coefficients ( $v$ ) which are time dependent.

During the fermentation, pH of the broth decreased almost exponentially, without a noticeable lag phase, due to accumulated organic acids (Figure 1). The acids were determined as the titratable acidity, which was expressed in grams of acetic acid per litre of the sample (Figure 2). Approximately 15% lower pH values were achieved at higher temperatures in samples that had been inoculated with larger quantity of inoculum solution. This is in accordance with the measured values of total quantity of generated acids,

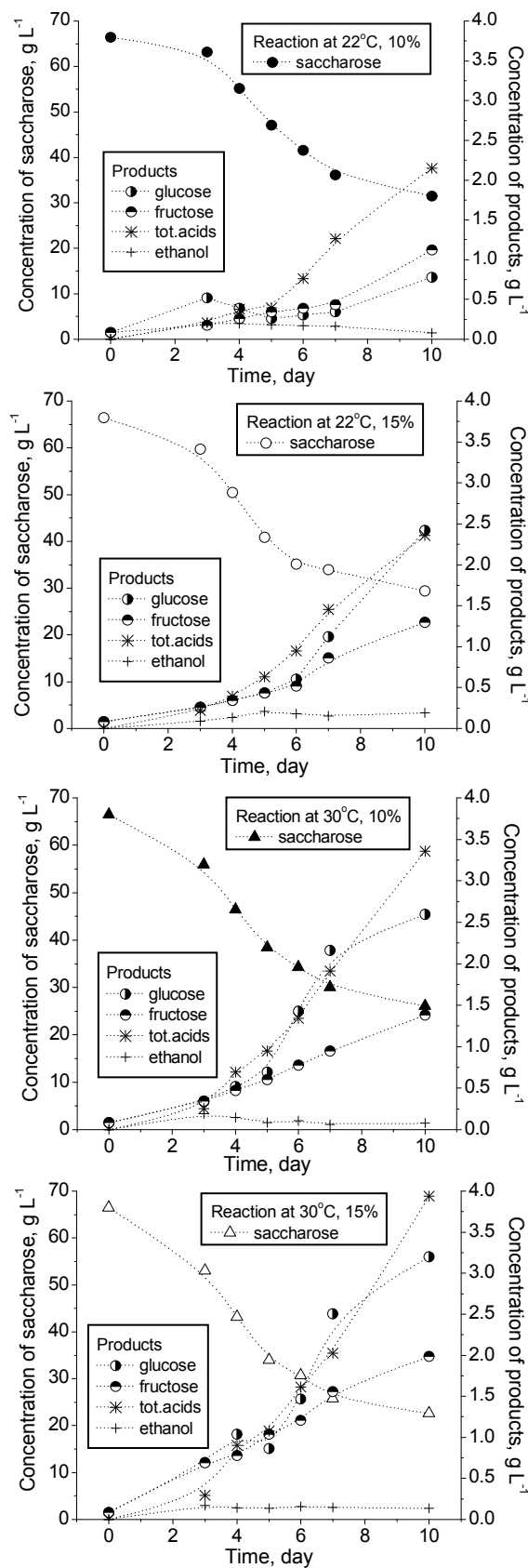


Figure 2. Kinetics of saccharose fermentation as a function of temperature and inoculum concentration; lines (B-spline) used only as guide to the eye.

which were found to be: 2.2 g L<sup>-1</sup> (22 °C and 10% (V/V)), 2.4 g L<sup>-1</sup> (22 °C and 15% (V/V)), 3.4 g L<sup>-1</sup> (30 °C and 10% (V/V)), and 3.9 g L<sup>-1</sup> (30 °C and 15% (V/V)) at the 10<sup>th</sup> day of fermentation. Apart from total acids, quantities of other intermediates/products were measured. It was found that the reaction mixtures contained 1.2-2 g L<sup>-1</sup> of glucose and 0.75-3.25 g L<sup>-1</sup> of fructose after 10 days of fermentation, showing that glucose was consumed more rapidly than fructose. The amount of ethanol produced was very small (0-0.5%) and could be tolerated in soft drinks. Greater quantities of all products were present in the samples obtained at 30 °C and inoculated with 15% (V/V) of starter culture.

Based on the mass balance, at the 10-th day of fermentation, it can be concluded that less than 15% of saccharose converts to the mentioned products (glucose, fructose, total acids and ethanol). Following amounts of saccharose were converted: 6.2% (22 °C and 10% (V/V)), 9.4% (22 °C and 15% (V/V)), 11.2% (30 °C and 10% (V/V)), 13.9% (30 °C and 15% (V/V)). The unconverted saccharose quantities were found at rather high levels: 47.4% (22 °C and 10% (V/V)), 44.2% (22 °C and 15% (V/V)), 39.2% (30 °C and 10% (V/V)) and 34% (30 °C and 15% (V/V)). It is typical of fermentation by Kombucha that a network of cellulose, which can be seen with the naked eye, is generated. The film from the surface, together with the attached cellulose fibres, was taken after the 10<sup>th</sup> day of batch fermentation. The obtained values at 22 °C with addition of 10 and 15% (V/V) of inoculum were 0.198 and 0.218 g L<sup>-1</sup>, respectively. At 30 °C and with 10 and 15% (V/V) of inoculum, the films total solid were 0.304 and 0.321 g L<sup>-1</sup>, respectively. Before the measurement, the mentioned system was washed with water and completely dried to constant mass.

### Saccharose concentration model

Following the trends of changes of saccharose quantity (Figure 2), it can be concluded that three stages exist: *i*) slight initial decrease at the beginning of fermentation, *ii*) very steep decrease in an intermediate stage and *iii*) slight decrease at the end of fermentation, with the slope similar to the initial one,

under all experimental conditions (temperatures and concentrations of inoculum). The best model to present the curve consisting of two retaining stages and very steep decline in-between is Boltzmann's (sigmoidal) function:

$$S(t) = A_2 + \frac{A_1 - A_2}{1 + e^{-\frac{t-t_0}{\Delta t}}} \quad (2)$$

where  $S$  denotes saccharose concentration (g L<sup>-1</sup>), which changes in time  $t$ . Parameters  $A_1$  and  $A_2$  correspond to the positions of two asymptotes to the  $S(t)$  curve (upper and lower),  $t_0$  is the  $t$ -coordinate of the point at which the slope has the highest value, while  $\Delta t$  is the width of the step of an exponential decrease. Four parameters ( $A_1$ ,  $A_2$ ,  $t_0$  and  $\Delta t$ ) were determined by applying the Levenberg-Marquardt method (Origin 6.1) over the experimental data (saccharose concentrations in Figure 2). The values presented in Table 1 were obtained.  $S(t)$  functions were determined for all investigated reactions at the chosen temperatures and in the presence of different inoculum concentrations. The achieved coefficients of determination are very high ( $R^2 > 0.99$ ), indicating a good fit of the data to the selected model within the entire interval of variable values.

Significance of each particular parameter of the concentration model (2) was assessed by statistically determined  $t$ -factors (Table 2). An analysis of  $t$ -factors gives a physical meaning to the parameters and to the model itself. Obviously, the most significant parameter is  $A_1$  (42-44.5%), which is closely related to the initial concentration of saccharose. Almost equally significant are parameters  $t_0$  (27-29%) and  $A_2$  (21-24%). They correspond to the middle point on the concentration curve (at which fast reaction starts to decelerate) and to the final concentration of saccharose, respectively. The least significant is parameter  $\Delta t$  (7-9%), which defines the width of the steepest part of  $S(t)$  curve, associated with the period of time when the most intensive consumption of saccharose occur.

Table 1. Parameters of sigmoidal (Boltzmann) concentration model

Parameters in Eq. (2)	Conditions			
	22 °C, 10% (V/V)	22 °C, 15% (V/V)	30 °C, 10% (V/V)	30 °C, 15% (V/V)
$A_1$	67.51	66.17	67.10	68.78
$A_2$	31.57	30.62	28.81	22.72
$t_0$	4.812	4.458	4.070	3.749
$\Delta t$	1.1000	0.7987	0.9215	1.248
$R^2$	0.99539	0.98793	0.99816	0.99740

Table 2. Statistical assessment of the parameters by *t*-factors

<i>t</i> -Factors for parameters in Eq. (2)	Conditions			
	22 °C, 10% (V/V)	22 °C, 15% (V/V)	30 °C, 10% (V/V)	30 °C, 15% (V/V)
<i>t</i> -Factor for $A_1$	44.40%	41.60%	41.80%	42.00%
<i>t</i> -Factor for $A_2$	21.90%	23.10%	23.60%	21.00%
<i>t</i> -Factor for $t_0$	26.70%	29.10%	27.70%	27.90%
<i>t</i> -Factor for $\Delta t$	7.03%	6.20%	6.96%	9.15%

### Reaction rate model

Because Kombucha fermentation is a complex process, an empirical approach to modelling of fermentation kinetics was used. The model is based on saccharose concentration change during fermentation. The first derivative of the concentration Eq. (2) defines the rate of reaction (1):

$$-\frac{dS(t)}{dt} = \frac{e^{\frac{t-t_0}{\Delta t}} (A_1 - A_2)}{\Delta t \left(1 + e^{\frac{t-t_0}{\Delta t}}\right)^2} \quad (3)$$

Since the derivative (3) is obtained analytically, its application does not cause any error in calculations. Derivatives, calculated from Eq. (3), are graphically presented in Figure 3 as the rate curves, along with the  $S(t)$ -curves (experimental and estimated). Also, experimental rate values were presented for all investigated cases. The experimental rates were calculated from the measured saccharose concentrations at different temperatures and inoculum concentrations. The rate estimates were done using the derivative of  $S(t)$  function at the middle point of each time interval,  $(S(t)_{i+1} - S(t)_i) / \Delta t$ . Errors in

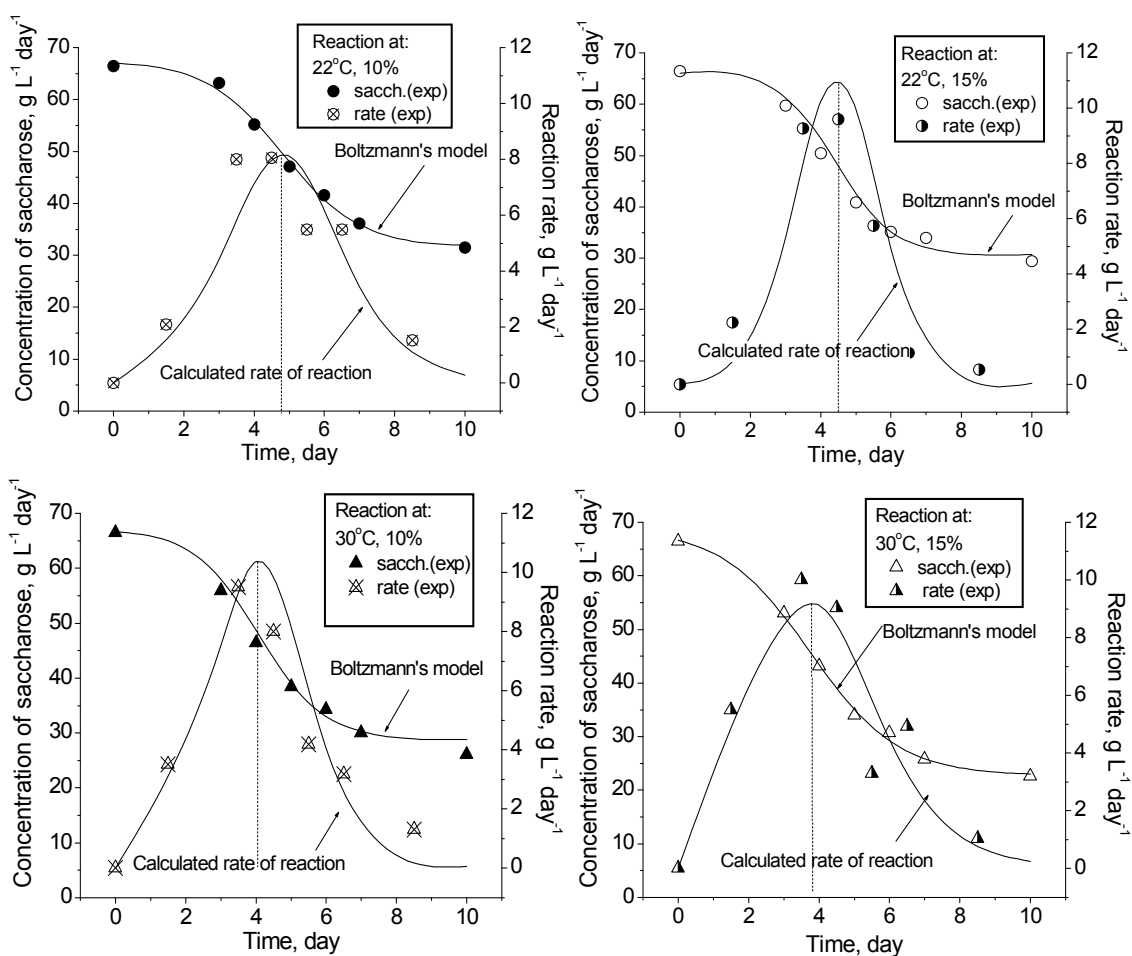


Figure 3. Reaction rate as a function of temperature and inoculum concentration; lines show mathematical models, symbols present experimental values.

such calculations could be significant, as depicted in Figure 4, which makes the numerical approximation of reaction rates less precise. When the deviations of experimental rates from the calculated ones (model 3) are expressed as a sum of squared differences divided by total number of experimental points, the following values are obtained: 1.35 (22 °C and 10% (V/V)), 1.92 (22 °C and 15% (V/V)), 1.67 (30 °C and 10% (V/V)) and 1.48 (30 °C and 15% (V/V)), all in  $\text{g L}^{-1} \text{day}^{-1}$ .

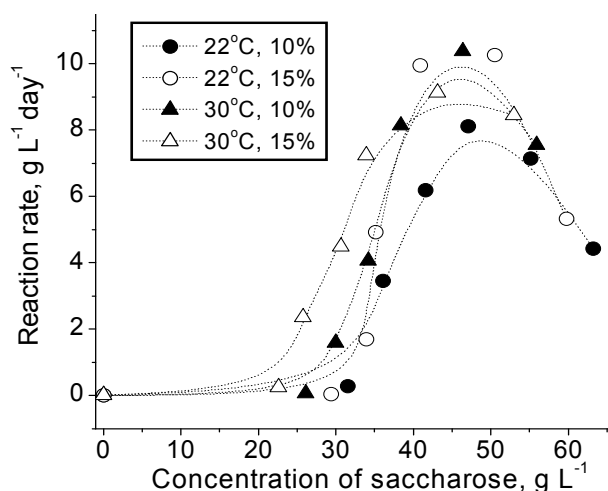


Figure 4. Saturation curves – reaction rates versus substrate concentration; lines (B-spline) used only as guide to the eye.

The analysis of the rate curves (Figure 3) shows that each one passes through a maximum, indicating that the rate of fermentation increases to a maximum after approximately 4-5 days. This acceleration phase is partly caused by the microbial composition of Kombucha cultures. Namely, yeast transforms glucose to ethanol, which in turn stimulates the production of acetic acid [7]. On the other hand, bacteria synthesize a floating cellulosic pellicle that entraps yeast cells and bacteria cells. The immobilization of microorganisms improves the fermentation process. A floating cellulosic pellicle could be observed as carrier for the Kombucha yeasts and bacteria. All these interactions contribute to a kind of reaction self-acceleration. After reaching a maximum, the rate of reaction decreases rapidly, probably due to an effect of over-acidity, typical of later phases of fermentation, which might suppress metabolic activity of the relevant cultures. This observation is supported by the results of Chen and Liu [31] who investigated prolonged fermentation of saccharose by Kombucha. They also found that accumulation of organic acids might reach harmful levels to health despite the fact that some valuable compounds may appear later in fermentation.

The highest rate ( $11 \text{ g L}^{-1} \text{day}^{-1}$ ) was achieved in the system at 22 °C, previously inoculated with 15% of starter culture. High rates ( $10.5$  and  $9.5 \text{ g L}^{-1} \text{day}^{-1}$ ) were obtained for reactions at 30 °C. The lowest maximum ( $8 \text{ g L}^{-1} \text{day}^{-1}$ ) occurred in the reaction at 22 °C, which was initiated with 10% (V/V) of starter culture. It can be concluded that the results are generally in agreement with the expectations related to the positive effect of temperature on kinetics of reactions. Also, higher quantity of inoculum does not increase the maximal rate of reaction, at 30 °C, suggesting that inoculum concentration of 10% (V/V) would be an acceptable level [29]. The fermentation conducted at 22 °C and 15% (V/V) starter cultures (Figure 3) is an exception in terms of both relevant factors - temperature and inoculum concentration. Namely, the rate of this reaction deviates from the expected high value. An exact explanation of reasons for such a departure of what is expected requires further investigation. As far as duration of reaction is concerned, a longer period of time (4.5-5 days) is required for achieving maximal rate at low temperature, while a shorter period of time (3.8-4 days) is enough at high temperature. Generally, reactions rates are higher at higher temperature. When the data related to the rate curves are known, the saturation curves (reaction rates versus substrate concentration) can be presented (Figure 4). The saturation curves show sigmoidal kinetics at low substrate concentrations, indicating a complex non-Michaelis-Menten type kinetics, which were successfully modelled by the empirical model.

One of the benefits of knowing the rate of fermentation is the possibility for determining the optimal duration of the process of Kombucha beverage production in order to achieve the required acidity (pH) of the final product. Various authors suggest different pH values, but none lower than pH 3.5. In our experiments, the fermentation broth reached this value between 6.5 and 10 days (Figure 1). On the other hand, if the fermentation were performed only within the range of accelerated rates (3.8-5 days), the achieved pH values would be approximately 4-4.3, respectively. From our point of view [32], these values are adequate.

## CONCLUSION

By processing the experimental results, two mathematical models for the kinetics of saccharose fermentation by Kombucha were obtained - one for the change of saccharose concentration during its fermentation, and the other for the rate of the mentioned fermentation. It has been shown that both

empirical models enable better insight into saccharose transformation, so the following conclusions can be drawn:

- rate of fermentation increases to a maximum within approximately 4-5 days for all investigated cases;
- after achieving maximum, rate of reaction decreases rapidly;
- rate of fermentation mostly increases with the increase of working temperature in the range of 22 to 30 °C;
- rate of fermentation is slightly affected by the concentration of inoculum in the range of 10 to 15% ( $W/V$ );
- optimal duration of fermentation, under the applied conditions, is 3.5-5 days;
- saturation curves show sigmoidal kinetics at the chosen concentration of substrate.

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NAUČNI RAD

## KINETIKA FERMENTACIJE SAHAROZE KOMBUHOM

*Mehanizmi reakcije tokom fermentacije saharoze uz primenu Kombuhe nedovoljno su objašnjeni. U radu je analizirana kinetika fermentacije saharoze Kombuhom korišćenjem predloženog empirijskog modela. Podaci su dobijeni na 1,5 g L<sup>-1</sup> crnog čaja, sa 66,47 g L<sup>-1</sup> saharoze i dodatkom 10 ili 15% (V/V) inokuluma Kombuhe. Ukupan broj vijabilnih ćelija bio je sledeći: približno 5×10<sup>5</sup> ćelija kvasaca po mL inokuluma i približno 2×10<sup>6</sup> bakterijskih ćelija po mL inokuluma. Uzorci su analizirani nakon 0, 3, 4, 5, 6, 7 i 10 dana. Odredene su pH vrednosti, sadržaj saharoze, glukoze, fruktoze i etanola kao i ukupna kiselost. Koncentracioni model saharoze definisan je kao sigmoidalna funkcija na 22 i 30 °C, i sa dodatkom 10 ili 15% (V/V) inokuluma. Determinisani koeficijenti funkcije bili su veoma visoki (R<sup>2</sup> > 0,99). Brzine reakcije izračunate su kao prvi izvodi Boltzmanove funkcije. Nije utvrđena jednostavna korelacija između brzine reakcije i nezavisno promenljivih (temperature i koncentracije inokuluma). Analiza empirijskog modela ukazuje da fermentacija saharoze Kombuhom podleže veoma kompleksnoj kinetici.*

*Ključne reči: Kombuha fermentacija saharoze, kinetika, empirijski model.*