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INFLUENCE OF WORKING CONDITIONS UPON KOMBUCHA CONDUCTED FERMENTATION OF BLACK TEA

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Local domestic Kombucha was used in fermentation of 1.5 g l^{-1} of black tea (Indian tea, 'Vitamin', Horgoš, Serbia and Montenegro), sweetened with 66.47 g l^{-1} of sucrose. Inoculation was performed by both 10% and 15% of fermentation broth from previous process. The fermentation was conducted alternatively, at 22°C and 30°C, for 10 days. The samples were analysed after 0, 3, 4, 5, 6, 7 and 10 days, so that their pH values, content of total acids, sucrose, glucose and fructose contents as well as quantity of ethanol were determined. At the same time, experiments were performed on tea inoculated with 10% of fermentation broth, at temperatures 22°C, 30°C and 35°C, for 21 days. The samples were taken after 0, 3, 7, 10, 14, 17 and 21 days and quantity of vitamin C was measured. Experimentally obtained results were analysed and the response functions of glucose, fructose, metabolites and biosynthesized vitamin C were defined. Process duration was found the most influencing on the composition of fermentation products, then temperature and inoculum concentration.

Keywords: Kombucha; response surface method; fermentation; metabolites; vitamin C.

INTRODUCTION

Most of the world population, especially people in highly developed countries, has demonstrated increased awareness and interest in functional food, i.e., food that positively effects upon bio-regulatory functions and human health. Such an interest lasts for a few decades, having great impact in the development of food industry. On the other hand, a beverage, that possesses characteristics of functional food, is known for a few thousand years. It originated in China, 220 BC, Korea and Japan. There, it was popular due to detoxifying and energizing properties as well as curing digestive problems (Dufresne and Farnworth, 2000). This refreshing beverage, testing like sparkling apple cider, appears during fermentation of sugared tea, caused by activity of a symbiotic association of bacteria and yeasts. The association is well known as Kombucha (Teoh *et al.*, 2004), very probably after Doctor Kombu who brought the tea fungus from Korea to Japan, in 414 (Dufresne and Farnworth, 2000).

The yeast cells hydrolyse sucrose into glucose and fructose, producing ethanol as metabolite (Reiss, 1994; Sievers *et al.*, 1995), while acetic acid bacteria convert glucose into gluconic acid and fructose into acetic acid. Two main metabolites (ethanol and acetic acid) catalyse actual reactions; acetic acid stimulates the yeast to produce ethanol, whereas ethanol stimulates acetic acid bacteria to grow and produce acetic acid (Liu *et al.*, 1996). Apart from the main metabolites, Kombucha beverage contains most of tea ingredients (such as carbohydrates, potassium, manganese and fluoride ions, vitamins E, K, A and B, amino acids, particularly theanine, and so on) as well as other compounds that appear as a result of numerous reactions; oxidation of tea polyphenols leads to the formation of catechins, theaflavins, theaflavinic acid, theasinensis and proanthocyanidin polymers (Balentine *et al.*, 1997; Hara *et al.*, 1995a); degradation of amino acids is involved in the genesis of terpenoids, carotenoids, lipids and linoleic acid (Hara *et al.*, 1995b). Fermentation process induces synthesis of the B complex of vitamins and folic acid (Roche, 1998).

Fermentation induces also biosynthesis of ascorbic acid—vitamin C—important natural antioxidant, which serves as human health protector and drug. One who has started with application of vitamin C as a drug is leading vitamin C clinician Frederick Klenner (1971), followed by Robert Cathcart, establisher of the Orthomolecular medicine (Cathcart, 1991, 1993). Petrović and Lončar (1996) and Malbaša *et al.* (2002a), have determined content of vitamin C in fermentative liquids of tea fungus. Activity of vitamin C as well as activities of other compounds present in Kombucha fermentation system is modified in a positive way by the chemical environment in the fermented beverage. For example, it has been reported that tocopherol

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and ascorbic acid exert strong synergetic effects on the antioxidant of tea catechins (Hara *et al.* 1995c). Therefore, vitamin C and other constituents of Kombucha beverage protect human health more efficient than the same isolated compounds. Vitamin C is also an essential component of the human diet. It enhances iron adsorption (Cook and Redd, 2001; Halberg and Hulthen, 2000), prevents megaloblastic anemia (Jacob, 1994) and reduces stomach cancer (Hemila and Herman, 1995). Ascorbic acid inhibits iron absorption by tannins. Decreased tannins and increased ascorbic acid concentration, in fermented tea, are very useful for iron absorption and improved digestion (Pasha and Reddy, 2005).

Production of vitamin C and other valuable compounds, in Kombucha lead fermentation process, is strongly related to the process duration, fermentation temperature, Kombucha symbiosis and inoculum concentration, source of carbon atoms and other working conditions (Petrović *et al.*, 1995–1996). It has been reported (Petrović *et al.*, 1995–1996) that the product of 7th day of fermentation possesses optimal sensory characteristics; increase of acidity during fermentation longer than 7 days makes liquor of the Kombucha too sour and unpleasant. However, some authors (Chen and Liu, 2000) have investigated changes in major compounds of tea fungus metabolites during prolonged fermentation of up to 60 days. Temperature of fermentation significantly influences kinetics of substrate intake and product formation. Some authors find 28°C optimal (Petrović *et al.*, 1995–1996). It was proved that the composition of fermented tea greatly depend on the individual Kombucha association (Reiss, 1994; Blanc, 1996) as well as on the concentration of inoculum solution. As far as sucrose substrate is concerned, it seems that concentration of 50 g l^{-1} gives optimal concentration of both ethanol and lactic acid (Reiss, 1994). Blanc (1996) reported about investigations on systems with 50, 70 and 100 g l^{-1} of sucrose, while Malbaša *et al.* (2002b) followed a kind of sucrose and inulin balance during tea fungus fermentation.

Effect of working conditions on the quantities of particular fermentation products can be analysed by application of the response surface methodology (RSM), which relies on an analysis of the response function, given mostly in polynomial form. Many examples can be found in published papers on processes similar to Kombucha fermentation. Tarabasz-Szymanska *et al.* (1999) optimized biosynthesis of pullulan by Pullularia pullulans. using second-order polynomial model, expressing the dependence of the pullulan productivity on medium component concentrations and pH. Adinarayana and Ellaiah (2002) optimize the fermentation medium for maximum alkaline protease production. Carvalho *et al.* (2004) applied screening design and quadratic model to determine adequate conditions for fermentation by *Candida guilliermondii* FTI 20037 cells in xylitol production. Optimization of bacteriocin production by *Lactobacillus plantarum* LPCO10 was explored by Leal-Sánchez *et al.* (2002) when experiments on culture medium, inoculum size, aeration of the culture and growth temperature were statistically processed. Bacteriocin production by *Lactobacillus pentosus* B96 was optimized by Delgadoa *et al.* (2005), who defined polynomial function of NaCl concentration and temperature. RSM was employed (Shih and Shen, 2005) to study the effect of yeast extract, glucose, ammonium sulfate, and initial medium pH on the production of poly-ε-lysine (ε-PL) by *Streptomyces albulus* IFO 14147 cultures. A second-order polynomial equation proved adequate both for analysis and for finding optimal ε-PL yield.

The main objective of work of Martin (2001) was to apply RSM to study the effect of the operating parameters on the production of the yeast as protein source. The independent variables studied were fermentation time, temperature and pH. By a second order multiple regression analysis, mathematical model was obtained. Another study was undertaken (Liew *et al.*, 2005) to optimize yeast extract, glucose, concentration of vitamins and culture pH for maximizing the growth of a probiotic bacterium, *Lactobacillus rhamnosus* by applying RSM. Wang and Lu (2005) used a polynomial regression model with cubic and quadratic terms in investigating the effects of time, temperature and broth content on mycelial growth,

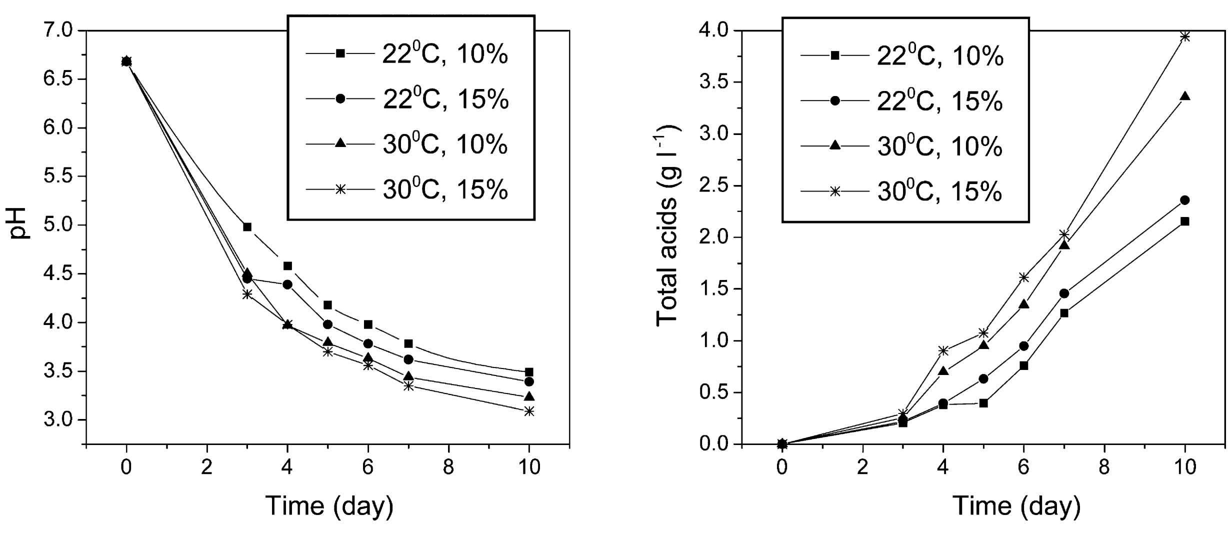

Figure 1. pH and total acids as functions of time, temperature and inoculum concentration.

in terms of dry cell weight and extracellular polysaccharide content produced by the fungus *Boletus* spp. ACCC 50328.

Kalathenos *et al.* (1995) studied the combined effect of pH, ethanol and fructose on the growth of *Saccharomyces cerevisiae* at 25°C by the standard RSM. Kalil *et al.* (2000) used RSM in combination with modeling and simulation to design and optimize alcoholic fermentation process composed of four continuous reactors. Operational conditions for maximal yield and productivity were determined with ten parameters under consideration. Echegaray *et al.* (2000) investigated process with *Sacchoromyces cerevisiae* in fermenter containing molasses with urea and penicillin. The parameters varied were: filling time, time constant, total reducing sugar concentration in the feeding mash and total amount of wet-pressed yeast in the inoculum. The quadratic equations for ethanol yield and residual invertase activity were established. By using RSM, Dragone *et al.* (2004) investigated the influences of different fermentation factors on ethanol production by *Saccharomyces cerevisiae*. The resultant functional relationship was second order polynomial with temperature and nutrient supplementation as variables.

This paper will focus on determining relationships between time, temperature and inoculum concentration, as independent variables, and quantity of glucose and fructose, as dependent variables, in Kombucha fermented black tea. Also, it will analyse influence of the mentioned variables upon metabolite quantity as well as quantity of biosynthesized vitamin C.

MATERIALS AND METHODS

Kombucha Culture

Local domestic Kombucha determined by Markov *et al.* (2001) was used for the fermentation.

Kombucha Fermentation

Tap water was boiled, sweetened with 66.47 g l^{-1} of sucrose and mass of 1.5 g l^{-1} of black tea (Indian tea, 'Vitamin', Horgoš, Serbia and Montenegro) was added and removed by filtration after 15 min. After cooling to room temperature, the tea was inoculated, alternatively, with 10% and 15% of fermentation broth from previous Kombucha fermentation. The beakers were covered with cheesecloth and incubated in a thermostat at 22°C and 30°C for 10 days. The samples from Kombucha

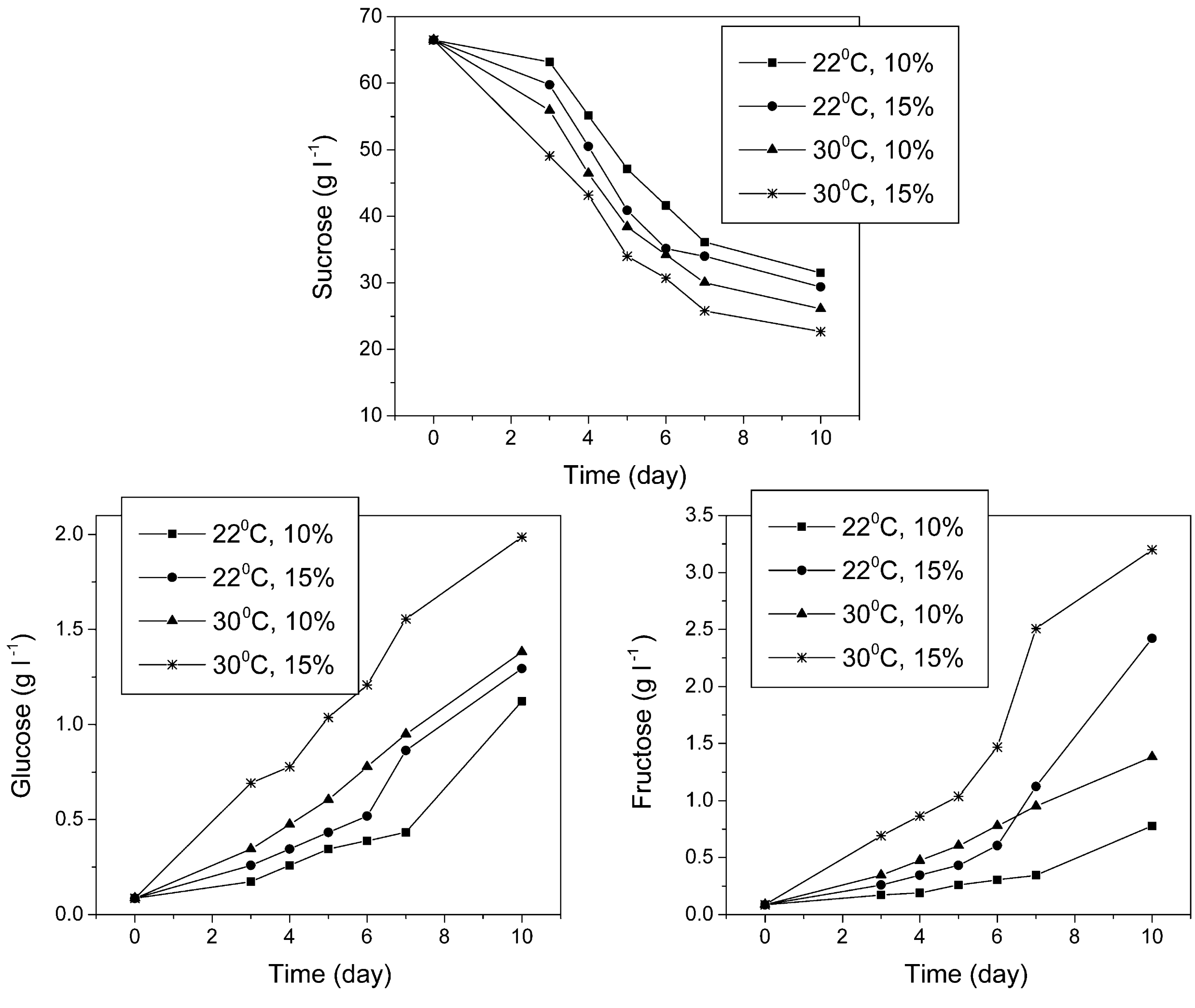

Figure 2. Sugar quantities as functions of time, temperature and inoculum concentration.

fermentation were taken after 0, 3, 4, 5, 6, 7 and 10 days and following quantities were measured: pH value, total acids, sucrose content, glucose content, fructose content and ethanol content.

Simultaneously, experiments were performed on tea inoculated with 10% of fermentation broth, at temperatures 22°C, 30°C and 35°C, for 21 days. The samples were taken after 0, 3, 7, 10, 14, 17 and 21 days and quantity of vitamin C was measured.

Methods of Analysis

pH values were measured with an electric pH meter.

Total acids content was determined by the volumetric method with sodium hydroxide and phenolphthalein as indicator.

Sucrose, glucose and fructose contents were determined using the test of Boehringer Mannheim (Cat. no. 716260).

Ethanol content was measured in accordance with the procedure of Boehringer Mannheim (Cat. no. 176290).

Vitamin C content was determined using the test of Boehringer Mannheim (Cat. no. 409677).

RESULTS

Measured Values: Presentation and Discussion

The results of measurements are presented in Figures 1–4. During fermentation, pH value of the obtained products decreased due to formation of acetic acid, gluconic acid and number of other organic acids (Figure 1). Quantities of generated acids were greater in samples obtained at higher temperatures. Also, they were greater in samples inoculated with greater concentration of starter. Intake of sucrose and formation of glucose and fructose (Figure 2) increased with the increase of all three independent variables (process duration, temperature and inoculum concentration), as it was expected. It seemed that effect of time on changes of quantities of all sugars dominated. Higher fermentation temperature caused steeper decrease of sucrose quantity as well as steeper increase of glucose and fructose quantity. Similar effect was noticed when inoculum concentration was concerned. As one of the products of biochemical process, small quantity of ethanol appeared, remaining within permitted values (0–0.5%). Ethanol and total acids compose metabolites whose dependence on fermentation time, temperature and inoculum concentration is presented in Figure 3. Quantities of metabolites strongly depend on time of reaction. Also, greater quantities of metabolite were present in samples obtained at higher temperatures and inoculated with greater concentration of starter.

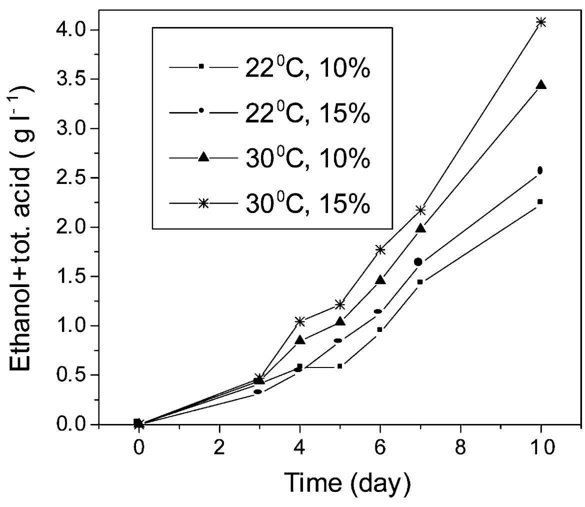

Figure 3. Metabolites concentration as function of time, temperature and inoculum concentration.

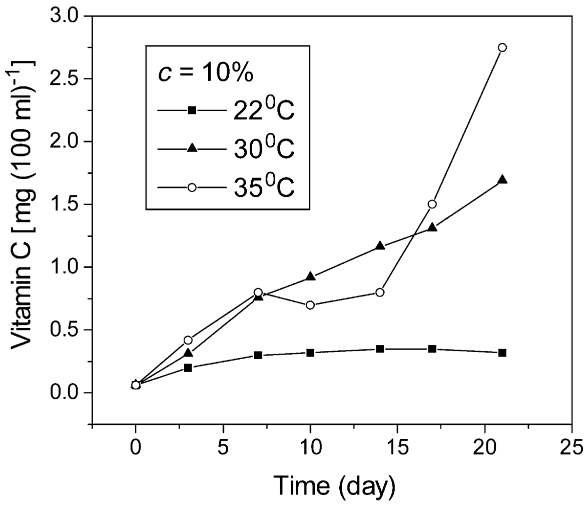

Figure 4. Vitamin C concentration as function of time and temperature, at inoculum concentration of 10%.

Although Kombuha fermented tea contains numerous valuable compounds, vitamin C is particularly monitored. Experiments at three temperatures, with inoculum concentration 10%, gave the results presented in Figure 4. Analysis of the results shows that quantity of vitamin C increases with the increase of both of independent variables, time and temperature.

It seems that all our results lead to the conclusion that long fermentation will give valuable product, particularly with as high level of vitamin C as possible. This can be supported by the results of Chu and Chen (2006) who have discovered that Kombucha average antioxidant activities raises to about 70% after fermenting for 15 days. However, defining optimal working conditions (which will guarantee product of optimal quality) is very complex problem. Despite noticing increased antioxidant activities, Chu and Chen (2006) did not recommend prolonged fermentation because of accumulation of organic acids, which might reach harmful levels. Sensory characteristics of the obtained product are also important. Since acidity increases in time, product becomes too sour and unpleasant. The lowest acceptable pH value should not decrease below pH = 3, value typical of digestive tract. Analysis of the measured values, acquired by this investigation, shows that 10-day fermentation, at 30°C and initiated by inoculum concentration 10%, matches the requirement. Corresponding quantity of vitamin C is around 1 mg per 100 ml of the fermented beverage.

The data basis obtained by experiments would be statistically processed in order to establish relationships between quantities of product components, as dependent variables,

Table 1. Parameters (b) and their significances (t) in mathematical models of glucose change and fructose change with time, temperature and inoculum concentration.

	b and t values for glucose function		b and t values for fructose function	
No.	b	t	b	t
1	−1.61	1.04	0.660	4.64 E−02
2	−0.203	1.62	−0.777	0.669
3	6.44 E−02	1.13	−2.05 E−03	3.91 E−03
4	9.37 E−02	0.82	−4.33 E−03	4.09 E−03
5	1.04 E−02	2.88	2.16 E−02	0.647
6	2.51 E−03	0.43	2.08 E−02	0.391
7	−3.54 E−03	0.84	−1.39 E−03	0.036
8	5.37 E−03	1.33	1.76 E−02	0.472
	$\delta = 0.008$		$\delta = 0.074$	

and time of reaction, temperature and inoculum concentration, as factors-independent variables.

Mathematical Model and Statistical Analysis

Mathematical modeling in this paper is based on application of the RSM. In process analysis of this kind, mathematical model is most frequently a polynomial, as reported by many scientists, on systems similar to Kombucha lead fermentation, who have found second order polynomial the most suitable. On one hand, it covers non-linearity in dependent-independent variables relationship and, on the other hand, the number of required experiments is rather small. So, following general form of function (y) was suggested in all our cases—when modelling: (1) increase of glucose concentration, (2) increase of fructose concentration and (3) increase of metabolites:

$$y = b_1 + b_2x_1 + b_3x_2 + b_4x_3 + b_5x_1x_2 + b_6x_1x_3 + b_7x_2x_3 + b_8x_1^2 \quad (1)$$

where x_1 denotes time, x_2 is temperature and x_3 denotes inoculum concentration. It is obvious that the response function (1) consists of linear terms and nonlinear terms, whereas nonlinear terms represent interaction of two variables (as their product) as well as quadratic time-term. As

Table 2. Parameters (b) and their significances (t) in mathematical model of metabolite change with time, temperature and inoculum concentration.

	b and t values for metabolite function	
No.	b	t
1	1.61	0.798
2	−0.464	2.81
3	−0.0547	0.733
4	−8.47 E−02	0.561
5	1.65 E−02	3.49
6	1.02 E−02	1.35
7	2.61 E−03	0.470
8	2.189 E−02	4.14
	$\delta = 0.01$	

Table 3. Influence of time, temperature and inoculum concentration on sugars and metabolite.

	Significance of variables in linear terms			Significance of variables in interaction terms		
System	Great	Medium	Small	Great	Medium	Small
Glucose	x_1	x_2	x_3	$x_1\ x_2$	$x_2\ x_3$	$x_1\ x_3$
Fructose	x_1	x_2, x_3		$x_1\ x_2$	$x_1\ x_3$	$x_2\ x_3$
Metab. in time	x_1	x_2	x_3	$x_1\ x_2$	$x_1\ x_3$	$x_2\ x_3$

a goodness-of-fit test of polynomial (1), applying of average deviation of N experimental values (y_{exp}) form N calculated values (y_{cal}), according to the relation:

$$\delta = \frac{\sum_{i=1}^{N} (y_{exp} - y_{cal})_i^2}{N} \quad (2)$$

was suggested. Number of parameters (b_1–b_8), which should be determined, was 8.

The available data basis was obtained from 28 measurements ($2^3 = 8$ according to the full factorial design and $5 \times 4 = 20$ due to additional measurements after 3, 4, 5, 6 and 7 days). After statistical processing of the measured data by the regression analysis method, the parameters were obtained, as presented in Tables 1 and 2. It has been proven that average deviations, for all our models, have acceptably small values (0.008 for glucose, 0.074 for fructose and 0.01 for total acids + ethanol), proving that second order polynomial, as a response function, was a good choice. So defined response functions were subjected to statistical analysis so that all their coefficients were tested for significance in comparison with the sample standard deviation by applying the Students t-test. The obtained t-factors in Tables 1 and 2 represent significance of each particular term—part of the whole mathematical model. A simplified review of significances is presented in Table 3, where one can find that process duration (time) is the most significant variable in all cases. Temperature is second important variable, while the least important variable is inoculum concentration, which does not mean that the concentration can be neglected, but (compared with time and temperature) has lower significance. Analysis of significances of interaction-terms shows that time–temperature interaction is the most significant, in all cases, time–inoculum concentration is less significant

Table 4. Parameters (b) and their significances (t) in mathematical model of vitamin C generation with time and temperature, at inoculum concentration of 10%.

	b and t values for vitamin C function	
No.	b	t
1	0.158	2.70
2	−0.139	22.8
3	−3.03 E−03	1.51
4	7.81 E−03	43
5	−1.03 E−03	7.32
	$\delta = 0.8\text{E}{-03}$	

Table 5. Influence of time and temperature, at inoculum concentration of 10%, onto vitamin C generation.

System	Significance of variables in linear terms: Great	Significance of variables in linear terms: Small	Significance of variables in interaction terms: Great
Vitamin C	x_1	x_2	$x_1\ x_2$

while temperature–inoculum concentration has the smallest significance.

Mathematical model, chosen for describing vitamin C generation depending on time (x_1) and temperature (x_2), has following form:

$$y = b_1 + b_2x_1 + b_3x_2 + b_4x_1x_2 + b_5x_1^2 \quad (3)$$

As a goodness-of-fit test of polynomial (3), applying of average deviation (2) was suggested. The number of parameters required for definition of model (3) was 5, while the number of available experimentally determined values was 21 ($2^2 = 4$ according to the full factorial design and $5 \times 2 = 10$ due to additional measurements after 3, 7, 10, 14 and 17 days plus seven measurements for the intermediate temperature).

Calculated *b*-parameters and their *t*-values are presented in Table 4. Average deviation of measured values from the calculated ones was acceptable small (0.8E-03), proving adequate choice of vitamin C response surface. The analysis of significances is presented in Table 5. It can be concluded that process duration is more important than temperature; interaction of these two is very significant.

CONCLUSION

Investigation of Kombucha conducted fermentation was performed with the aim to determine effects of fermentation time, temperature and inoculum concentration on the composition of fermentation product (particularly, on glucose, fructose, total acids + ethanol and vitamin C). For this purpose, response functions for all mentioned components of product were derived, as second order polynomials, adequateness of which was proven.

Once defined, mathematical models were applied for analysis of responses of: glucose, fructose, metabolites and vitamin C to changes of independent variables: time, temperature and inoculum concentration. It was concluded that process duration has been most influencing variable. As none of the response surfaces has extreme within the investigated area, a standard optimisation was impossible. So, as controlling variable pH value of the product, which should not drop below predetermined value, according to sensory and nutritive requirements, might be accepted.

Defined mathematical models could be extended by including new, relevant variables. Particularly important is volume of the reactor. When taken into account, it will give a response surface which might be applied in scaling-up fermentation equipment.

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