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Factors influencing kombucha production: effects of tea composition, sugar, and SCOBY

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Abstract

Kombucha is a functional beverage that lacks studies toward standardization of process parameters; thus, the present work aims to evaluate the effect of sucrose content, initial SCOBY mass, and tea composition on several characteristics of the final beverage. For this purpose, a factorial design was performed, and the effect of the three variables was statistically evaluated. All kombuchas were within Brazilian regulation standards (higher than 2.5 and lower than 130 mgEq/L) and can be classified as non-alcoholic beverages. Kombucha yield ranged from 87.3% to 96.4%; SCOBY growth, from 41.11 to 62.08%; turbidity, from 108 to 421.5 NTU; acetic acid, from 3.78 to 5.33 mg/mL; glycerol, from 0.25 to 0.52 mg/mL; and ethanol content, from 1.90 to 3.21 mg/mL. Results showed that kombucha yield, pH, and SCOBY growth were significantly influenced by the SCOBY mass; acidity was greatly affected by the tea composition; kombucha's turbidity was significantly affected by the sucrose content and the interaction of the sugar content and tea composition; and glycerol, alcohol, and acetic acid were not affected by the parameters evaluated. Results bring science-based information about this global trending beverage, seeking high quality and standardization.

Keywords: green tea; black tea; factorial design; non-alcoholic beverage.

Practical application: Results show process parameters significantly influencing important kombuchas' features.

1 INTRODUCTION

Kombucha is a functional beverage obtained by the fermentation of *Camellia sinensis* and sugars by a symbiotic culture of active bacteria and yeasts, also known as SCOBY (Miranda et al., 2022; Vargas et al., 2021). Its functional properties are credited to the presence of polyphenols, minerals, and compounds with antioxidant, antimicrobial, anticancer, and anti-inflammatory activities, among other important health-promoting compounds (Bortolini et al., 2022; Cardoso et al., 2020; Miranda et al., 2022; Vargas et al., 2021; Villarreal-Soto et al., 2019).

The global market for functional beverages was valued at over US$ 110 million in 2020, and it is expected to grow 5.9% annually until 2030 (Allied Market Research, 2022). Kombucha is a millenarian drink, but it has been produced on a large scale in the past decade for commercial purposes. It is a homemade/handcraft production that helps popularize fermented beverages (Miranda et al., 2022; Vargas et al., 2021). Due to the lack of standardized production, efforts have been made to evaluate the process parameters of kombucha's features in these contexts. For example, Lobo et al. (2017) studied a black tea kombucha produced with an infusion of 7.5 g/L added 50 g/L of sugar and fermented for 7 days at 25°C; Ivanišová et al. (2019) also used a black tea infusion (5 g/L) added 30 g/L of sugar at 22°C for 7 days. Jakubczyk et al. (2020), on the contrary, made black, green, white, and red teas at a ratio of 8 g/L, added 100 g/L of sugar, and fermented for 14 days at 28°C. However, these authors did not inform the methodology of the amount of SCOBY inoculated. Cardoso et al. (2020) fermented for 10 days at 25°C black and green tea (12 g/L) kombuchas with 50 g/L of sugar and 30 g of SCOBY per liter of infusion. Other conditions are reported by Miranda et al. (2022), showing a large range of bioprocess parameters used in the literature to produce kombucha. However, many efforts must be made to deeply understand how those independent variables impact beverage profiles, mainly through standard large-scale processes.

Most studies perform the fermentation at a temperature range of 20–30°C, close to room temperature (Miranda et al., 2022). Meanwhile, the effect of time is more suitable to be evaluated by kinetic analysis. Green and black tea are the main *C. sinensis* infusions used, and the combination of both is also studied (Neffe-Skocińska et al., 2017). Sugar addition is another important process parameter since it is the source of carbohydrates needed to perform the fermentation process. Among the beverage's characteristics, Brazilian regulation establishes that non-alcoholic kombucha must present a maximum of 50 mL of ethanol per liter (0.5% v/v), volatile acidity in the range of 30–130 mg of acetic acid equivalent per liter (mgEq/L), and

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pH in the range of 2.5–4.2 (Brazil, 2019). However, other parameters, such as yield and turbidity, have not been reported.

Evaluation of the impact of different parameters on characteristics of complex food matrices by the conventional "one-at-a-time-approach" may lead to a critical analysis and ignore the importance of the interaction of the independent variables. On the contrary, factorial designs are powerful statistical tools since they are particularly suited to solve and evaluate complex interactions among parameters in which products are involved and have been used in several fields of food science and technology (Girelli et al., 2023; Kotta et al., 2021; Polat et al., 2020; Yeddes et al., 2020), including bioprocess techniques (Mir et al., 2021; Valiyan et al., 2021). Thus, this article aimed to evaluate the effect of the kombucha mass, black and green tea infusion ratio, and sugar concentration on several kombuchas' characteristics.

2 MATERIALS AND METHODS

2.1 Chemicals and ingredients

Green and black teas (CháMais, Xanxerê, SC, Brazil), sugar (Alto Alegre, SP, Brazil), and mineral water (Água da Pedra, Lajeado, Brazil) were purchased from a local market (Lajeado, RS, Brazil). The HPLC chemicals were obtained from Sigma-Aldrich (>99.9% of purity, Saint Louis, Missouri, USA) and others from Êxodo Científica (Sumaré, SP, Brazil).

2.2 Kombucha production and treatments

Green and black teas were prepared separately at 95°C for 5 min, where 7 g of tea was mixed for each 1 L of mineral water, and the combination of the different amounts of green tea and black tea was evaluated according to the factorial design in Table 1. After cooling to room temperature, infusions were transferred to 500 mL glass bottles, where sugar concentration and SCOBY mass were added. To evaluate the influence of SCOBY mass, 340 g of SCOBY were cut into cubes and manually homogenized before weighing and transferring into the infusions. A complete factorial design with three coded levels and three independent variables was used to evaluate the combined effect of the SCOBY ratio (X_1), sugar concentration (X_2), and green:black tea ratio (X_3). Coded and real levels and variables are shown in Table 1, which were chosen based on previous literature (Miranda et al., 2022), as well as the initial values of pH and acidity of infusions.

Table 1. Kombuchas' pH and acidity at time zero of fermentation for factorial design trials.

Trial	Scooby weight (X_1)	Sucrose (X_2)	Green:black tea ratio (X_3)	Acidity (mgEq/L)	pH
1	15.03 (-1)	6.81 (-1)	20.2 (-1)	6.5	3.42
2	22.47 (+1)	6.81 (-1)	20.2 (-1)	7.5	3.16
3	15.03 (-1)	9.19 (+1)	20.2 (-1)	8	3.33
4	22.47 (+1)	9.19 (+1)	20.2 (-1)	8.6	3.12
5	15.03 (-1)	6.81 (-1)	79.8 (+1)	6.6	3.25
6	22.47 (+1)	6.81 (-1)	79.8 (+1)	7.2	3.20
7	15.03 (-1)	9.19 (+1)	79.8 (+1)	4.8	3.50
8	22.47 (+1)	9.19 (+1)	79.8 (+1)	7.2	3.07
9	18.75 (0)	8.00 (0)	50 (0)	5.3	3.11
10	18.75 (0)	8.00 (0)	50 (0)	6.1	3.15
11	18.75 (0)	8.00 (0)	50 (0)	6.2	3.18
12	18.75 (0)	8.00 (0)	50 (0)	4.8	3.13

The tops of the jars were closed with gauze and elastic and kept at 25°C (Solab S-101, Piracicaba, SP, Brazil) for 10 days. Temperature and time were kept constant in the microbiological chamber (Solab S-101), and the values used were the average reported (Miranda et al., 2022). After the fermentation, the beverage was filtered through TNT fabric, and kombucha samples were collected, transferred to Eppendorf microtubes, and stored at -18°C until further analysis.

2.3 Yield, pH, total acidity, turbidity, and SCOBY growth

The volumes of kombucha were measured in a 500-mL flask and compared to the initial infusion volume. The pH analysis was performed on a benchtop digital pHmeter (PHOX P1000, Colombo, PR, Brazil). The total acidity was determined by titration with standardized 0.01 N sodium hydroxide, and the result was expressed as mg acetic acid equivalent per liter (mgEq/L). Turbidity was measured in a digital turbidimeter (DLT-WV, Dellab, Araraquara, SP, Brazil) and expressed in nephelometric turbidity units (NTU). The SCOBY growth was measured by drying at 105°C (S-100, Solab, Piracicaba, SP, Brazil) and weighing in an analytical balance (Shimadzu, AUX220, Japan) and expressed as a percentage by comparing the initial with the final dried mass.

2.4 Alcohol, acetic acid, and glycerol by the HPLC

Ethanol, acetic acid, and glycerol concentrations were determined by the HPLC (Shimadzu, Japan) equipped with a refractive index detector and Bio-Rad HPX-87H column (300×7.8 mm, 9 μm particle size, and 8% cross-linkage) using 5 mM sulfuric acid as eluent at 45°C at a flow rate of 0.6 mL/min. Kombucha samples were centrifuged at 10,000 *g* and filtered (0.45 μm filter) before injecting 20 μL aliquots. The standards of the compounds analyzed were used for identification (retention time) and quantification (external standard). The results were expressed as mg/L.

2.5 Statistical analysis

The results were analyzed by the standard design analysis of the Statistica 10.0 software (Statsoft, Tulsa, OK, United States), where the data were fitted to Equation 1 by regression analyses. In addition, determination coefficients (R^2) were evaluated, and variance analysis (ANOVA) was performed to evaluate the effect of the independent variable on responses as well as the significance of the model.

$$Y = b_0 + b_1x_1 + b_2x_2 + b_3x_3 + b_{12}x_1x_2 + b_{13}x_1x_3 + b_{23}x_2x_3 \quad (1)$$

where:

Y: the dependent variable (yield, SCOBY growth, acidity, pH, glycerol, turbidity, alcohol, or acetic acid);

b_0: intercept;

b_1, b_2, and b_3: linear coefficients;

b_{12}, b_{13}, and b_{23}: interaction coefficients.

3 RESULTS AND DISCUSSION

Table 2 shows the results of the factorial design. The yield of kombucha ranged from 89.1 to 96.4% of the initial volume. The reduction of the tea volume after fermentation is due to the SCOBY absorption, whose growth in dried mass ranged from 41.11 to 62.08%. From a large-scale point of view, these results are essential since yield impacts beverage productivity, and the SCOBY mass impacts the planning of waste management by the industry, leading to the correct dimensioning of equipment and effluent treatment stations.

The total acidity and pH values were according to the Brazilian legal standards of 30–130 mgEq/L and 2.5–4.2 (Brazil, 2019), showing the adequacy of the fermented beverage production procedure. The pH varied from 2.7 to 2.9, which is within the acceptable range as described by Değirmencioğlu et al. (2020) when evaluating the fermentation of kombucha from white, green, oolong, and black teas. In a comparison of the pH values of non-fermented beverages (Table 1) and the final product (Table 2), there was a reduction in the range of 0.24 (trial 9) and 0.64 (trial 7). Compared to previously published green and black teas, kombucha had pH values of 3.2 and 3.5, respectively. The values below pH 2.5 have a high concentration of acetic acid, and pH values higher than 4.2 may compromise the beverage's microbiological safety (Cardoso et al., 2020). In this study, volatile acidity varied from 49.9 mgEq/mL (trial 7) to 91.2 mgEq/mL (trial 1), which is in accordance with Cardoso et al. (2020) and Ivanišová et al. (2019). Among the organic acids to enhance acidity and decrease pH, the most important acids produced during fermentation are glucuronic, gluconic, lactic, malic, citric, tartaric, folic, malonic, oxalic, pyruvic, and glucuronic acids, which is one of the most valuable health acids in kombucha (Jayabalan et al., 2014). Differences between the initial acidity conditions of the trials (Table 1) and at the end of fermentation (Table 2) show an enhancement of the parameter in the range of 46.3 mgEq/L (trial 7) and 84.4 mgEq/L (trial 12). pH reduction and acidity enhancement are related to the production of D-glucuronic, lactic, tartaric, acetic, and citric acids through fermentation. Process conditions impact the production of these compounds by the microbial consortium (Neffe-Skocińska et al., 2017).

The turbidity values varied considerably from 107 to 423 NTU. The clarity of kombuchas is related to the amount of cellulose produced by the acetic acid bacteria in the biomass. Increased turbidity may be related to more cellulose and other fibrous matter being released into the broth and microbial growth in the fermented infusion (Amarasinghe et al., 2018; Goh et al., 2012; Zofia et al., 2020).

The alcohol content values varied from 5.02 to 1.47 mg/L, which transformed into % (v/v) (considering an ethanol density of 789 g/L at 20°C) as requested by the Brazilian regulation; the results ranged from 0.40 to 0.19%, which indicates that the kombucha produced is suitable to be called a non-alcoholic beverage (Brazil, 2019). The observed acetic acid concentration was between 3.78 and 5.33 mg/L, and the glycerol concentration was between 0.25 and 0.84 mg/L. Acetic acid has been reported to be the main acid produced in kombuchas (Ivanišová et al., 2019; Cardoso et al., 2020; Jakubczyk et al., 2020). Cardoso et al. (2020) observed acetic acid concentrations close to 3 g/L for both green and black tea kombuchas. Meanwhile, Ivanišová et al. (2019) observed 1.55 g/L for black tea kombucha.

The substrate used by microorganisms was commercial sucrose, and the symbiosis of bacteria and yeast into SCOBY is responsible for the extracellular enzymatic hydrolysis of sucrose into glucose and fructose and the transformation of glucose and fructose into ethanol and carbon dioxide by the yeasts, while the bacteria convert glucose into gluconic acid and fructose into acetic acid as a result of metabolic chain reactions (Spedding, 2015). Glycerol is the major by-product of ethanol production by yeasts, and its concentration in yeast-fermented beverages contributes to body and fullness, also influencing flavor intensity. Thus, increasing glycerol yield benefits both flavor and

Table 2. Factorial design and responses for the production of kombuchas.

Trial	Scooby weight (X_1)	Sucrose (X_2)	Green:black tea ratio (X_3)	Yield (%)	SCOBY growth (%)	Acidity (mgEq/L)	pH	Glycerol (mg/mL)	Turbidity (NTU)	Alcohol (mg/mL)	Acetic acid (mg/mL)
1	15.03 (-1)	6.81 (-1)	20.2 (-1)	96.4	55.172	91.2	2.85	0.476	298.5	2.849	4.955
2	22.47 (+1)	6.81 (-1)	20.2 (-1)	96.4	62.076	78.1	2.79	0.339	282.5	2.328	4.961
3	15.03 (-1)	9.19 (+1)	20.2 (-1)	96.0	48.083	80.0	2.89	0.423	240.5	2.587	5.039
4	22.47 (+1)	9.19 (+1)	20.2 (-1)	87.3	62.027	83.4	2.72	0.300	300.0	1.468	4.780
5	15.03(-1)	6.81 (-1)	79.8 (+1)	96.0	41.111	62.1	2.88	0.395	378.5	2.557	4.824
6	22.47 (+1)	6.81 (-1)	79.8 (+1)	92.7	58.053	70.8	2.81	0.406	421.5	2.827	5.328
7	15.03 (-1)	9.19 (+1)	79.8 (+1)	96.4	42.126	49.9	2.86	0.411	108.0	2.952	4.341
8	22.47 (+1)	9.19 (+1)	79.8 (+1)	92.4	60.272	65.0	2.77	0.391	215.5	2.671	4.609
9	18.75 (0)	8.00 (0)	50 (0)	94.5	57.204	81.9	2.87	0.400	211.5	2.268	4.307
10	18.75 (0)	8.00 (0)	50 (0)	89.1	61.193	77.6	2.86	0.517	268.0	3.209	4.843
11	18.75 (0)	8.00 (0)	50 (0)	94.5	62.003	63.0	2.82	0.250	322.5	1.896	3.783
12	18.75(0)	8.00 (0)	50 (0)	94.5	57.125	89.2	2.80	0.267	399.5	2.205	4.378

ethanol reduction in non-alcoholic fermented beverages (Zhao et al., 2015).

Results from the statistical analysis of the factorial design indicated that linear models of independent variables (X_1, X_2, and X_3 – SCOBY mass, sucrose, and tea ratio) were not suitable to model the behavior of any of the responses. Furthermore, R^2-values lower than 0.70 and F-values calculated lower than critical F-values at 5% significance show less data adequacy for Equation 1. Thus, the effect of each independent variable and their interaction with the process performance parameters was evaluated (Calado & Montgomery, 2003).

Table 3 shows that kombucha yield was affected by the SCOBY mass used at the beginning of fermentation ($p<0.10$). However, dried biomass enhancement was positively affected by the initial dried weight of SCOBY used in the kombucha and the tea composition. The heavier the initial SCOBY, the more dry mass was produced at the end of the fermentation process ($p<0.05$); meanwhile, the tea ratio negatively affected the enhanced dried mass SCOBY: less green tea in the composition of the fermented tea led to higher biomass growth ($p\leq0.10$). Additionally, Villarreal-Soto et al. (2019) observed that higher surface area contact in kombuchas vessels accelerates the fermentation kinetics, leading to higher ethanol production and bioactive compound amounts.

Tea composition and SCOBY's initial weight negatively affected acidity and pH ($p<0.05$). Results indicate that heavier SCOBY at the beginning of fermentation leads to a lower pH. Meanwhile, higher concentration of green tea leads to lower acidity. Cardoso et al. (2020) observed no significant differences in acidity between black and green tea. However, Coton et al. (2017) verified a high predominance of different acetic and lactic bacteria species among the green and black tea kombuchas and consequent variation in the production of organic acids. Also, in the present work, higher biomass at the beginning of fermentation leads to lower pH values, possibly due to a higher population of acetic and lactic bacteria producing organic acids, whose concentrations were captured by the pH meter. Also, it is important to highlight that each trial presented different pH and acidity conditions (Table 1), and differences between the initial and final values of the kombucha may also influence other results.

Turbidity was affected by the sucrose concentration and the interaction of sucrose concentration and tea ratio ($p<0.10$). Thus, keeping the same values of SCOBY mass and tea composition, a higher sucrose concentration leads to low turbidity. Goh et al. (2012) observed that the amount of sucrose added to black tea broth affects microbial cellulose production by kombucha SCOBY. However, when changing the values of tea composition, there is a significant effect on the interaction with the sucrose content on the beverage's turbidity. These are important results since the conventional "one-at-a-time-approach" ignores the importance of the interaction of the independent variables, which in the present case is significant in the response, and does not lead to an accurate analysis.

The glycerol, alcohol, and acetic acid contents were not affected by the process variables studied, which means that within the evaluated SCOBY mass range, the sucrose range, and the tea's range, glycerol, alcohol, and acetic acid contents did not vary statistically ($p>0.05$). However, considering that the production of these compounds proceeds through a network of reactions, including the hydrolysis of sucrose to less carbohydrates, such as glucose, and their utilization in the production of ethanol by yeasts, and that this alcohol is used as a substrate by acetic bacteria to produce acetic acid, this reaction chain could be too complex to be detected by the modeling procedure proposed in the present work to reach significant effects in the conditions studied.

Review articles about kombucha show that researchers use different tea compositions, sugar concentrations, SOCBY weight, temperatures, and fermentation times (Bortolomedi et al., 2022; Miranda et al., 2022), bringing another hurdle to the international comparison of results. Thus, the results from this study also contribute to this scenario, in which much effort must be taken to better standardize kombucha production on laboratory and industrial scales.

4 CONCLUSION

Thus, this article presented that the kombuchas produced were within Brazilian legal parameters and may be called a non-alcoholic beverage. Statistical analyses showed that kombucha yield, pH, and SCOBY growth are influenced by the SCOBY mass used at the beginning of fermentation. These results show that the weight of the initial inoculum is a critical parameter for kombucha production, although much attention has not been given to this variable. Acidity is affected by the tea composition; meanwhile, kombucha's turbidity is affected by the sucrose concentration and the interaction of the sugar content and tea composition. A significant interaction between process variables presented in the present work demonstrates great relevance. It shows the importance of factorial design

Table 3. Regression coefficients of the factorial design and effect of independent variables on kombuchas' characteristics.

	Yield	SCOBY	Acidity	pH	Turbidity	Glycerol	Alcohol	Acetic
X_1	-0.020‡	0.13984*	3,525	-0.0975*	47.75	-0.06725	-0.41275	0.12975
X_2	-0.011	-0.00976	-5,975	-0.0225	-129.75‡	-0.02275	-0.22075	-0.32475
X_3	0.0018	-0.06449*	-21,225*	0.0175	2.75	0.01625	0.44375	-0.15825
$X_{1\,x}X_2$	-0.0018	0.02061	5,725	-0.0325	35.25	-0.00425	-0.28725	-0.12525
$X_{1\,x}X_3$	0.0018	0.0356	8,375	0.0175	27.75	0.06275	0.40725	0.25625
$X_{2\,x}X_3$	0.0120	0.02593	-3,025	-0.0075	-110.75‡	0.02325	0.34025	-0.27625

X_1: initial SCOBY mass; X_2: sucrose concentration; X_3: green:black tea ratio; $X_{i\,x}X_j$: interaction effect; *statistically significant at 5%; ‡statistically significant at 10%.

analysis in evaluating kombucha's production since the one-by-one experimental procedure hardly indicates this behavior. Glycerol, alcohol, and acetic acid were not affected by the parameters evaluated in the ranges studied. Despite being an ancestral beverage, the production of kombucha on an industrial scale in Western countries is new. More studies about the complex interaction between microorganisms for the product's fermentation, the different teas that compose it, and other process parameters are necessary to allow its proper application at an industrial level, aiming at a higher quality and standardized product.

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