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Investigation of different aeration strategies for the production of kombucha with defined co-cultures

Kombucha fermentation is a complex process influenced by many different process parameters and the dynamics of the microorganisms present. Since no universally established standards exist for large-scale production of kombucha, manufacturers still need to acquire the knowledge to establish controllable and reproducible processes. The aim of this study was to investigate the extent to which the kombucha fermentation process can be influenced by varying the oxygenation regime and the initial pH value. For this purpose, co-culture fermentations with the yeast *Zygosaccharomyces bailii* and the acetic acid bacterium *Komagataeibacter hansenii* were carried out under defined conditions, varying the type and intensity of aeration using various fixed aeration rates and controlled oxygen saturations. The pH values were varied in ranges between 4.1 and 7.5. The fermentations were comprehensively characterized via online and offline analytics, so that both the processes and the products could be chemically, microbiologically, and sensorially evaluated over time. By splitting the process into a sufficiently long anaerobic phase of 4 days followed by an active aeration phase at 0.1 vvm for three to four days, a sufficient oxygen supply was achieved under the given conditions to adequately convert the ethanol produced by the yeast into acetic acid by the bacteria. Sensory properties of the products were not negatively affected by oxidation reactions. Compared to reference processes conducted under traditional, static fermentation conditions, a process acceleration was achieved with equivalent sensory quality but significantly reduced alcohol concentrations (<0.08 %vol vs. 0.47 – 0.50 %vol. after 7 days). This study provides an example of how to produce a kombucha product under defined and accelerated conditions that includes an aerobic phase, and which can be used as basis for further production process developments.

Descriptors: kombucha, tea fermentation, aeration, co-culture, yeast, acetic acid bacteria

Abbreviations: AA: acetic acid, AAB: acetic acid bacteria, BR: bioreactor, LAB: lactic acid bacteria, DO: dissolved oxygen, GA: gluconic acid, GMP: good manufacturing practice, onl.: online, offl.: offline, SCOBY: symbiotic culture of bacteria and yeast, V: fermentation variation, vvm: volume of air per volume of medium per minute, WNGB: wide neck glass bottle, YED: yeast extract dextrose

1 Introduction

Kombucha is a complex fermentation product, produced from sugared tea, that is experiencing enormous market growth worldwide. In 2024, the global kombucha market was estimated at US$ 2.97 billion, and is expected to grow to US$ 4.65 billion by 2029, corresponding to a compound annual growth rate (CAGR) of 9.48% [1].

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An essential initial step in kombucha fermentation is that, under micro- to anaerobic conditions, the yeasts metabolize the available sugar into ethanol and carbon dioxide, while simultaneously forming numerous aromatic fermentation by-products. Acetic acid bacteria (AAB), which are typically found in this fermentation system, can then oxidize the ethanol to acetic acid (AA) and glucose to gluconic acid (GA). This requires the presence of oxygen, which under static fermentation conditions is only available at the surface of the liquid and is very limited due to its rapid consumption. This complex interplay can result in an aromatic tea beverage that is well balanced in terms of sweetness and sourness. In traditional production processes, an undefined symbiotic culture of bacteria and yeast (SCOBY), containing different species of yeast, AAB, and sometimes lactic acid bacteria (LAB) is used for fermentation and the process is designed in a way that part of the finished fermentation batch is used as inoculum for the next kombucha production, a process called backslopping.

Although kombucha is produced commercially, from an industrial perspective it is still a relatively young beverage category without a standard good manufacturing practice (GMP). Current production practice relies mostly on traditional, largely undefined processes,

and individual, inaccessible industry knowledge, thus creating a need for standardized and reproducible production processes.

In recent years, research on kombucha has increased enormously, with many studies focusing on characterizing the microbial composition [2, 3] and general sensory evaluation [4–6], as well as potential health-promoting effects [7–9]. With increasing knowledge and commercial relevance, there has also been an increase in studies systematically examining the kombucha fermentation process, defined starter cultures [10–12], tea varieties and different infusion conditions [13–17], sugar compositions [18–20], and process parameters such as pH [21], temperature [21, 22], and varying oxygen availability [23, 24]. However, based on the large differences in manufacturing practices, and the significant influence of process parameters on product quality, there is a continuing demand for systematic, verifiable studies that provide guidance for manufacturing practices.

With this background, the present study aimed to investigate the extent to which the kombucha production process can be influenced and accelerated through targeted aeration regimes. Using a defined yeast-AAB co-culture, various aeration types and intensities were specifically adjusted in combination with different starting pH values, and the corresponding processes and kombucha products were comprehensively characterized.

2 Material and methods

2.1 Tea preparation

For kombucha tea preparation, 8 g/L of green tea leaves (*Camellia sinensis*; China sencha, Ökotopia GmbH, Germany) in filter bags were added to a 20-L Speidel Braumeister brewing vessel (Speidel Tank- und Behälterbau GmbH, Germany) for 10 min at 75 °C, with final batch volumes of 10 or 15 L water. After removing the tea bags, the volume of the remaining liquid was determined using measuring cups. Based on the volume determined, 40 g/L sucrose (Carl Roth GmbH, Germany) and 20 g/L glucose (AppliChem GmbH, Germany) were added to the hot tea.

2.2 Pre-culture management

The yeast *Zygosaccharomyces bailii* Zs-1303 and the acetic acid bacterium (AAB) *Komagataeibacter hansenii* Ko-0201, both cultures from the VLB strain collection, were used for the fermentation studies (Fig. 1). Propagation of pre-cultures occurred for each in two steps. First, 250 µL cryo-culture was inoculated into 25 mL of YED (yeast extract (Carl Roth), 10 g/L, glucose (AppliChem), 50 g/L) medium in a sterile 250 mL shake flask with vented cap. Cultures were cultivated at 30 °C for 48 h with 130 rpm in an incubator shaker (New Brunswick™ I26, Eppendorf AG, Germany). For the second pre-culture, 50 mL of YED medium was inoculated with 500 µL of the first pre-culture in a 500 mL shake flask and incubated under the same conditions for 72 h. For the inoculation of the prepared tea, the cell concentration of the second preculture was determined (see 2.5.1) followed by separation via centrifuge (4750 x g, 5 min; Rotina 420 R, Hettich AG, Germany) and washing step with 0.9% sodium chloride solution.

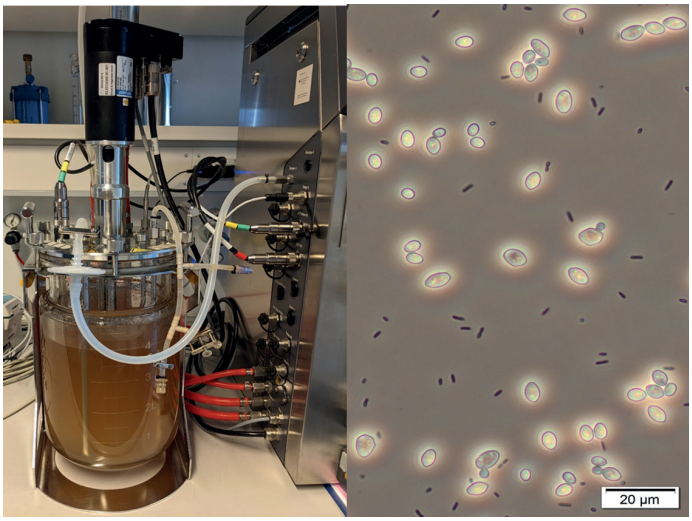


Fig. 1 **Bioreactor used for controlled kombucha fermentation (left) and microscopic phase contrast picture of the bacteria and yeast co-culture (right)**

2.3 Kombucha fermentation

Kombucha fermentations were started with 10^6 yeast and 10^7 bacteria cells per mL (see 2.5.1 for cell concentration determination) and incubated at 30 °C for 10 – 14 days in 5-L bioreactors (Biostat® B (Fig. 1) and A, Satorius Stedim Systems GmbH, Germany) or a 5-L wide neck glass bottle (WNGB). The WNGB and the bioreactors including probes were sterilized for 20 min at 121 °C at 1 bar overpressure, respectively. The prepared tea was then poured in. For tea acidification, 1 mol/L acetic acid (AA; Carl Roth) was used. The volume required to achieve the desired pH was determined in preliminary tests by titration. The acid was added to the tea before transferring it to the cultivation vessel. Samples were taken at regular intervals for various analyses. In the case of the bioreactor fermentations, the stirrer was switched on for 1 minute at 300 rpm to obtain a thoroughly mixed sample. All steps of pre-culture treatment, bioreactor preparation, and sampling were performed under aseptic conditions to prevent contamination.

2.4 Process variations

The following study consists of ten different variations (V1-V10) of kombucha fermentations (Table 1). The respective fermentation settings are the result of an iterative study process and partly served to demonstrate the process behavior of extreme setting variations. In summary, the process variations can be described as follows: Fermentation V1 represents a static fermentation in a WNGB with no mixing, and with oxygen input only possible via the liquid surface. Fermentation V2 was carried out in a bioreactor with a similar specific interfacial area (Table 1, [25]) and without mixing. However, in V2 an active exchange of the atmosphere in the reactor headspace took place through headspace aeration. Both fermentations can be considered, in different ways, as reference fermentations for static process conditions. Fermentations V3 and V4 were carried out with a relatively strong, constant submerged aeration rate of 1 vvm with moderate stirring, with V3 starting at a pH of 7.5 (without adjustment) and V4 at a pH of 4.1 (adjusted with AA). This pH range represents conditions without any pH adjustment in the neutral range, e.g., using pure starter cultures without

acidified medium, as well as conditions common in practice with an acidic pH range, e.g., in a backslopping process. In V5 and V6, the dissolved oxygen (DO) was maintained at 10 % air-saturation using an integrated PID controller, with the respective adjustment being achieved by the aeration rate at a constant stirrer speed. Due to the aeration and stirring process, an adjustment of the liquid volume from 5 L to 4 L was necessary from V3 onwards. In V5, the DO was automatically controlled at the start of fermentation, and in V6 only from day 4. The delayed start was intended to ensure an anaerobic fermentation phase combined with alcohol and aroma production by the yeasts. In V7 and V8, fermentation was started at a constant and moderate aeration rate of 0.1 vvm from day 4, with the fermentation being carried out once without (V7) and once with (V8) an initial pH reduction. In V9, identical process conditions were set as in V8, with the difference that constant moderate gassing began on day 2. In V10, the setting of V7 was used in triplicate, with the difference that the pH value was lowered to 5.8.

2.5 Analytical methods

2.5.1 Determination of total cell concentration by Coulter Counter®

The total cell concentration was determined via an electrical impedance-based particle counting using a Beckman Multisizer™ 3 Coulter Counter® (Beckman Coulter GmbH, Germany). For the measurement, 10 µL of the sample was diluted in 10 mL ISOTON II (Beckman Coulter), and subsequently 50 µL were used for analysis. The Coulter Counter® was equipped with a 30 µL capillary and the data were converted to size features with the Multisizer™ 3 Software Version 3.53 (Beckman Coulter). Particles with a size of 0.6 – 2.0 µm were considered to be bacteria cells, whereas particles with a size of 2.0 – 6.0 µm were considered to be yeast cells, enabling precise analysis of cell concentration and size distribution within the co-culture (see example in the supplementary data file).

2.5.2 Determination of low molecular weight sugars, organic acids and alcohol

The analyses of glucose, fructose, sucrose, ethanol, glycerol, and the organic acids, acetic acid and gluconic acid, were conducted via HPLC (Knauer Wissenschaftliche Geräte GmbH, Germany) applying isocratic elution of 10 µL sample on a Nucleogel® Ion 300 OA column (Macherey-Nagel GmbH & Co. KG, Germany) with 5.0 mmol/L H_2SO_4 at a flow rate of 0.4 mL/min and 40 °C column temperature. Detection was performed in a refractive index detector. Sugars, ethanol and glycerol were detected using a refractive index detector. Organic acids were detected via multiple wavelength detector at a wavelength of 210 nm. Chromatograms were evaluated using the ClarityChrom® Chromatography data system software (Knauer). Total fermentable sugar concentration was expressed as the sum of the quantified monosaccharides and disaccharides.

2.5.3 Analyses of dissolved oxygen, pH and redox potential

Dissolved oxygen (DO) was recorded online using a probe (VisiFerm DO 325, Hamilton Bonaduz AG, Switzerland) whose 100% value corresponds to that of the medium completely saturated with air oxygen at atmospheric pressure. A combination probe (Easyferm Plus pH/RX 325, Hamilton Bonaduz AG) was used for online analysis of pH and redox values. The pH value was also analyzed offline using a laboratory pH meter (766 Calimatic, Knick Elektronische Messgeräte GmbH & Co. KG, Germany). All probes were calibrated according to the manufacturer's specifications.

2.5.4 Sensory analysis

Sensory properties of the kombucha products were analysed after 7 and 14 days of fermentation by a previously established descriptive and evaluative analysis scheme including ten taste attributes (alcoholic, astringent, bitter, fruity, solvent-like, sour, sparkling,

Table 1 Investigated process variations of the kombucha fermentations

Process variation	System	Starting pH	Aeration condition	Stirring during aeration	Vol.	a
V1	5 L-WNGB	7.3	no aeration	none	5 L	0.052 cm^{-1}
V2	5 L-BR	7.1	surface aeration, 1 vvm	none	5 L	0.040 cm^{-1}
V3	5 L-BR	7.5	1 vvm	300 rpm	4 L	0.050 cm^{-1}
V4	5 L-BR	4.1	1 vvm	300 rpm	4 L	0.050 cm^{-1}
V5	5 L-BR	4.1	controlled, DO = 10%	300 rpm	4 L	0.050 cm^{-1}
V6	5 L-BR	4.1	controlled, DO = 10%, starting at day 4	300 rpm	4 L	0.050 cm^{-1}
V7	5 L-BR	7.2	0.1 vvm, starting at day 4	300 rpm	4 L	0.050 cm^{-1}
V8	5 L-BR	4.1	0.1 vvm, starting at day 4	300 rpm	4 L	0.050 cm^{-1}
V9	5 L-BR	4.1	0.1 vvm, starting at day 2	300 rpm	4 L	0.050 cm^{-1}
V10	5 L-BR	5.8	0.1 vvm, starting at day 4	300 rpm	4 L	0.050 cm^{-1}

WNGB = Wide-neck glass bottle, BR = Bioreactor, DO = Dissolved oxygen, Vol. = Working volume,
a = Specific interfacial area (surface area / working volume)

sweet, tea base, yeasty) scored on a scale from 1 (imperceptible) to 9 (very pronounced), and six odor attributes (acetic, alcoholic, fruity, yeasty, solvent-like and sulfurous) rated from 1 (not perceptible) to 3 (strongly perceptible), respectively. Additionally, overall rating plus the rating of taste and odor were scored between 1 (very bad) to 9 (very good) [26]. Sensory analysis was conducted in a non-blinded setting by a trained panel comprising three to four experienced assessors. Nevertheless, it should be noted that a statistical evaluation is not adequately possible due to the available data and the relatively small panel size.

3 Results and discussion

3.1 Fermentation processes

In fermentation variation V1, a WNGB was used in static fermentation mode, which allowed natural gas exchange in the headspace through a cotton plug. Although this mode of operation takes place with a defined co-culture and temperature, it most closely corresponds to classic kombucha production, as it also occurs in home and small-scale production.

The offline data illustrate the steady increase in yeast and bacterial cells with a simultaneous decrease in pH (Fig. 2, V1(c)). This resulted in rapid ethanol formation coupled with the formation of GA, followed by a slightly delayed formation of AA starting on day 4 (Fig. 2, V1(a)). This indicates that the yeast began fermentation and ethanol production immediately. The AAB, in turn, started converting the glucose present into GA early and, after day 4, began to convert a part of the simultaneously produced ethanol into AA.

V2 represents a mainly static cultivation in the bioreactor. Here, the liquid was briefly mixed using a stirrer only before sampling, and the headspace was aerated throughout the runtime; this was intended to deplete the CO_2 layer above the liquid surface. The DO fell to 0% immediately after inoculation and was thus completely consumed by the yeast and bacteria (Fig. 2, V2(b)). This trend is also reflected in the redox value – as indicator for the oxidation or reduction capacity of a solution – which drops rapidly in the anaerobic environment and remains between –20 and –80 mV over the whole fermentation period. The slight redox peaks during the samplings (Fig. 2 V2(b)) are a result of the slightly increased oxygen input via the liquid interface caused by mixing. However, this input is not high enough resp. the DO probe is not sensitive enough, to allow a noticeable signal of the DO value. This shows that the redox signal under anaerobic and microaerobic conditions provides information that could allow conclusions to be drawn about the fermentation status. Here, even the information about the timing of the change is relevant. However, specifically attributing the causes of a signal change, e.g., an increase in signal due to a sudden decrease in oxidative metabolic activity within the culture, requires further investigation.

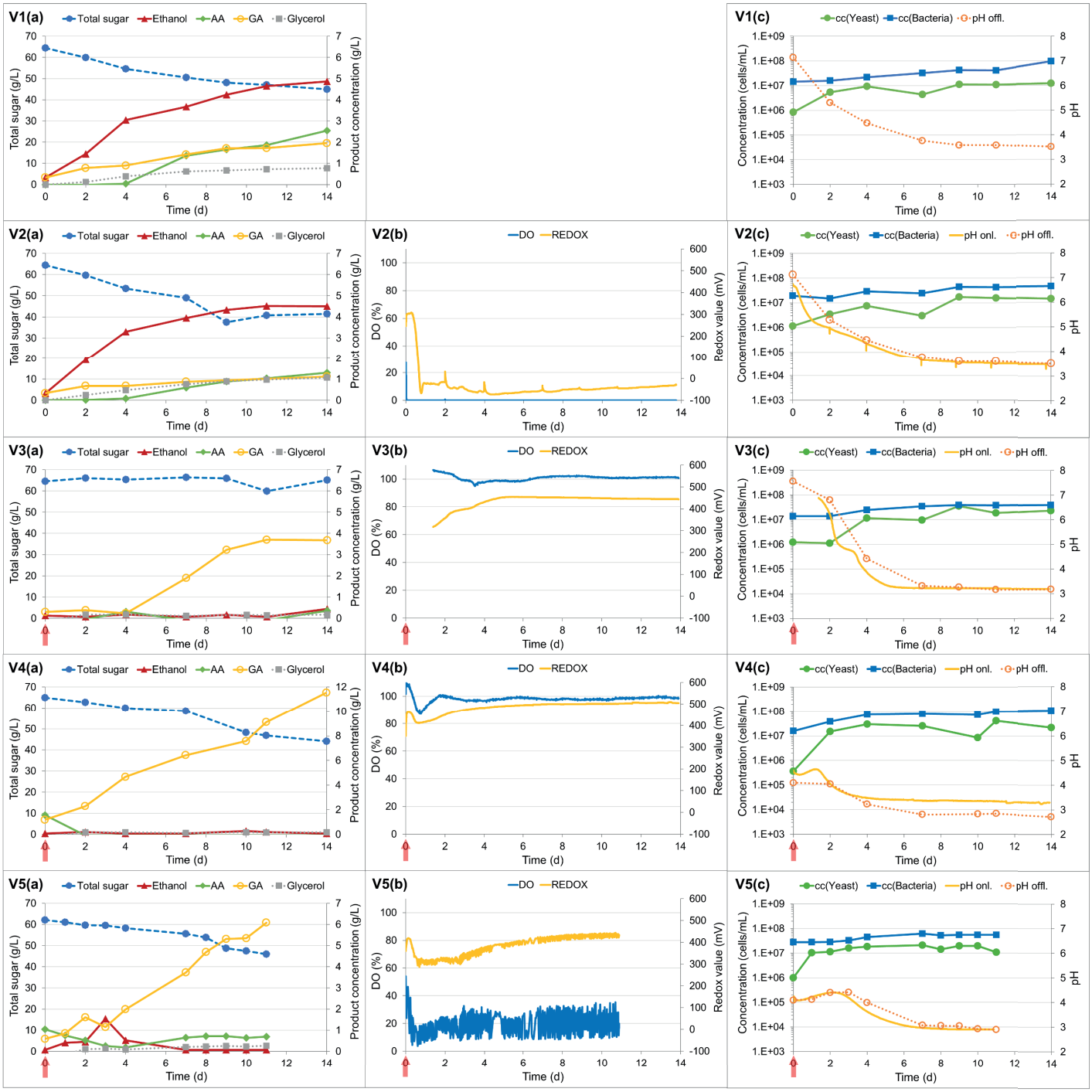
In fermentations V3 and V4, the fermentation batches were aerated from the beginning in such a way that oxygen was not limited. Both processes, with the aeration rate set at 1 vvm, exhibited a consistent oxygen saturation of around 100% (Fig. 2 V3(b)). Both processes are characterized by the fact that almost exclusively GA is identifiable as a metabolic product, with formation occurring significantly faster with initial acidification (V4) than in the process with a neutral initial pH value (V3). The almost complete absence of sugar consumption and the lower increase in cell count further suggest that there was generally less microbial activity in V3 (Fig. 2 V3, V4). Also, there is hardly any yeast activity in the form of ethanol or glycerol production.

In V5 and V6, an oxygen saturation of 10% was maintained from the beginning (V5) or after a four-day anaerobic phase (V6). Fig. 2 V5(b) and Fig. 3 V6(b) show that, with the existing control settings, DO values fluctuate within a range of 10-30% (V5) and 10-20% (V6) saturation, respectively. This is probably a result of suboptimal PID (proportional–integral–derivative) controller settings and could be caused, for example, by a too high KP (proportional gain) value or a too low T_I (integral time constant) value. However, for the objective of the experiments, these DO overshoots can be considered uncritical, as the target constant saturation at a lower range has been created. In V5, the existing oxygen supply results in GA production by the AAB from the beginning with a relatively linear increase over time. Under the prevailing conditions, a net production and accumulation of ethanol is also evident, peaking on day 3, before it is oxidized to AA by the AAB (Fig. 2 V5(a)). This trend is also consistent with the pH decrease starting on day 3 (Fig. 2 V5(c)).

Compared to V5, a significantly higher amount of ethanol was produced in V6 during the 4-day anaerobic phase, with a peak of 3.1 g/L. With the start of aeration, this was then effectively metabolized by the AAB to AA within 4 days. At the same time, GA was also formed from the glucose present. After aeration, a characteristic acid-induced decrease in pH occurs, which is already at 3.0 after 9 days. Interestingly, there is also a brief increase in pH, which is detectable by the frequent online pH measurement (Fig. 3 V6(c)). This phenomenon may be explained by the fact that dissolved CO_2 is released during aeration. The associated shift in equilibrium to the detriment of carbonic acid leads to a reduction in the associated protons and a brief increase in pH. This behavior can also be observed in the other aerated fermentations to varying degrees (e.g., Fig. 2 V4(c), V5(c); Fig. 3 V7(c), V8(c), V9(c)).

In V7 to V10, constant aeration rates of 0.1 vvm (here 0.4 L/h) were set after a certain anaerobic fermentation phase. This type of aeration has the advantage of being feasible in practice without the need for appropriate sensor technology and control loop systems. Within the experiments, the starting pH values were varied, and in V9, the initial anaerobic phase was reduced from 4 to 2 days.

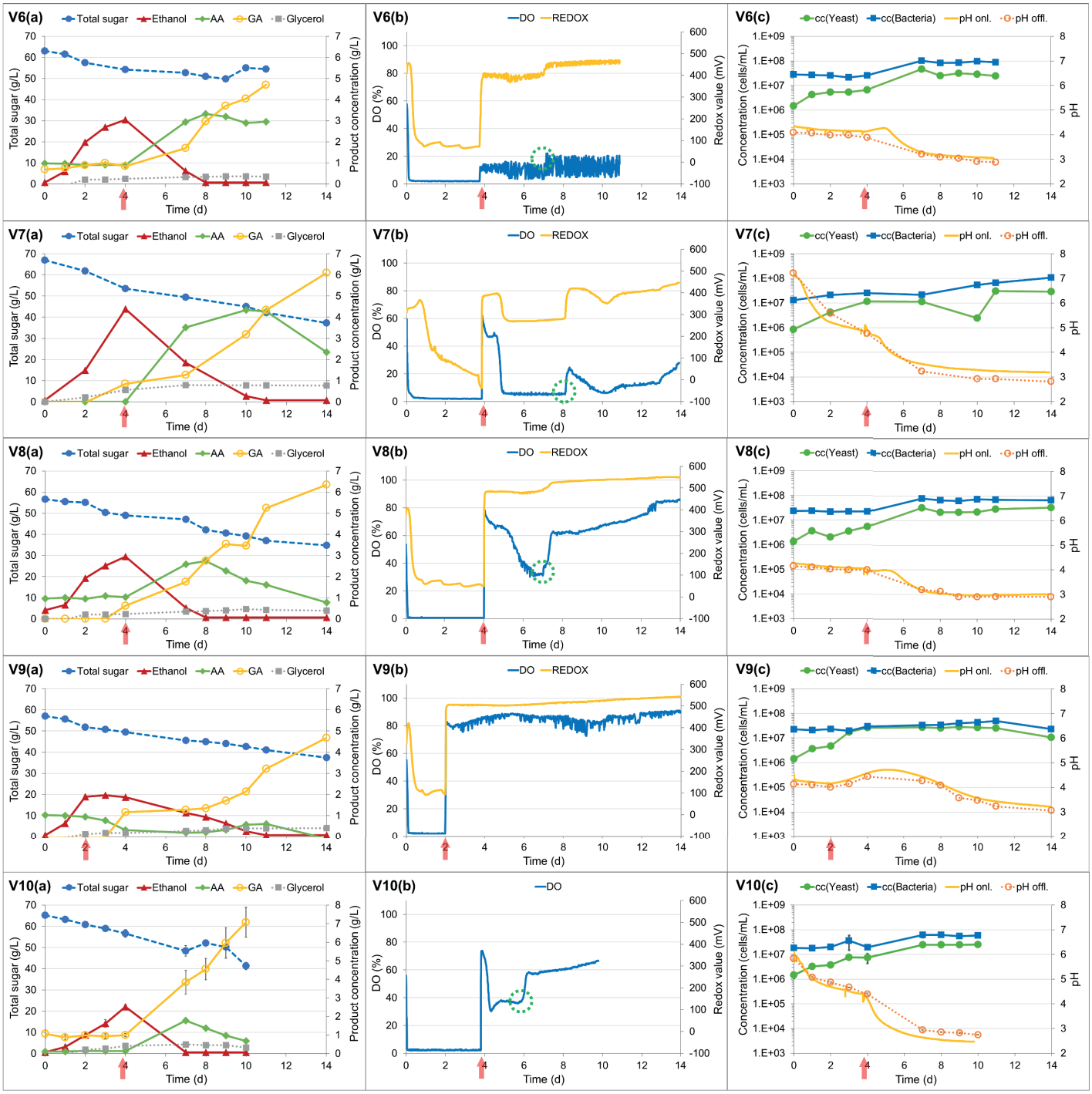
As expected, all processes showed the rapid consumption of DO after inoculation, which created the target anaerobic conditions (Fig. 3). This was accompanied by the formation of ethanol, which was further oxidized to AA after the onset of aeration. The highest concentration of AA was measured on day 10 at a concentration of 4.3 g/L (Fig. 3 V7(a)). Once again, a parallel and sustained formation of GA was observed in all conditions until the process was terminated. This indicates the ongoing activity of *Komagataeibacter hansenii* and the availability of glucose, which is the basis for GA generation. In addition to the very high GA formation in V4 of 11.5 g/L after 14 days, a very pronounced GA formation was observed in V10 with a concentration of 6.6 ± 0.8 g/L (Fig. 2 V4(a), Fig. 3 V10(a)).



Notes: (i) For the fermentation in wide neck glass bottle (V1), no online data exist
(ii) In V3, recording of online data started at day one

Fig. 2 **Process data of the varied kombucha fermentations V1 to V5 (see Tab. 1). Illustrated are (a) the course of the total sugar concentration and the metabolites EtOH, acetic acid (AA), gluconic acid (GA) and glycerol, (b) the dissolved oxygen (DO) and redox value, and (c) the cell concentration of yeast and bacteria cells and the pH value measured online (onl.) and offline (offl.). Data are derived from single fermentation, respectively. The red arrow illustrates the start of aeration and stirring**

A closer look at the trend of AA formation in V7, V8, and V10 reveals that the maximum is reached after the consumption of ethanol, followed by a steady decrease. This indicates that, despite the availability of sugar, no noticeable ethanol is produced by the Crabtree-positive *Z. bailii* [27], as this would in turn be further oxidized to AA by the AAB. At the same time, the course also demonstrates that *Z. bailii* under the given aerobic conditions is able to metabolize AA via the TCA cycle, even in the presence of alternative sugars, which in turn contributes to its good acid tolerance [28]. At the same time, under the given conditions, reduced yeast growth can be observed after reaching the AA peaks, evident by the lack of change or slight decrease in yeast cell concentrations from about day 7 (Fig. 3 V7(c), V8(c), V10(c)). Comparing the four fermentations V7 to V10, V9 represents an exception relative to the others. Despite the rapid increase in yeast cell concentration in the first days and the associated ethanol production, the



Note: (iii) Redox values for V10 are not available

Fig. 3 **Process data of the varied kombucha fermentations V6 to V10 (see Tab. 1). Illustrated are (a) the course of the total sugar concentration and the metabolites EtOH, acetic acid (AA), gluconic acid (GA) and glycerol, (b) the dissolved oxygen (DO) and redox value, and (c) the cell concentration of yeasts and bacteria and the pH value measured online (onl.) and offline (offl.). The data from V6 to V9 each originates from single fermentations, data from V10 originates from triplicate fermentations. Offline data of V10 are mean values ± SD of triplicate fermentations. The red arrow illustrates the start of aeration and stirring; the green dotted circle illustrates a characteristic decrease in the oxygen uptake rate (here referred to as DO breakpoint).**

co-culture appears to exhibit lower activity after the start of aeration on day 2. In addition to the slower degradation of ethanol, the delayed and slow formation of GA, and the delayed, low net formation of AA, this is also evident from the comparatively low oxygen consumption (Fig. 3 V9(a and b)). It is notable that the aeration rate set at 0.1 vvm at V7, V8 and V10 ensured an oxygen supply in which complete oxygen saturation did not occur and in which there were phases of oxygen limitation (e.g. Fig. 3 V7(b), between days 5 and 8). Due to these conditions, process- and product-relevant time-points/phases can be determined from the online measured oxygen value and, under certain conditions, also the redox value. Thus, the online values in V7, V8 and V10 can be used to describe a characteristic progression during the aerobic phase with high metabolic activity. After the start of aeration and stirring, the DO value rises rapidly in all three fermentations. Even

before it reaches the full saturation value of 100%, the activated or redirected metabolism of the aerobic cultures leads to rapid oxygen consumption. In the case of V7, the consumption was so high that between days 5.0 and 8.1, with constant DO values of around 5%, oxygen-limiting conditions can be assumed (Fig. 3 V7(b)). The subsequent rapid increase in DO (and simultaneously in the redox value) suggests a reduction in the oxygen consumption rate and thus a sudden drop in the metabolic activity of the co-culture, here referred to as DO breakpoint. This characteristic is also evident, to a lesser extent, in V8 and V10, and possibly in the DO-controlled process V6 (highlighted by green dotted circles in Fig. 3, respectively). The first three processes differ only in starting pH values of 7.2 (V7), 4.1 (V8), and 5.8 (V10) (Table 1). Interestingly, a decrease in the time span between the start of aeration and the characteristic DO increase is evident in this sequence, from 4.1 d (V7), 3.0 d (V8), and 2.1 d (V10). These temporal differences are also reflected in the comparison of other characteristic points of the process, such as attainment of the maximum AA peak, the consumption point of ethanol and the reduction in the rate of the pH reduction. The latter is illustrated by the time needed to reach a pH value of 3.1, which was the case in V7 after 9.2 d, in V8 after 7.3 d, and in V10 after 5.1 d (Fig. 3).

Since variations V7, V8, and V10 also produced different concentrations of ethanol and, depending on this, AA, the results of these processes can be viewed as a framework for further process developments. Within this, control options for process acceleration and refinement AA concentrations are suggested. This knowledge can be used, for example, to adjust the profile of the organic acids, here GA and AA, which in turn can have an impact on the quality-determining sensory product properties.

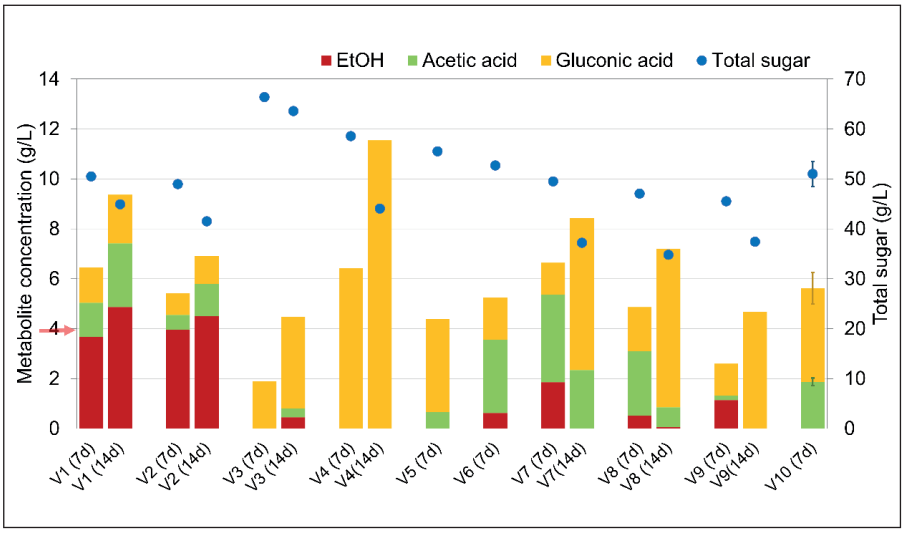


Fig. 4 **Key components in the fermentation products after 7 and 14 days. The red arrow illustrates the value of 3.95 g/L which corresponds to 0.5 %vol ethanol. Data from V1 to V9 correspond to the analyses of single fermentations, data from V10 correspond to mean values ± standard deviation from triple fermentations**

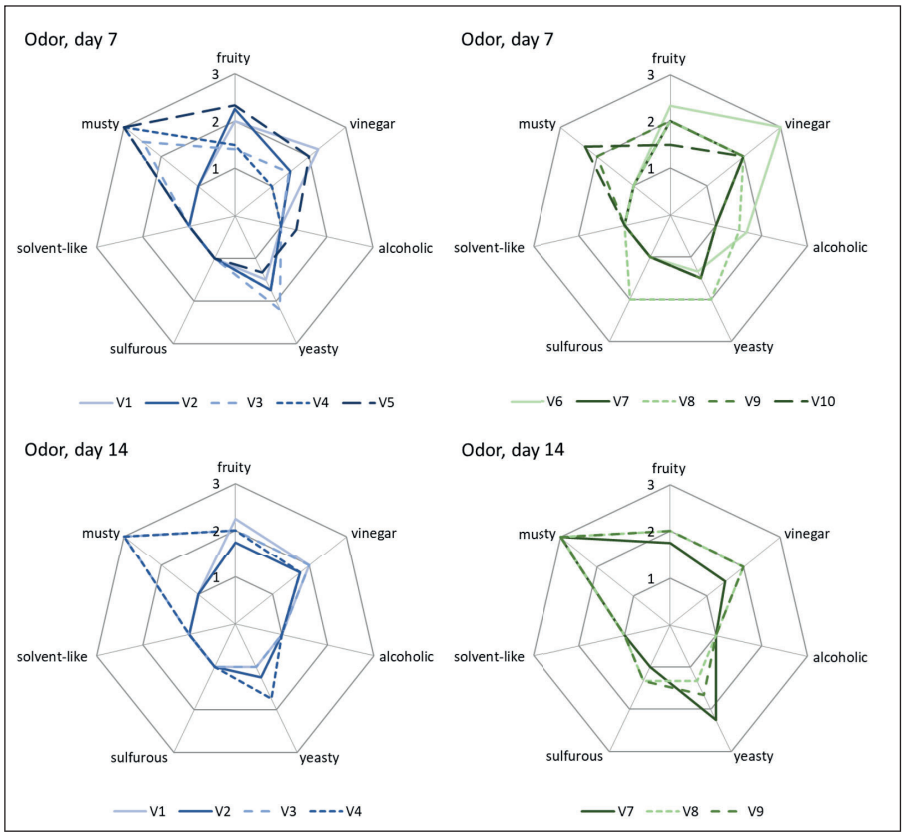


Fig. 5 **Descriptive evaluation of the smell of the different kombucha products after 7 and 14 days. The samples were evaluated on a scale from 1 (not perceptible) to 3 (strongly perceptible)**

3.2 Sensory evaluation

Samples were taken from all fermentations of the various process variants after 7 days, as well as from V1, V2, V3, V4, V7, V8, and V9 after 14 days for sensory evaluation. The remaining fermentations (V5, V6, V10) were terminated before day 14. To facilitate comparison, Figure 4 shows the residual sugars and key metabolites of the kombucha samples. The descriptions of odor and taste are shown in Figures 5 and 6. The respective overall ratings can be found in Figure 7.

The comparison in Figure 4 illustrates that the samples vary greatly in terms of ethanol, AA, GA, and residual sugar concentrations. This is a result of the different processing conditions set, which led to very different metabolic activities and fermentation dynamics in the respective cultures, as discussed above.

These differences are also reflected in the sensory description of the samples. It is striking that after 7 days, only 4 of 10 samples exhibited no musty odor; after 14 days, only the two non-aerated batches V1 and V2 were free of this negative characteristic (Fig. 5).

Fig. 6 **Descriptive evaluation of the taste of the different kombucha products after 7 and 14 days. The samples were evaluated on a scale from 1 (imperceptible) to 9 (very pronounced)**

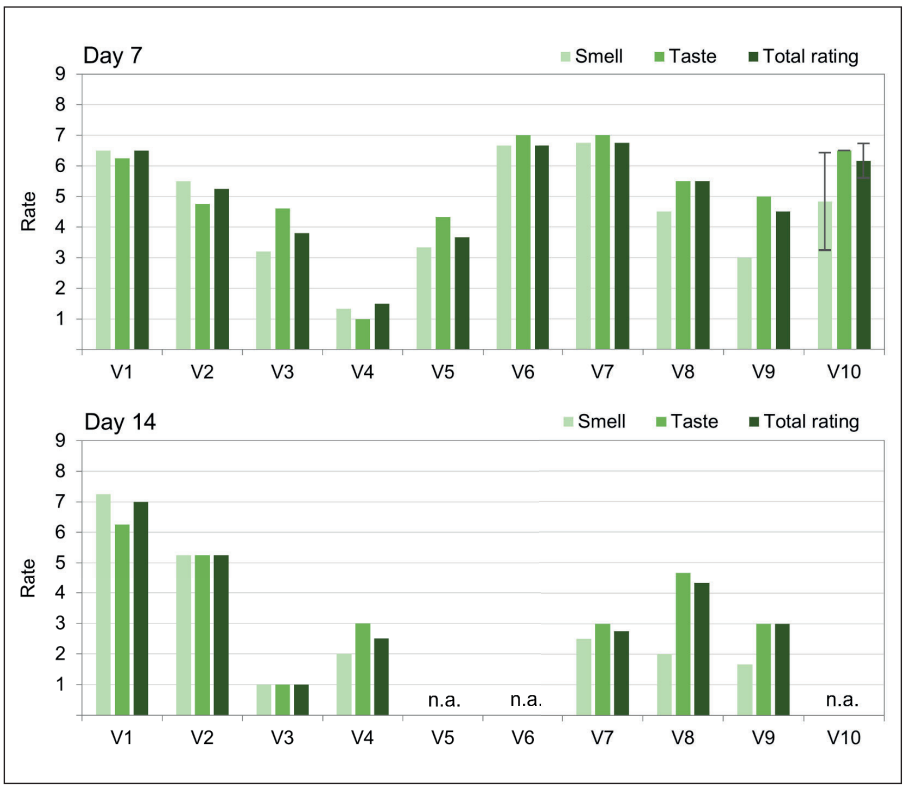


Fig. 7 **Sensory rating of the different kombucha products after 7 and 14 days. The samples were evaluated on a scale from 1 (= very bad) to 9 (= very good), respectively. n.a.: not analyzed. Data from V1 to V9 correspond to the analyses of single fermentations, data from V10 correspond to mean values ± standard deviation from triple fermentations**

This attribute, which is obviously caused by the aeration and mixing process, also significantly determines the overall product evaluation (Fig. 7). Overall, only 4 of the samples after 7 days (V1, V6, V7, V10), and only one sample after 14 days (V1), achieved an overall rating of more than 6 out of 9 points (Fig. 7). These samples have in common that they have a fruity and acetic acid odor and are (largely) free of typical off-flavors such as solvent-like or sulfurous notes. The slightly musty odor, which in this case is only perceptible in sample V10, is discussed further below. These olfactory impressions correspond well with the taste assessments of the samples. The five samples rated as "good" are also characterized by pronounced acidity and fruitiness in taste (Fig. 6).

The significant differences in the chemical profiles and sensory evaluations of the samples examined here, with otherwise identical tea formulations and inoculation conditions, underscore how strongly the product characteristics depend on the production conditions—in particular, the oxygen regime, the initial pH value, and the fermentation time. The results clearly show that process changes such as active aeration and mixing can dramatically alter the complex dynamics of the microorganisms and thus lead to products with completely different quality and properties. Nevertheless, the high variance in the evaluation of the odor within the triple V10 fermentations evident here also illustrates that a more comprehensive database, in combination with a larger panel of tasters, is favorable for stable process and product development.

In general, excessive aeration led rapidly to products with degraded sensory properties. This is particularly evident in the olfactometric evaluation as a musty odor, which was characteristic of both samples with intensive aeration of 1 vvm (V3(7d, 14d); V4(7d, 14d)), and samples with aeration of more than three days (V5(7d); V7(14d); V8(14d); V9(14d)). Interestingly, even after moderate aeration of 0.1 vvm, a slight musty note was already noticeable after a 3 day aerobic phase in V10. Since this product was generally rated positively in the overall result, it can be assumed that the sampling point on the seventh day represents a phase in which the concentration of off-flavors exceeded the sensorially negative range. This would also be consistent with the previously discussed assumption that, under the process conditions prevailing in V10, the general process dynamic is slightly accelerated compared to V7 and V8. Considering the sampling time in the promis-

ing process variations V6, V7, V8, and V10, it is noteworthy that in V6 and V7, this time occurs before the previously discussed DO breakpoint (Fig. 3, V6(b), V7(b); green dotted circles). In contrast, the sampling time in V8 falls precisely in this critical phase, or in V10, sampling took place approximately 25 hours after the changed oxygen demand of the co-culture. Due to the lack of sampling and sensory analysis at times before and after this characteristic DO breakpoint, unfortunately, no more precise statement can be made regarding the sensory alteration in this phase.

3.3 Technological consideration

Although *Z. bailii* is known to be very tolerant to weak acids and especially resistant to acetic acid [29, 30], the conditions at the DO breakpoint described above could be sufficiently harsh to cause excessive stress for the cells. For example, the pH values of the discussed batches V6, V7, V8, and V10 by day 6 are already between 2.8 and 3.6, thus not only below the pK_a of acetic acid (pK_a(AA) = 4.76) but also of gluconic acid (pK_a(GA) = 3.86). Under these conditions, AA and GA are predominantly in the undissociated and thus membrane-permeable form, resulting in increased toxicity for the yeast cells. Additionally, it is known for *Z. bailii* that ethanol inhibits the uptake resp. transport of acetate into the cell and thus plays a crucial role in the acid resistance of this species [28, 31]. The complete conversion of ethanol to AA, forced here by the aerobic regime, could thus lead to conditions in which *Z. bailii* is less adapted and thus more stressed. The microbial analysis that is based on particle detection does not allow for a sufficiently reliable determination of the extent to which stress-induced autolysis of the yeast cells occurred. Nevertheless, it can generally be assumed that, under the elevated temperature of 30°C and the acidic conditions prevailing here, increasingly stressful conditions and an increasing formation of fermentation by-products such as alcohols, acids, esters, and aldehydes can occur over time. Regardless of whether cell-autolysis products such as lipids, fatty acids, amino acids, and nucleotides are available or not, chemical and enzymatic oxidation reactions can be favored under present conditions, resulting in the formation of aldehydes, ketones, and sulfur compounds, which in turn can lead to a yeasty, meaty, and sometimes musty off-flavor [32–34]. However, the fermentation process and the products from V7 provide a very interesting indication. After the DO breakpoint on day 8, there was a noticeable decrease in yeast cell concentration, which was reduced from $1.1*10^7$ to $2.4*10^6$ cells/mL from day 7 to 10 (Fig. 3 V7(c)). This markable decrease in yeast cell count suggests a high proportion of lysed cells. Looking at the two sensory-assessed samples taken well before and after the DO breakpoint, a clear sensory change is evident. The sample on day 7 received a good rating of approximately 7 out of 9 points both in terms of taste and odor, and no prominent off-flavor attributes could be identified (Figs. 4-6). In contrast, the product after 14 days exhibited a more pronounced yeasty taste and a very yeasty and musty odor, resulting in a relatively poor rating of 4 points on average (Fig. 4).

The possible relationship discussed above between the availability of ethanol to maintain acid tolerance in *Z. bailii* is also consistent with the sensory evaluations of the two statically cultivated batches V1 and V2. In both cases, relatively high concentrations of ethanol were present in the samples tasted (0.47-0.62 %vol, Fig. 4) and no significant off-flavor formation was perceptible. Thus, considering the positive product evaluation evident in V1, ethanol would have a certain relevance not only from a sensory perspective (Fig. 7), but also regarding its technological function in the use of *Z. bailii* under acidic conditions for the production of kombucha.

As a result, it would be very interesting to examine in more detail the influence of certain process-characteristic times – using the example of V10, these would be days 5, 6, and 7. One can only speculate as to the extent to which, under the given process conditions, there is a connection between the detectable characteristic DO breakpoint of the co-culture and an optimal harvest time for the kombucha product. Targeted studies of this relationship, while simultaneously considering the microorganisms used and co-culture specificity, could lead to a relevant harvesting control point for this production process, especially when it is accelerated by aeration.

Systematic studies in which process control is specifically achieved through active aeration in kombucha production are rare. Czarnecki et al. investigated increased oxygen availability during kombucha fermentation with SCOBY and by using air-permeable silicone bags under static conditions [24]. Even though the processes took significantly longer than in the present study, significantly higher GA concentrations were achieved with increased oxygen availability than in conventional glass vessels. This is consistent with the observed increase in GA production under increased oxygen availability. The working group also reported a positive influence of the addition of glucose and fructose on the final organic acid profile in the product. Similar results, meaning a mixture of both acids AA and GA, were achieved here through the targeted use of sucrose and glucose as carbon sources, which presumably had a positive effect on product quality (Figs. 4 and 7). Unfortunately, the evaluation basis in the study of *Czarnecki et al.* was based solely on chemical analyses, and there was no sensory evaluation of the produced kombucha products. Therefore, statements regarding any negative effects due to metabolic shifts within the mixed culture or oxidation reactions cannot be made. *Błaszak et al.* investigated constant aeration within a coffee-kombucha fermentation using an undefined mixed culture (SCOBY) [35]. While they claimed that aeration accelerated the process from 8 to 4 days, their evaluation was based solely on parameters such as pH shift, increase in bacterial cellulose, polyphenol content, and antioxidant capacity in the samples. Therefore, no conclusions can be drawn about the actual sensory quality of the products. It is likely that strong aeration could negatively impact sensory properties, thus questioning the practical relevance of these studies.

4 Conclusions

In this study, sensorially appealing kombucha products could be produced using the co-culture of *Komagataeibacter hansenii* Ko-0201 and *Zygosaccharomyces bailii* Zs-1303 under various process conditions. Process optimization through the separation of the traditional static fermentation into two distinct phases—an initial anaerobic phase followed by an aerobic phase—led to an accelerated production.

Good results were achieved with a pre-established green tea recipe and co-culture-specific inoculation densities of 10^7 bacterial and 10^6 yeast cells per mL. A four-day static incubation at 30 °C followed by aerobic conditions (aeration rate of 0.1 vvm, moderate agitation) resulted in a desirable product within seven days. Future studies, incorporating more frequent sampling, are expected to provide greater insight into process dynamics and product quality, facilitating further refinement of fermentation parameters, with high potential to further shorten the production time.

Under the conditions used, it was found that the duration of the anaerobic phase is critical for the formation of aromatic compounds and ethanol. The second aerobic phase should be long enough to achieve the desired degree of ethanol degradation and acid formation while minimizing the development of off-flavors presumably based on oxidation reactions.

By considering very different process conditions and using different online analyses, not only characteristic fermentation dynamics but also process-specific time points were identified, which indicate a characteristic change in metabolic activity of the co-culture with potentially important consequences for product sensory perception. Although the phenomena identified here are process- and microorganism-specific, suggestions are provided for possible process control strategies that are particularly relevant for industrial processes using defined starter cultures and aerated systems. In further developments, more frequent samplings and analyses will allow better process and product evaluation and would reveal possibilities for general and co-culture specific process acceleration and optimization.

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Conflict of interest

The authors declare there are no conflicts of interest.

CRediT authorship contribution statement

Marie Ludszuweit: Conceptualization, Formal analysis, Investigation, Writing – review & editing, Visualization. Leon Bender: Formal analysis, Investigation, Writing – review & editing. Gayatri Mehta: Conceptualization, Formal analysis, Investigation. Martin Hageböck: Writing – review & editing. Brian Gibson: Writing – review & editing, Supervision. Martin Senz: Conceptualization, Formal analysis, Writing - Original Draft, Writing – review & editing, Visualization, Supervision, Funding acquisition.

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Supplementary data

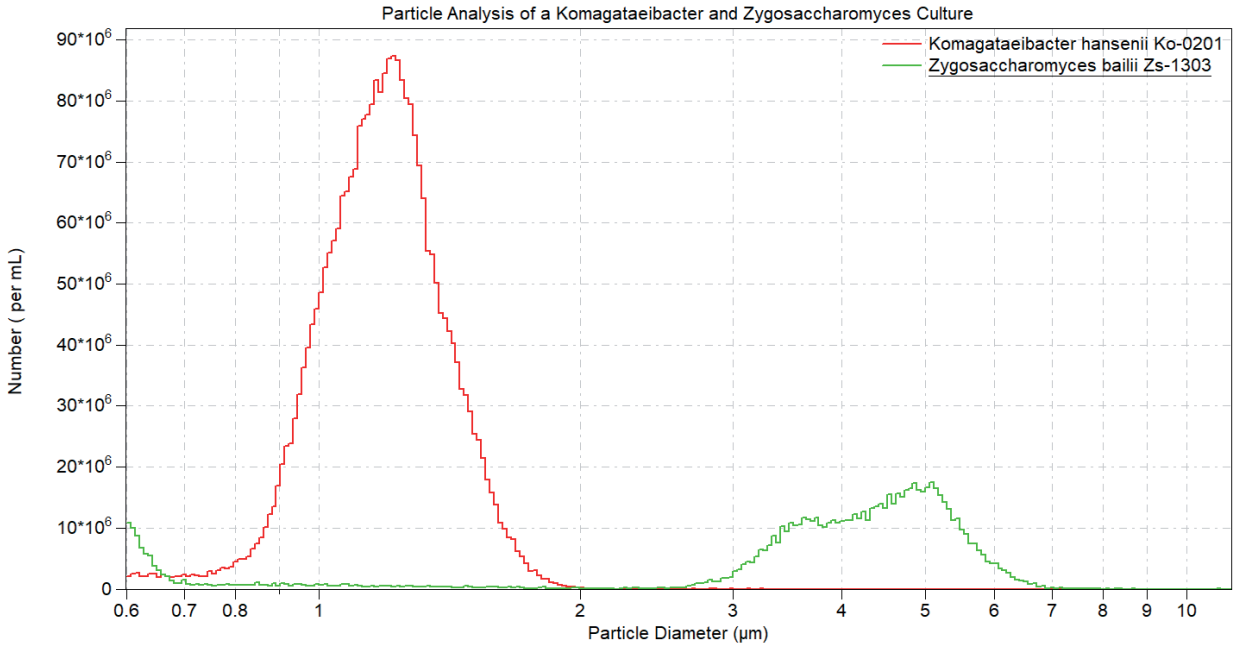

Figure S-1: Illustration of the particle analysis of a *Komagataeibacter* and a *Zygosaccharomyces* culture. Samples of the respective pre-cultures are shown as examples. Measurements were performed using the Beckman Multisizer™ 3 Coulter Counter® device (Beckman Coulter GmbH, Germany) and the Multisizer™ 3 software version 3.53 (Beckman Coulter).