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Original Article

Multi-Response Desirability Optimization of Sucrose Charge in Black-Tea Kombucha: Ethanol, Acetic Acid, and Biocellulose as Critical Quality Attributes

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ABSTRACT

Background: Kombucha is a functional beverage made by acetic acid bacteria and osmotolerant yeasts. Its acceptability depends on keeping residual ethanol below 0.50% w/v with sufficient acidity. The initial sucrose charge governs both outcomes, yet it is rarely optimized as a critical process parameter.

Objective: To evaluate four initial sucrose charges for their effects on ethanol, acetic acid, pH, titratable acidity and pellicle thickness, and to identify an operating window by desirability analysis.

Methods: Black-tea kombucha was fermented statically for 14 days at 25–28 °C with 5, 10, 15 or 20% w/v sucrose. The three vessels of each level were pooled into one composite, assayed in analytical triplicate by GC-FID (ethanol), ion-exclusion HPLC (acetic acid), potentiometry (pH) and alkalimetry (titratable acidity), and pellicle thickness was measured with a caliper. Analysis used one-way ANOVA, Tukey's HSD, polynomial models and Derringer–Suich desirability.

Results: Sucrose charge affected every attribute ($p \leq 0.021$; $\eta^2 = 0.685–0.998$). Ethanol was biphasic. It peaked at 0.341 ± 0.007% w/v (95% CI 0.324–0.359) at 10% sucrose (68% of the threshold). Acetic acid fell and the acetification index decreased 9.9-fold from 5% to 20%. Pellicle thickness increased linearly over the tested range ($R^2 = 0.974$). Composite desirability peaked (D = 0.538) at a predicted 19.1% w/v, within 15–20% w/v, with 15% preferred for ethanol headroom.

Conclusion: Sucrose charge is a control lever for halal-compliant kombucha manufacture. Ethanol should still be verified by gas chromatography. Biological replication is needed, because dispersion reflects analytical repeatability of pooled composites.

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1. Introduction

Kombucha is a lightly effervescent, acidic beverage. It is made by fermenting sweetened tea with a symbiotic culture of bacteria and yeast (SCOBY), and over the past decade it has grown into one of the fastest-growing categories of the global functional-beverage market[1,2]. Consumer interest rests on the reported antioxidant, antimicrobial and metabolic activities of the fermented matrix, and on its organic acids, polyphenol transformation products and live microorganisms[3–5]. In Indonesia the category has expanded rapidly through small and medium enterprises and home brewers, where fermentation is typically conducted in open vessels under ambient conditions, with no in-process analytical control, so acidity and residual alcohol differ widely from one batch to the next[6–8]. The problem goes beyond taste. It is a matter of regulation and religious compliance, because a beverage whose ethanol drifts above the permitted threshold cannot lawfully be marketed as a non-alcoholic product.

The biochemistry behind this risk is well described. Yeasts of the genera *Saccharomyces*, *Zygosaccharomyces*, *Brettanomyces* and *Pichia* secrete extracellular invertase, which hydrolyzes sucrose to glucose and fructose, and the released hexoses are fermented to ethanol and carbon dioxide through the Embden–Meyerhof–Parnas pathway[9,10]. Next come the acetic acid bacteria. Dominated in most consortia by *Komagataeibacter* and *Acetobacter*, they oxidize the ethanol to acetic acid through membrane-bound pyrroloquinoline-quinone (PQQ)-dependent alcohol dehydrogenase and aldehyde dehydrogenase, which acidifies the medium and confers antimicrobial protection[11,12]. The same organisms also oxidize free glucose directly to gluconic and glucuronic acids through membrane-bound glucose dehydrogenase. They polymerize glucose, too, into the extracellular cellulose pellicle that floats at the air–liquid interface[13,14]. Ethanol is an obligate intermediate, not an end product. The residual ethanol measured at harvest reflects the instantaneous imbalance between yeast production and bacterial consumption[15].

Because sucrose is the sole carbon input in a conventional formulation, its initial concentration sets the ceiling on ethanol production, the substrate supply for acetification, the osmotic pressure experienced by the consortium, and the carbon available for cellulose biosynthesis[16,17]. Published work has repeatedly shown that fermentation time, tea type, temperature and oxygen availability shape the kombucha metabolome[18–20]. Several studies, most of them Indonesian, varied sugar concentration and fermentation duration together while reporting total acidity or sensory acceptance[6,21,22], and recent optimization studies of green-tea/pineapple, Pu-erh tea and black-aged garlic kombucha addressed chemical, sensory and bioactive profiles without treating residual ethanol as a constrained response[16,23,24]. One thing remains under-addressed. It is the treatment of the sucrose charge as a formally optimized critical process parameter, evaluated against several conflicting quality

attributes at once. Maximizing acidity alone drives the process toward conditions that also raise ethanol. Minimizing ethanol alone sacrifices acidity, biocellulose yield and microbiological protection. A defensible manufacturing setpoint requires the conflicting responses to be reconciled quantitatively.

A second gap concerns the analytical interpretation of acidity itself. Titratable acidity and specifically quantified acetic acid are often treated as the same quantity in kombucha quality reports, because titratable acidity is routinely expressed as acetic acid equivalents[25,26]. They measure different things. The former is the total neutralizable acid capacity, the latter a single analyte. When both are measured on the same sample, the difference between them quantifies the non-acetic organic-acid pool generated by the direct glucose-oxidation shunt[13], and that difference carries mechanistic information about how the consortium partitions carbon. It has rarely been reported as an explicit quality descriptor.

The Master's Program in Pharmacy of the Faculty of Pharmacy, Universitas Sanata Dharma, Yogyakarta, conducts research at the interface of natural-product chemistry, formulation science and pharmaceutical quality systems, and Indonesia's considerable biological and fermentation-culture diversity provides a strong resource base for applied fermentation biotechnology[27,28]. Pharmaceutical quality by design supplies the logic. Its critical quality attribute (CQA), critical process parameter and design-space vocabulary is codified in ICH guideline Q8(R2). Applying that logic to an artisanal food fermentation is an appropriate translational contribution from a pharmacy graduate program, because the same framework that governs a solid dosage form governs a fermented beverage that must meet a numerical compliance limit. In Indonesia that limit is set by Fatwa No. 10 of 2018 of the Indonesian Council of Ulama. The fatwa holds a fermented beverage to be permissible when its naturally formed ethanol content remains below 0.50% and the product is not medically harmful. A beverage that breaches that threshold falls instead into the alcoholic-beverage category governed by Regulation No. 5 of 2021 of the National Agency of Drug and Food Control[29,30].

This study had three aims. The first, (i), was to quantify the effect of four initial sucrose charges (5, 10, 15 and 20% w/v) on residual ethanol, acetic acid, pH, total titratable acidity and pellicle thickness in black-tea kombucha harvested at day 14. The second, (ii), was to derive and model two carbon-partitioning indices, the acetification index and the non-acetic acid fraction, that point to the underlying metabolic partitioning. The third, (iii), was to identify, by multi-response Derringer–Suich desirability analysis, the sucrose charge and the surrounding design space that jointly maximize acidity, biocellulose yield and microbiological protection while holding residual ethanol below the Indonesian halal compliance threshold. The novelty is simple to state. A descriptive artisanal variable becomes a statistically defined process setpoint with an explicit compliance margin.

The work aligns directly with the scope of the Natural Sciences Engineering and Technology Journal in biotechnology and green chemistry. It uses a self-renewing microbial consortium, not a chemical catalyst. No organic solvent is needed. The process runs at ambient temperature and pressure, and it converts an inexpensive agricultural sugar into an acidified beverage and a recoverable bacterial-cellulose biomaterial in a single step[31,32].

2. Methods

2.1. Materials

Commercial black tea (*Camellia sinensis*, Tjatoet brand, Indonesia) came from a local retailer in Yogyakarta. Refined commercial cane sucrose (food grade, ≥99.8% sucrose) and bottled steam-sterilized mineral water were used as received. The starter culture was a mature SCOBY pellicle together with its liquor, both taken from a single mother culture that the laboratory maintained, so every vessel received an inoculum of equivalent genetic and physiological makeup. Ethanol 70% v/v (technical grade) served for surface sanitation. The contract laboratory was supplied with sodium hydroxide volumetric standard solution and phenolphthalein indicator, both of analytical grade. Glassware was borosilicate throughout.

2.2. Study design

The experiment followed a single-factor, four-level, completely randomized design, with the initial sucrose charge as the sole manipulated factor: 5, 10, 15 and 20% w/v (coded S5, S10, S15 and S20). Three independent fermentation vessels were prepared per level (three independent vessels per level, 12 vessels in total, pooled by level before analysis; Section 2.5). Nothing else varied. Tea concentration, water source, working volume, inoculum mass, starter fraction, temperature, light exposure and harvest day were all held constant. Figure 1 illustrates the complete five-phase protocol, including the point at which composite pooling occurs. The study took place at the Faculty of Pharmacy, Universitas Sanata Dharma, Yogyakarta, Indonesia, and all analytical determinations were subcontracted to an accredited third-party testing laboratory (PT Saraswanti Indo Genetech, Bogor, Indonesia).

2.3. Preparation of the fermentation medium

Jars, measuring cylinders and stirring rods were all washed with detergent, rinsed with potable water, sprayed internally with 70% v/v ethanol and air-dried before use. The working bench was sanitized in the same way. Mineral water was boiled to 100 °C, and black tea was infused in it at 10 g L^{-1} for 15 min to extract polyphenols and assimilable nitrogen. The infusion was then clarified through filter paper and a fine mesh sieve. Each jar received nine hundred milliliters of hot infusion (≥70 °C, to guarantee complete dissolution), and the assigned sucrose mass was weighed on an analytical balance and dissolved with a sterile glass rod: 45, 90, 135 and 180 g per jar, corresponding to 5, 10, 15 and 20% w/v in the 900 mL of sweetened tea. Jars were covered loosely and left to cool to 25–28 °C before inoculation, since residual heat is lethal to the consortium.

2.4. Inoculation and fermentation

Each cooled jar received 100 mL of mature kombucha starter liquor (10% v/v of the final 1000 mL working volume), followed by one SCOBY pellicle of constant mass (50 ± 1 g) that was transferred with sanitized forceps. Jars were closed with a double layer of clean, porous cotton cloth secured with a rubber band. This permits oxygen transfer for the obligately aerobic acetic acid bacteria. It also excludes insects and coarse particulates. Fermentation ran statically for 14 days, without agitation, in a dark cabinet held at 25–28 °C. At harvest, the parent and newly formed pellicles were lifted out with sterile forceps, the liquid phase was filtered through clean cloth, and the samples were bottled at once and sent to the contract laboratory under chilled conditions.

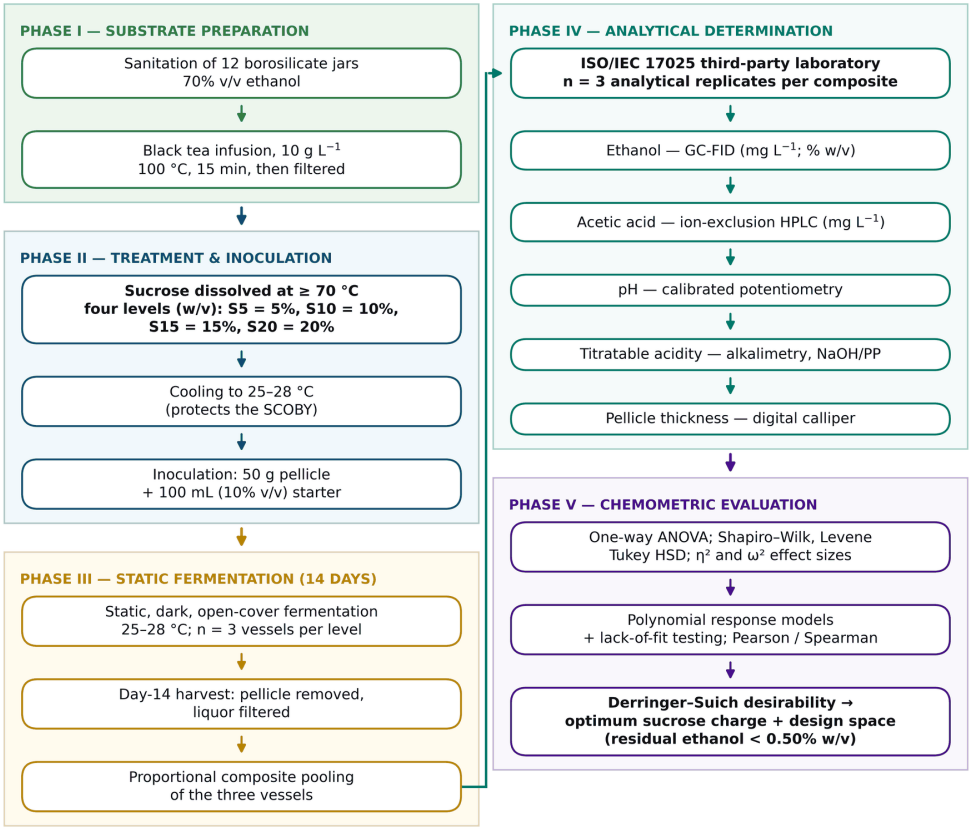

Figure 1. Experimental workflow for the multi-response optimization of sucrose charge in black-tea kombucha, from substrate preparation through static fermentation, third-party analytical determination, and chemometric quality-control evaluation.

2.5. Sampling strategy

Within each treatment level, proportional composite sampling was applied. Equal volumes were withdrawn from each of the three independent vessels of a level and combined into one homogeneous composite before instrumental analysis, following the Codex Alimentarius general guidelines on sampling (CAC/GL 50-2004). Physical pooling works as a mechanical averaging step: it dissolves inter-vessel biological variability into a single representative unit, and it is widely used when high-cost instrumental assays would otherwise be prohibitive. Each composite was then measured in analytical triplicate (n = 3 independent determinations per composite). Dispersion statistics here therefore describe analytical repeatability, not biological variance. Section 4.8 addresses explicitly what this design choice means for inference.

2.6. Analytical determinations

Ethanol. Residual ethanol was determined by gas chromatography with flame-ionization detection (GC-FID). The filtered beverage was injected directly, and an internal-standard calibration bracketing 0.01–1.00% w/v was used. Gas chromatography is the technique of reference for low-level ethanol in fermented beverages, because it resolves ethanol from water, higher alcohols and the acidic matrix and avoids the strong positive bias that hydrometry and refractometry show in sugar-rich media[33,34]. Results were expressed both as mg L^{-1} and as % w/v.

Acetic acid. Acetic acid was quantified by high-performance liquid chromatography (HPLC) on an ion-exclusion column with ultraviolet detection at 210 nm. This separation is needed because gluconic, glucuronic, lactic, malic and citric acids co-occur with acetic acid in kombucha, and without it all of them would be counted together[13]. An external calibration curve of acetic acid reference standard supported quantification. Results were expressed in mg L^{-1}.

pH. Each composite was measured at 25 °C with a digital pH meter. The meter was calibrated immediately before use against pH 4.01 and pH 7.00 certified buffers, and the electrode was rinsed with deionized water between determinations.

Total titratable acidity (TTA). Alkalimetry was used to determine titratable acidity. A measured aliquot of beverage was titrated with standardized sodium hydroxide to a stable faint pink phenolphthalein endpoint, and the result was expressed as % w/v acetic acid equivalents according to $CH_3COOH + NaOH \rightarrow CH_3COONa + H_2O$. Titratable acidity measures the total neutralizable acid capacity, whereas pH reflects only the instantaneous free hydrogen-ion activity. The two parameters thus carry different information and are not redundant[18,28].

Pellicle thickness. Each vessel's newly synthesized cellulose pellicle was blotted free of adhering liquor. Thickness was then measured with a digital caliper at five evenly distributed points, and the vessel value was the mean of those five readings.

2.7. Derived bioprocess indices

Three descriptors were computed to expose carbon partitioning. The acetification index is the molar ratio of acetic acid to residual ethanol, $(c_{acetic}/60.05)/(c_{ethanol}/46.07)$, and it expresses how completely the ethanol pool has been oxidized at harvest. The non-acetic acid fraction is the difference between titratable acidity expressed as mg L^{-1} acetic acid equivalents and the HPLC-determined acetic acid concentration; it estimates the pool of gluconic, glucuronic and other secondary organic acids. The regulatory headroom is residual ethanol expressed as a percentage of the 0.50% w/v halal compliance threshold. Yields were also normalized. Each metabolite was expressed per gram of sucrose charged (mg metabolite per g sucrose).

2.8. Statistical analysis

All data are reported as mean ± standard deviation (SD) of n = 3 analytical replicates, with two-sided 95% confidence intervals (CIs). Analyses were performed in Python 3.11 using NumPy 2.4.4 and SciPy 1.17.1. Normality of within-group values and of the pooled residuals was tested by the Shapiro–Wilk test. Homogeneity of variance was tested by Levene's test (mean-centered), with Bartlett's test as a supporting check. Treatment effects were assessed by one-way analysis of variance (ANOVA). Where Levene's test indicated heteroscedasticity, ratio-scale indices were $\log_{10}$-transformed before analysis, and the Alexander–Govern heteroscedastic test and the Kruskal–Wallis test were computed as sensitivity checks. Pairwise comparisons used Tukey's honestly significant difference (HSD) procedure at $\alpha = 0.05$, and the compact letter display that resulted was generated from the maximal cliques of the non-significant-difference graph. Effect sizes are reported as η^2 and as the less biased ω^2. Dose–response behavior was modeled by ordinary least squares polynomial regression of degree one to three, and the models were compared by adjusted R^2, the Akaike information criterion and an explicit lack-of-fit F test against pure error, which the replicated design makes estimable. Associations were quantified by Pearson r with Fisher z-transformed 95% confidence intervals, and Spearman ρ was computed as a rank-based check. Because the 12 observations are analytical replicates of four composites, the p-values and confidence intervals from the ANOVA, regression and correlation analyses describe analytical repeatability and do not test biological variation. Exact p-values are reported to three decimal places; values below 0.001 are shown as $p < 0.001$.

Multi-response optimization relied on the Derringer–Suich desirability approach. Six individual desirability functions were defined on the interpolated sucrose domain. Acetic acid, titratable acidity and pellicle thickness were larger-the-better. Residual ethanol was smaller-the-better, with a hard constraint setting desirability to zero above 0.50% w/v. pH was target-the-best: fully desirable between 2.80 and 3.20, and zero outside 2.50–4.20. Microbiological integrity was modeled as a logistic function of sucrose, centered on the observed osmotic-protection threshold. Weights of 1.0 were assigned to acetic acid, titratable acidity, pH and pellicle thickness, 1.5 to ethanol and 2.0 to microbiological integrity, reflecting the relative severity of failure of each attribute. The composite desirability was the weighted geometric mean, $D = (d_1^{w_1} \times d_2^{w_2} \times \ldots \times d_6^{w_6})^{1/W}$, in which $W = w_1 + w_2 + \ldots + w_6$. The design space was defined as the contiguous sucrose interval over which D remained within 90% of its maximum.

3. Results

3.1. Assumption diagnostics

Normality held for the residuals of every primary response (Shapiro–Wilk $p = 0.457$ for ethanol, 0.362 for acetic acid, 0.105 for pH, 0.138 for titratable acidity and 0.302 for pellicle thickness; all $p > 0.05$). Variances were homogeneous as well (Levene $p = 0.131$, 0.439, 0.996, 0.999 and 0.154, respectively). The acetification index was the exception. Untransformed, it violated homoscedasticity (Levene $p = 0.037$), because its dispersion scales with its magnitude. After $\log_{10}$ transformation, both normality (Shapiro–Wilk $p = 0.376$) and variance homogeneity (Levene $p = 0.486$) were restored, so all inference on this index is reported on the transformed scale, with back-transformed means given for interpretation. Parametric and non-parametric tests agreed in direction and significance for every response.

3.2. Primary quality attributes

The initial sucrose charge affected all five measured attributes. Table 1 gives the complete descriptive and inferential results, and Figure 2 shows the four primary CQAs. Residual ethanol did not rise monotonically but followed a biphasic profile. It was lowest at S5 (0.056 ± 0.001% w/v; 95% CI 0.053–0.059) and rose sharply to a maximum at S10 (0.341 ± 0.007% w/v; 95% CI 0.324–0.359), after which it fell significantly at S15 (0.215 ± 0.005% w/v) and climbed again at S20 (0.308 ± 0.006% w/v). The overall effect was large ($F(3,8) = 1707.5$, $p < 0.001$, $\eta^2 = 0.998$, $\omega^2 = 0.998$), and Tukey's test separated all four levels into distinct groups, as the four different letters in the ethanol rows of Table 1 and above the bars of Figure 2A show. The dashed regulatory line in Figure 2A makes the compliance picture plain: every level stayed below the 0.50% w/v threshold. The least favorable level, S10, reached only 68.3% of the ceiling, with a one-sided 95% upper confidence bound of 0.354% w/v and a margin of 31.7%.

Table 1. Physicochemical and biomaterial quality attributes of black-tea kombucha after 14 days of static fermentation at four initial sucrose charges (mean ± SD of n = 3 analytical replicates on the treatment composite).

Quality attribute	S5 (5% w/v)	S10 (10% w/v)	S15 (15% w/v)	S20 (20% w/v)	F(3,8)	p	η^2
Residual ethanol (mg L^{-1})	557.39 ± 11.71^{d}	3414.28 ± 71.70^{a}	2150.78 ± 45.17^{c}	3081.76 ± 64.72^{b}	1707.5	< 0.001	0.998
Residual ethanol (% w/v)	0.056 ± 0.001^{d}	0.341 ± 0.007^{a}	0.215 ± 0.005^{c}	0.308 ± 0.006^{b}	1707.5	< 0.001	0.998
Acetic acid (mg L^{-1})	664.87 ± 10.64^{a}	462.93 ± 7.41^{b}	344.17 ± 5.51^{d}	369.64 ± 5.91^{c}	1089.6	< 0.001	0.998
pH at harvest	3.080 ± 0.011^{a}	2.910 ± 0.010^{b}	2.930 ± 0.010^{b}	2.880 ± 0.010^{c}	223.1	< 0.001	0.988
Titratable acidity (% w/v)	0.1850 ± 0.0033^{b}	0.1900 ± 0.0034^{ab}	0.1950 ± 0.0035^{a}	0.1950 ± 0.0035^{a}	5.8	0.021	0.685
Pellicle thickness (mm)	1.50 ± 0.11^{d}	2.65 ± 0.20^{c}	3.85 ± 0.29^{b}	5.15 ± 0.39^{a}	103.8	< 0.001	0.975

Note. *Different superscript letters within a row indicate means that differ significantly by Tukey's HSD test (α = 0.05). Rows 1 and 2 give the same measurement in two units.*

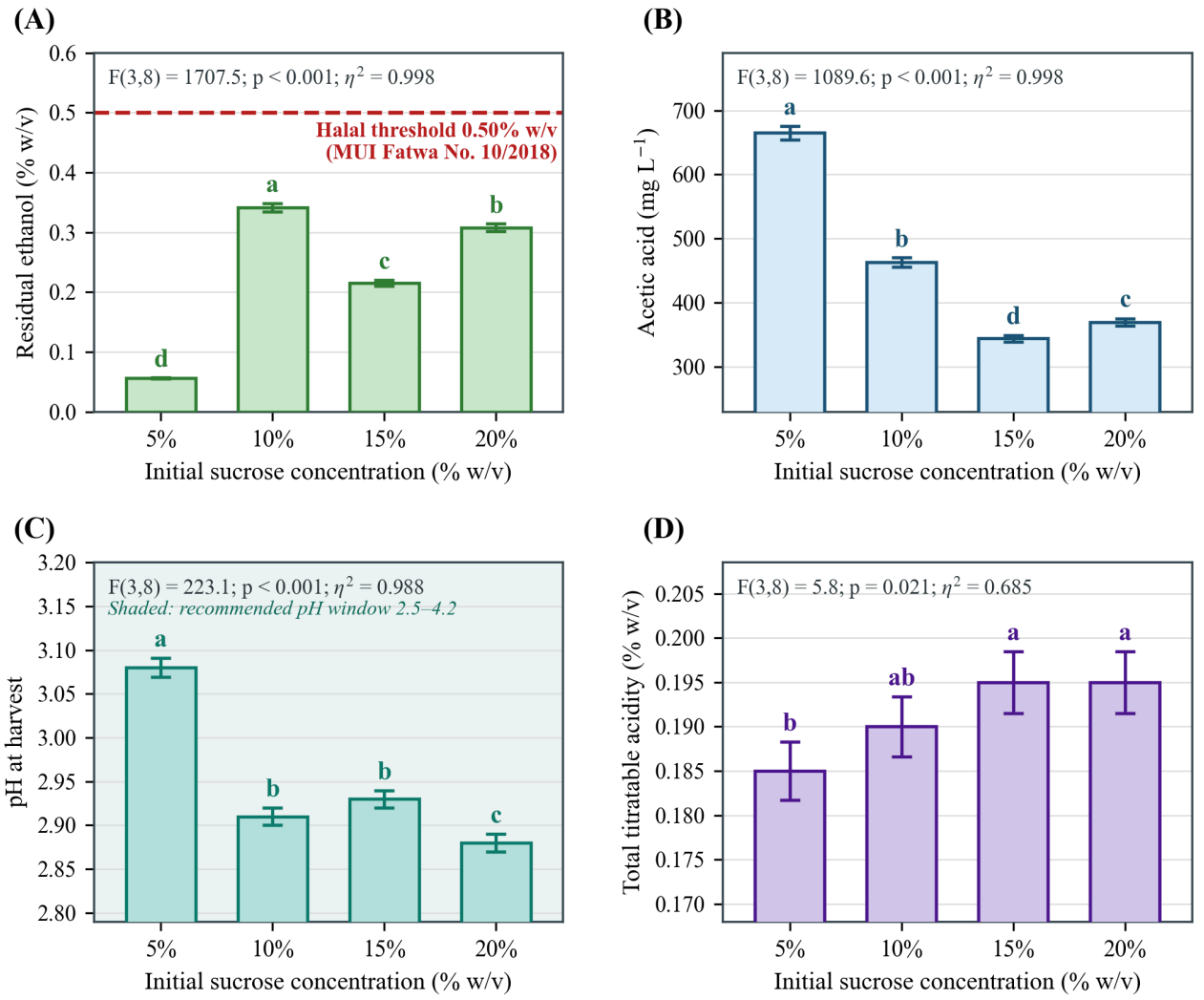

Figure 2. Primary critical quality attributes of black-tea kombucha after 14 days of static fermentation at four sucrose charges: (A) residual ethanol, (B) acetic acid, (C) pH at harvest, and (D) total titratable acidity. Bars are mean ± SD of three analytical replicates; different lowercase letters above the bars denote means that differ significantly by Tukey's HSD test (α = 0.05). The dashed red line in panel A is the 0.50% w/v halal threshold for naturally formed ethanol established by Fatwa No. 10 of 2018 of the Indonesian Council of Ulama (MUI).

Acetic acid moved in the opposite direction. It decreased monotonically from 664.87 ± 10.64 mg L^{-1} at S5 to 344.17 ± 5.51 mg L^{-1} at S15, then recovered slightly but detectably to 369.64 ± 5.91 mg L^{-1} at S20 (third row of Table 1; Figure 2B; $F(3,8) = 1089.6$, $p < 0.001$, $\eta^2 = 0.998$). All pairwise contrasts were significant, including S15 versus S20 ($p = 0.015$). The pH fell from 3.080 ± 0.011 at S5 to 2.880 ± 0.010 at S20 ($F(3,8) = 223.1$, $p < 0.001$, $\eta^2 = 0.988$). S10 and S15 were statistically indistinguishable from one another (they share the letter b in Table 1), and all values fell inside the 2.50–4.20 window recommended for safe kombucha[35,36], as the shaded band in Figure 2C shows. Total titratable acidity (Figure 2D) rose from 0.1850 ± 0.0033% w/v at S5 to a plateau of 0.1950 ± 0.0035% w/v shared by S15 and S20 ($F(3,8) = 5.8$, $p = 0.021$, $\eta^2 = 0.685$). S5 differed from S15 and S20 ($p = 0.030$ for both) but not from S10 ($p = 0.349$). That is why S10 carries the shared grouping ab in Table 1 and in Figure 2D. Pellicle thickness increased across every level, from 1.50 ± 0.11 mm at S5 to 5.15 ± 0.39 mm at S20 ($F(3,8) = 103.8$, $p < 0.001$, $\eta^2 = 0.975$), and all four levels differed significantly from each other (final row of Table 1).

3.3. Carbon partitioning and derived indices

Table 2 gives the three derived carbon-partitioning indices, the substrate-normalized yields and the regulatory headroom. They clarify what the primary attributes only imply. The acetification index is the molar ratio of acetate produced to ethanol remaining. It was close to unity at S5 (0.915 ± 0.019 mol mol^{-1}). At every higher charge it collapsed by nearly an order of magnitude, to 0.104 ± 0.003 at S10, 0.123 ± 0.003 at S15 and 0.092 ± 0.003 at S20, a 9.9-fold reduction between the extremes (log_{10}-scale ANOVA $F(3,8) = 4755.7$, $p < 0.001$, $\eta^2 = 0.999$; all four levels separated by Tukey's test). The proportion of titratable acidity attributable to acetic acid fell in parallel, from 35.9% at S5 to 17.7% at S15. The non-acetic acid fraction rose from 1185.1 ± 22.7 mg L^{-1} to a plateau of 1605.8 ± 29.8 mg L^{-1} ($F(3,8) = 96.0$, $p < 0.001$, $\eta^2 = 0.973$). Higher sucrose charges, in other words, did not reduce total acid output. They redirected it away from acetic acid and towards secondary organic acids. The biomaterial phase mirrored this redirection, with a strong correlation between the non-acetic fraction and pellicle thickness ($r = 0.880$, 95% CI 0.618–0.966, $p < 0.001$); both variables also rise with sucrose charge, so this association does not by itself isolate a shared metabolic origin. Figure 3 illustrates the metabolic logic behind the redistribution, namely the coupling of the ethanologenic and oxidative fluxes through the shared ethanol pool and the competing PQQ-dependent glucose-oxidation shunt, and Figure 4 shows the structures of the substrate and of every metabolite named in this section.

Table 2. Derived bioprocess and carbon-partitioning indices (mean ± SD, n = 3).

Derived index	S5	S10	S15	S20	F(3,8)	p
Acetification index (mol acetate mol^{-1} ethanol)	0.915 ± 0.019^{a}	0.104 ± 0.003^{c}	0.123 ± 0.003^{b}	0.092 ± 0.003^{d}	4755.7	< 0.001
Acetic acid share of titratable acidity (%)	35.94 ± 0.08^{a}	24.37 ± 0.82^{b}	17.65 ± 0.09^{d}	18.96 ± 0.58^{c}	584.2	< 0.001
Non-acetic acid fraction (mg L^{-1})	1185.1 ± 22.7^{c}	1437.1 ± 41.5^{b}	1605.8 ± 29.8^{a}	1580.4 ± 39.1^{a}	96.0	< 0.001
Ethanol yield (mg g^{-1} sucrose)	11.15 ± 0.23^{c}	34.14 ± 0.72^{a}	14.34 ± 0.30^{b}	15.41 ± 0.32^{b}	1703	< 0.001
Acetic acid yield (mg g^{-1} sucrose)	13.30 ± 0.21^{a}	4.63 ± 0.07^{b}	2.29 ± 0.04^{c}	1.85 ± 0.03^{d}	6431	< 0.001
Regulatory headroom (% of 0.50% w/v ceiling)	11.15 ± 0.23^{d}	68.29 ± 1.43^{a}	43.02 ± 0.90^{c}	61.64 ± 1.29^{b}	1708	< 0.001

Note. *Superscript letters denote Tukey groupings (α = 0.05); the acetification index and the acetic-acid share were tested on the log10 scale. Regulatory headroom is residual ethanol as a percentage of the 0.50% w/v threshold.*

Yield normalization reinforced the point (fourth and fifth rows of Table 2). Ethanol yield per gram of sucrose charged peaked at S10 (34.14 ± 0.72 mg g^{-1}), and at every other level it was two- to three-fold lower, whereas acetic acid yield declined steadily from 13.30 ± 0.21 mg g^{-1} at S5 to 1.85 ± 0.03 mg g^{-1} at S20 ($F(3,8) = 6431$, $p < 0.001$). The practical consequence is simple. Sucrose added beyond about 15% w/v bought progressively less acetic acid per unit of substrate while continuing to buy pellicle.

3.4. Response modeling and correlation structure

Figure 5 shows the polynomial models fitted to the replicate-level data panel by panel, and Table 3 gives their goodness-of-fit statistics. Pellicle thickness (Figure 5D) was the only response adequately described by a straight line. The linear model explained 97.4% of the variance with no evidence of lack of fit ($F(2,8) = 0.12$, $p = 0.887$), giving a slope of 0.243 mm per percentage point of sucrose. Titratable acidity was also adequately linear (lack-of-fit $p = 0.427$), although its coefficient of determination was modest ($R^2 = 0.610$), in line with its early plateau. The remaining responses were less accommodating. Acetic acid (Figure 5B), pH (Figure 5E), ethanol (Figure 5A) and the acetification index (Figure 5F) each showed significant lack of fit for linear and quadratic forms (acetic acid quadratic lack-of-fit $p = 0.015$; ethanol quadratic lack-of-fit $p < 0.001$), and each required the saturated cubic form, which reproduces the four treatment means exactly and therefore serves for interpolation only, never for extrapolation beyond 20% w/v. The non-acetic acid fraction (Figure 5C) was the one intermediate case. A quadratic model described it adequately, with no detectable lack of fit ($p = 0.243$).

Table 4 gives the full correlation structure, which agreed with the proposed mechanism. Sucrose charge correlated almost perfectly with pellicle thickness ($r = 0.987$, $p < 0.001$). Its correlations with acetic acid and pH were strong and negative (acetic acid $r = -0.890$, $p < 0.001$; pH $r = -0.836$, $p < 0.001$), while its correlation with the non-acetic acid fraction was positive ($r = 0.894$, $p < 0.001$). Ethanol and acetic acid were inversely related ($r = -0.727$, $p = 0.007$; Spearman $\rho = -0.196$, Table 4, so the Pearson value reflects mainly the contrast between S5 and the other levels). This is the expected signature of a consortium in which incomplete acetification leaves ethanol unconsumed. Ethanol also tracked pH inversely and closely ($r = -0.939$, $p < 0.001$).

3.5. Multi-response optimization and regulatory margin

Figure 6A shows the individual and composite desirability functions across the whole sucrose domain, and Figure 6B and Table 5 give their values at the four tested levels. Composite desirability was negligible at S5 (D = 0.002): although this level maximized acetic acid and minimized ethanol, it failed the titratable-acidity, microbiological-integrity and pellicle criteria outright ($d_{micro} < 0.001$, $d_{pellicle} < 0.001$; the same holds for titratable acidity, see the note to Table 5). S10 also scored poorly (D = 0.093). It combined the highest ethanol burden ($d_{ethanol} = 0.003$) with incomplete osmotic protection. The two high-sucrose levels were nearly equivalent and far superior. The two green bars in Figure 6B show D = 0.494 at S15 and D = 0.480 at S20, a difference of only 0.014 desirability units (2.9%). The two levels reach that score by different routes. S15 retains more regulatory headroom ($d_{ethanol} = 0.444$ versus 0.119), whereas S20 maximizes biocellulose yield ($d_{pellicle} = 1.000$ versus 0.644).

Across the interpolated domain, composite desirability peaked at 19.1% w/v sucrose (Dmax = 0.538). At this point the models predict 352.5 mg L^{-1} acetic acid, 0.196% w/v titratable acidity, 0.250% w/v residual ethanol, pH 2.90 and a 4.92 mm pellicle. The interval over which D remained within 90% of its maximum was contiguous. It spanned 18.1–20.0% w/v (the shaded band in Figure 6A), whereas the practical design space supported by the tested levels is 15–20% w/v. Every level met the regulatory constraint. The margin was wide: one-sided t-tests against the 0.50% w/v ceiling gave $p < 0.001$ even for the worst-case level, and the 95% upper confidence bounds were 0.058%, 0.354%, 0.223% and 0.319% w/v for S5, S10, S15 and S20 respectively.

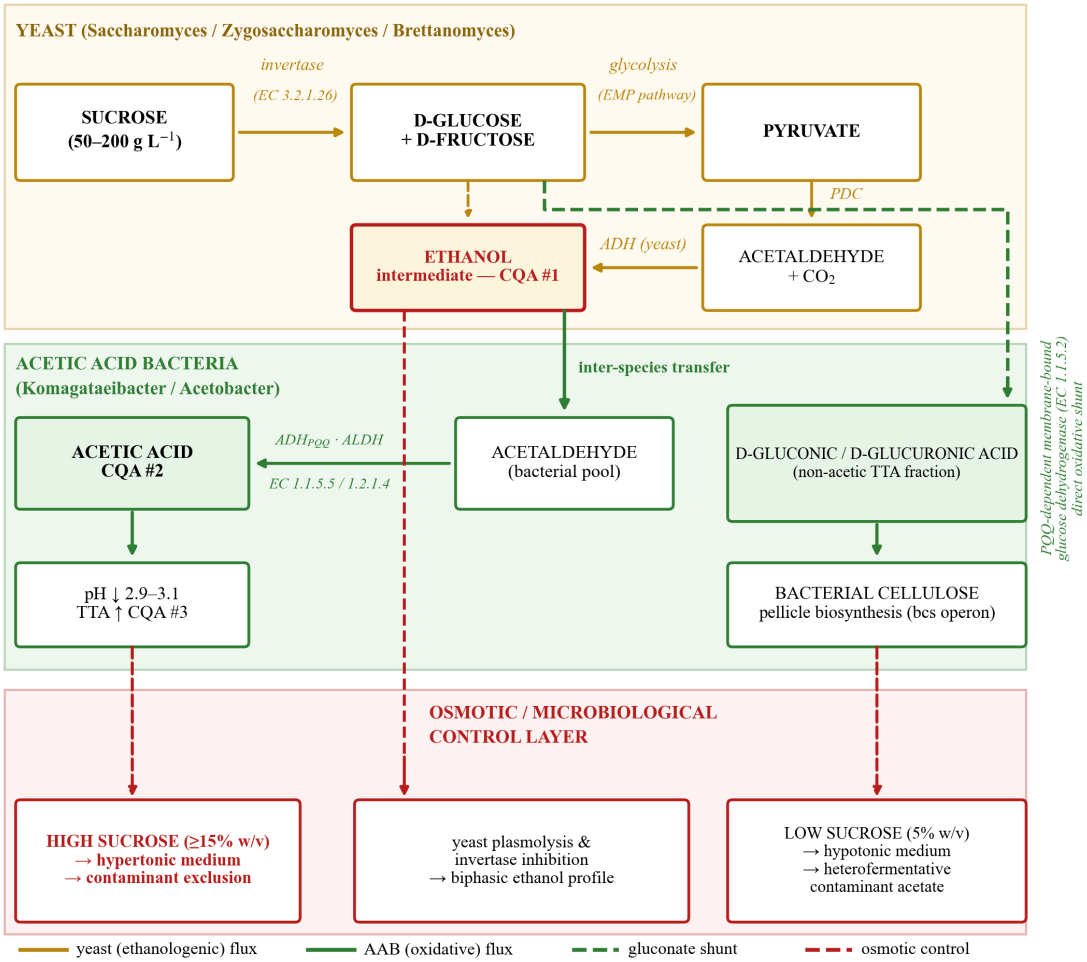

Figure 3. Proposed mechanism by which the initial sucrose charge governs carbon partitioning in the kombucha consortium. The ethanologenic (yeast) flux and the oxidative (acetic acid bacteria) flux are coupled through the ethanol pool, while the PQQ-dependent membrane-bound glucose dehydrogenase shunt diverts free glucose towards gluconic and glucuronic acids and biocellulose. The osmotic control layer summarizes how the sucrose charge simultaneously modulates contaminant exclusion and yeast stress. Abbreviations: AAB, acetic acid bacteria; ADH, alcohol dehydrogenase; ALDH, aldehyde dehydrogenase; CQA, critical quality attribute; EMP, Embden–Meyerhof–Parnas; PDC, pyruvate decarboxylase; PQQ, pyrroloquinoline quinone; TTA, total titratable acidity.

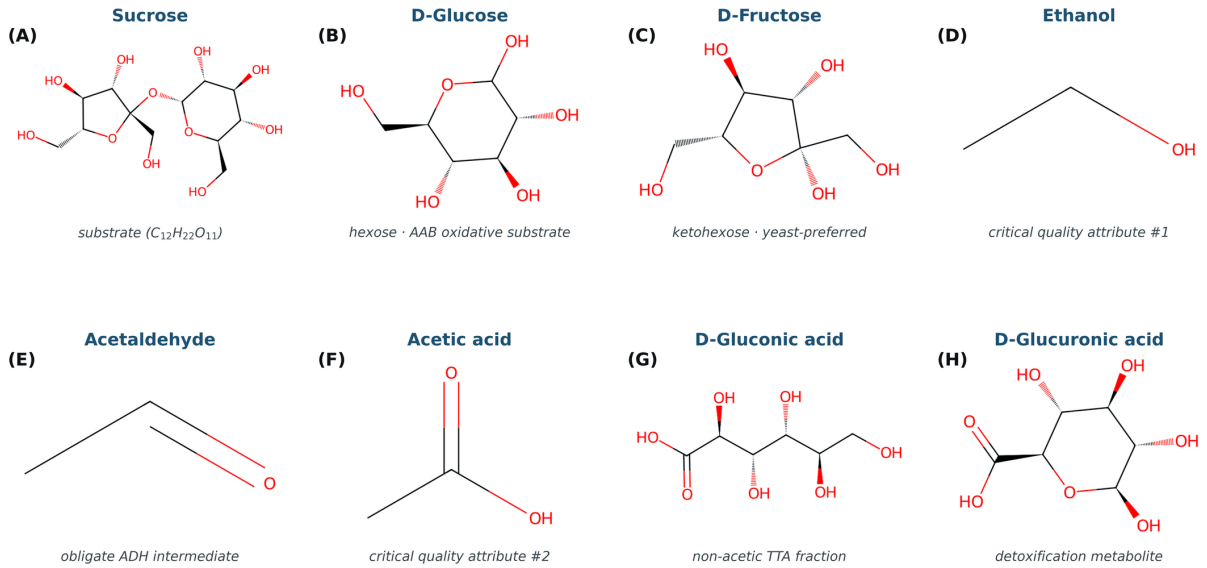

Figure 4. Chemical structures of the substrate and the principal metabolites quantified or inferred in this study: (A) sucrose, (B) D-glucose, (C) D-fructose, (D) ethanol, (E) acetaldehyde, (F) acetic acid, (G) D-gluconic acid and (H) D-glucuronic acid.

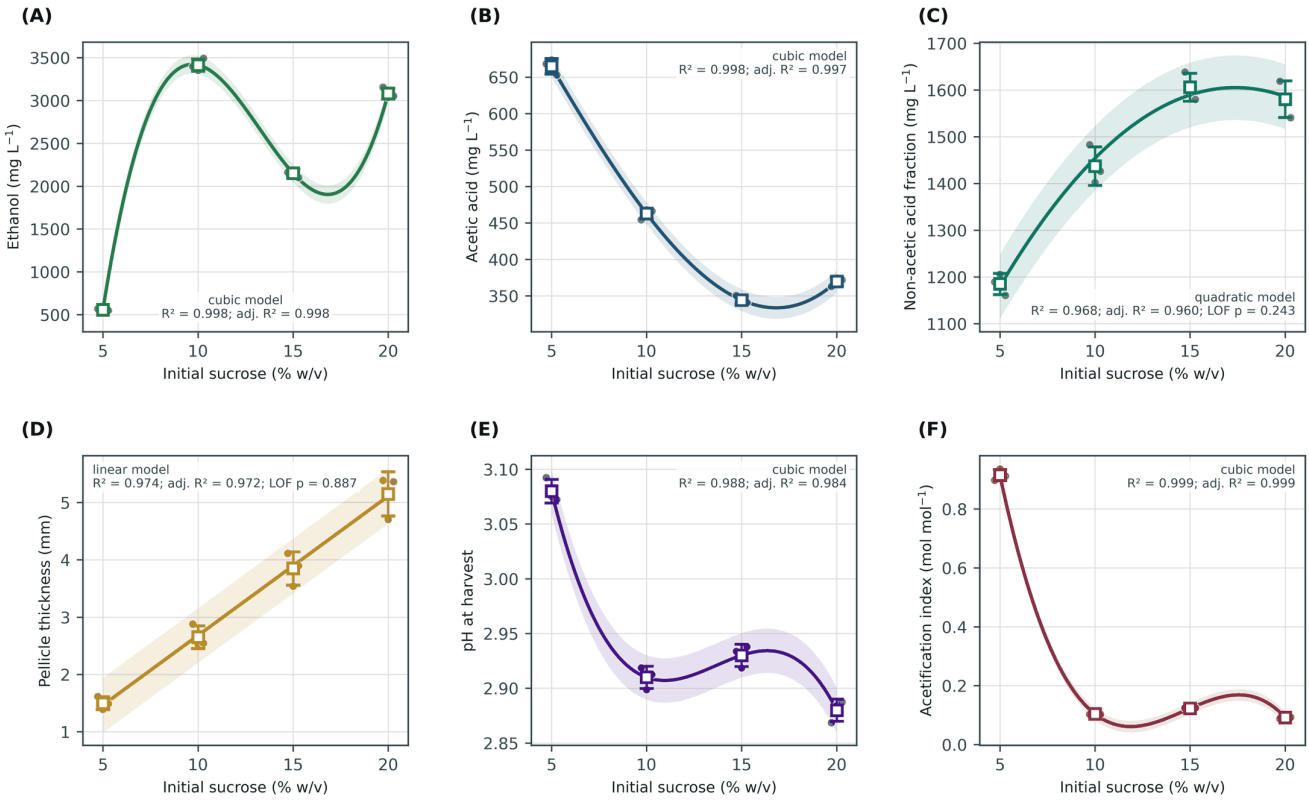

Figure 5. Polynomial response models relating the initial sucrose charge to (A) ethanol, (B) acetic acid, (C) the non-acetic titratable acid fraction, (D) pellicle thickness, (E) pH and (F) the acetification index. Open squares are treatment means ± SD, filled circles are individual analytical replicates, solid lines are the fitted models and shaded envelopes are ±1.96 × residual standard error.

Table 3. Polynomial response models relating the initial sucrose charge to each attribute, with lack-of-fit testing against pure error.

Response (selected model)	R^2	Adjusted R^2	RMSE	Regression p	Lack-of-fit F	Lack-of-fit p
Pellicle thickness — linear	0.974	0.972	0.242	< 0.001	0.12	0.887
Titratable acidity — linear	0.610	0.571	0.0034	0.003	0.95	0.427
Non-acetic acid fraction — quadratic	0.968	0.960	35.2	< 0.001	1.59	0.243
Acetic acid — cubic (saturated)	0.998	0.997	7.64	< 0.001	not estimable	—
Ethanol — cubic (saturated)	0.998	0.998	53.6	< 0.001	not estimable	—
pH — cubic (saturated)	0.988	0.984	0.0103	< 0.001	not estimable	—
Acetification index — cubic (saturated)	0.999	0.999	0.0100	< 0.001	not estimable	—

Note. *The selected model is shown in the first column (bold).*

Table 4. Pearson correlation structure among the process parameter and the measured attributes (n = 12 replicate-level observations), with Fisher-transformed 95% confidence intervals and Spearman ρ as a rank-based confirmation.

Variable pair	r	95% CI for r	r^2	p	Spearman ρ
Sucrose charge vs pellicle thickness	+0.987	+0.953 to +0.996	0.974	< 0.001	+0.972
Sucrose charge vs acetic acid	−0.890	−0.969 to −0.645	0.792	< 0.001	−0.777
Sucrose charge vs non-acetic acid fraction	+0.894	+0.658 to +0.970	0.800	< 0.001	+0.842
Sucrose charge vs pH	−0.836	−0.953 to −0.503	0.698	< 0.001	−0.799
Sucrose charge vs titratable acidity	+0.781	+0.376 to +0.936	0.610	0.003	+0.777
Sucrose charge vs acetification index	−0.782	−0.936 to −0.377	0.611	0.003	−0.777
Sucrose charge vs ethanol	+0.636	+0.098 to +0.886	0.405	0.026	+0.389
Ethanol vs acetic acid	−0.727	−0.918 to −0.264	0.529	0.007	−0.196
Ethanol vs pH	−0.939	−0.983 to −0.792	0.882	< 0.001	−0.720
Acetic acid vs pH	+0.898	+0.670 to +0.971	0.807	< 0.001	+0.406
Pellicle thickness vs non-acetic acid fraction	+0.880	+0.618 to +0.966	0.774	< 0.001	+0.853

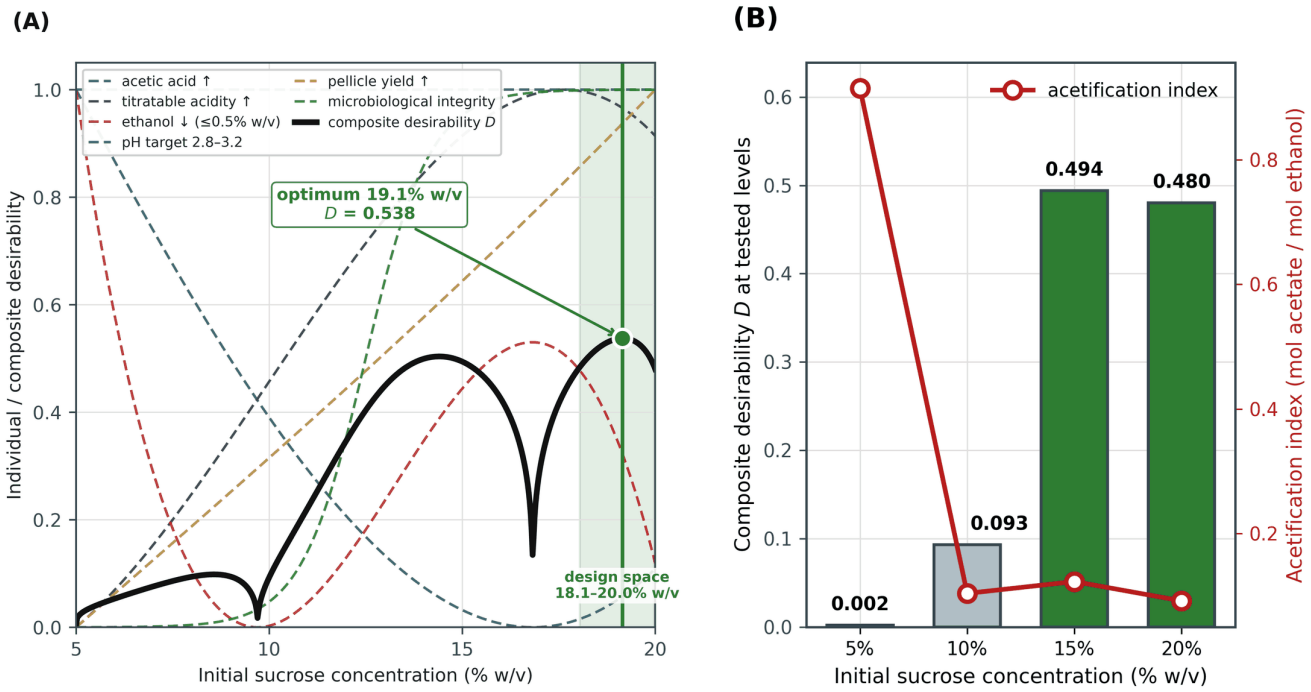

Figure 6. Multi-response optimization of the sucrose charge. (A) Individual desirability functions (dashed) and the composite Derringer–Suich desirability D (solid black) across the interpolated sucrose domain; the vertical line marks the predicted optimum and the shaded band the design space within 90% of Dmax. (B) Composite desirability at the four tested levels (bars, left axis) and the acetification index (red line, right axis).

Table 5. Multi-response Derringer–Suich desirability analysis at the four tested sucrose charges and at the interpolated optimum, together with the regulatory ethanol margin.

Criterion (weight)	S5	S10	S15	S20	Optimum (19.1%)
Acetic acid, larger-better (1.0)	1.000	0.391	0.032	0.109	0.057
Titratable acidity, larger-better (1.0)	0.000	0.457	0.914	0.914	0.966
Residual ethanol, smaller-better (1.5)	1.000	0.003	0.444	0.119	0.323
pH, target 2.80–3.20 (1.0)	1.000	1.000	1.000	1.000	1.000
Pellicle thickness, larger-better (1.0)	0.000	0.315	0.644	1.000	0.937
Microbiological integrity (2.0)	0.000	0.047	0.953	1.000	1.000
Composite desirability D	0.002	0.093	0.494	0.480	0.538
Ethanol 95% upper confidence bound (% w/v)	0.058	0.354	0.223	0.319	0.250
Margin below the 0.50% w/v ceiling (%)	88.9	31.7	57.0	38.4	50.1

Note. *Individual desirabilities range from 0 (unacceptable) to 1 (fully desirable). Values printed as 0.000 are below 0.0005; because a weighted geometric mean is zero only when an individual desirability is exactly zero, D = 0.002 at S5 implies that every individual desirability at S5 is greater than zero.*

Desirability results depend on the weights chosen. A sensitivity analysis was therefore run over five alternative weighting schemes (Table 6). The interpolated optimum moved only between 18.7 and 19.6% w/v across every scheme, and the 90%-of-maximum region always lay inside 17.7–20.0% w/v, so the location of the operating window is stable across these schemes. What the weights do change is the ordering of the two acceptable levels, and they change it in the direction the mechanism predicts. Emphasizing the ethanol constraint (weight 3.0) makes S15 clearly superior to S20 (D = 0.485 versus 0.380). Relaxing it (weight 1.0), or emphasizing acetic acid or pellicle yield, reverses the ranking in favor of S20. Removing the microbiological-integrity term altogether left the optimum unchanged at 19.1% w/v. It also did not rehabilitate S5 (D = 0.007), because that level fails the titratable-acidity and pellicle criteria as well, so the exclusion of low sucrose charges is over-determined rather than an artifact of the contamination term. In practice, the acceptable region is stable. The choice within it is a legitimate manufacturing decision rather than a statistical one.

Table 6. Sensitivity of the multi-response optimization to the weighting scheme.

Weighting scheme	Optimum (% w/v)	90%-of-max region (% w/v)	D at S15	D at S20	Preferred level
Base case (ethanol 1.5, microbiology 2.0)	19.1	18.1–20.0	0.494	0.480	S15
Ethanol weight reduced to 1.0	19.4	18.3–20.0	0.498	0.531	S20
Ethanol weight raised to 3.0	18.7	17.7–19.6	0.485	0.380	S15
Acetic acid weight raised to 2.0	19.6	18.6–20.0	0.358	0.403	S20

Weighting scheme	Optimum (% w/v)	90%-of-max region (% w/v)	D at S15	D at S20	Preferred level
Pellicle weight raised to 2.0	19.2	18.1–20.0	0.510	0.523	S20
Microbiological term removed	19.1	18.2–19.9	0.389	0.368	S15

Note. *The interpolated optimum and the 90%-of-maximum region are stable; only the ranking of the two acceptable levels responds to the weights.*

4. Discussion

4.1. Principal findings

Three findings emerge. First, the link between sucrose charge and residual ethanol in a static, open kombucha fermentation is not monotone: ethanol peaked at an intermediate charge of 10% w/v and was lower at both 5% and 15% w/v. Second, acetic acid moved opposite to sucrose. Total titratable acidity rose and then plateaued, so the acetic-acid share of total acidity fell from roughly one third to less than one fifth as sucrose increased, and the acid budget was redistributed, not reduced. Third, and of most use in practice, desirability analysis reconciled these conflicting responses. The optimum was a broad plateau between approximately 15 and 20% w/v, not a knife edge. Within that plateau, the choice of setpoint depends on whether the manufacturer prioritizes regulatory headroom or biocellulose yield.

4.2. Why ethanol is biphasic

Two opposing osmotic effects, acting on different members of the consortium, best explain the biphasic ethanol profile. Raising sucrose from 5 to 10% w/v relieves carbon limitation and lets yeast glycolytic flux approach its maximum, the classical substrate-saturation response reported for kombucha and other tea ferments[22]. Then the trend reverses. Water activity was not measured, but above 10% w/v the medium is likely to become hypertonic enough to impose water stress on the yeast population, with declining invertase activity and glycolytic flux and depressed ethanol output, which would explain the effect observed at 15% w/v. Supporting evidence from a different system comes from high-gravity fermentations: raising wort osmolality measurably depresses yeast viability and ethanol productivity unless osmoprotectants are supplied[37]. Ethanol rose again at 20% w/v. One possible explanation, not tested here, is selective enrichment of osmotolerant yeasts such as *Zygosaccharomyces* over the 14-day incubation, combined with a disproportionately greater inhibition of the acetic acid bacteria, whose oxidative metabolism is limited both osmotically and by oxygen at the air–liquid interface[15,26]. The acetification index supports this reading. At 20% w/v it was the lowest of all four levels (first row of Table 2; red trace in Figure 6B). Ethanol therefore appears to have accumulated not because more was produced per unit substrate, since the ethanol yield per gram of sucrose at S20 (15.4 mg g^{-1}) was less than half that at S10 (34.1 mg g^{-1}), but because less of it was oxidized away.

This matters for quality control. On this evidence, the ethanol risk in a high-sugar kombucha is more a consumption problem than a production problem. Interventions that improve oxygen transfer at the surface, such as a larger surface-to-volume ratio, a more permeable closure, or a short aerated secondary phase, should therefore reduce residual ethanol more effectively than reducing the sugar charge, and, as a hypothesis to be tested, they would do so without sacrificing acidity or pellicle yield[15,38].

4.3. The redistribution of the acid budget

The decoupling of acetic acid from total titratable acidity is the most analytically useful result of this study. Acetic acid fell by 48% between 5 and 15% w/v sucrose while titratable acidity rose by 5%, so the non-acetic pool expanded by approximately 421 mg L^{-1}. In kombucha that pool is expected to consist largely of gluconic and glucuronic acids (not measured here), which acetic acid bacteria generate by direct periplasmic oxidation of free glucose through membrane-bound, PQQ-dependent glucose dehydrogenase, entirely bypassing the ethanol intermediate[11,13,14]. When glucose is abundant, this oxidative shunt competes successfully with ethanol oxidation for the same respiratory chain. It also feeds the UDP-glucose pool used for cellulose synthesis[14,39]. The strong correlation between the non-acetic acid fraction and pellicle thickness (Table 4; r = 0.880) is in line with such a shared origin. One practical corollary follows. Titratable acidity alone can be an inadequate quality specification for kombucha, because two batches with identical titratable acidity can differ almost twofold in acetic acid, and therefore in the sharp vinegar note that defines the product's sensory identity[22,40]. Specifications should state both.

4.4. Comparison with published kombucha studies

The ethanol concentrations measured here (0.056–0.341% w/v) sit within the range reported for artisanal and commercial kombucha. Surveys of commercial products have repeatedly found a minority of retail samples above the 0.5% non-alcoholic threshold while most sit well below it[1,2], and controlled laboratory fermentations of tea and fruit substrates likewise report residual ethanol that remains sub-threshold under moderate conditions but rises with warmer or longer fermentation[7,23,41]. Every one of our levels stayed below 0.5% at day 14. That held even at 20% w/v sucrose. It indicates that a well-balanced consortium with adequate surface aeration can tolerate a high sugar charge without breaching the limit, a more permissive conclusion than the conventional recipe of 5–10% w/v sucrose[42,43], which is usually presented as yielding a non-alcoholic or low-alcohol beverage.

The inverse relationship between sucrose and acetic acid contrasts with several Indonesian reports in which acidity rose with added sugar, but those studies generally quantified total acidity by titration, not acetic acid by chromatography[6,21,28]. Our data reconcile the apparent conflict. Measured by titration, our results agree with theirs, since titratable acidity did increase with sucrose, whereas measured by HPLC the acetic acid component fell. The disagreement is analytical, not biological. Studies that have applied chromatographic speciation to kombucha have similarly found that gluconic acid can exceed acetic acid, in some consortia at high sugar loadings[13,44].

The pellicle response was linear over the tested range. It rose by 0.243 mm per percentage point of sucrose, with no detectable curvature up to 20% w/v (Table 3 and Figure 5D), and this extends observations that carbon availability is the principal determinant of bacterial cellulose productivity in kombucha and related *Komagataeibacter* cultures[14,45,46]. The absence of substrate inhibition across this range deserves attention. It suggests that the cellulose-forming population tolerates osmotic conditions that measurably stress the ethanologenic yeasts. The physicochemical values reported here fall within the ranges given for black-tea kombucha at comparable fermentation times[3,7,18,27]: pH 2.88–3.08 and titratable acidity 0.185–0.195%. This supports the external validity of the dataset despite its composite sampling design.

4.5. Mechanistic synthesis

Figure 3 integrates these observations in a control scheme. The sucrose charge acts on four levers at once. First, it sets the total carbon available, which drives all downstream fluxes. Second, it sets the water activity of the medium, which selectively suppresses contaminating microorganisms at high concentrations while stressing the resident yeasts. Third, it sets the free-glucose concentration, which determines how much flux is diverted through the PQQ-dependent glucose dehydrogenase shunt to gluconic acid and cellulose rather than through the ethanol–acetate axis. Fourth, via the resulting acidity, it sets the pH. That feeds back on the enzymatic activity of the whole consortium. Because these levers pull in opposite directions on different quality attributes, no single-response optimization can identify a sound setpoint, which is precisely the argument for the multi-response approach used here.

The behavior at 5% w/v deserves particular comment. It is the level at which the naive single-response reading is most misleading. As detailed in Table 1 and Table 2, that level gave the highest acetic acid (664.9 mg L^{-1}) and an acetification index close to unity, which would ordinarily indicate near-complete conversion. Yet its titratable acidity was the lowest, its pH the highest and its pellicle the thinnest, and the medium may have been too dilute to offer an osmotic barrier to airborne contaminants in an open vessel (not tested). An acetification index near one in a system where ethanol is barely detectable is equally compatible with heterofermentative organisms generating acetate directly from glucose without an ethanol intermediate, a route open to lactic acid bacteria and to several contaminant genera[19,47,48]. Ratio indices computed on very small denominators must therefore be interpreted alongside absolute concentrations. The desirability framework accounts for this by weighting microbiological integrity most heavily, although that term itself rests on observation (Section 4.8).

4.6. Green chemistry and biotechnology significance

The process examined here embodies several green-chemistry principles that bear directly on the scope of this journal. A self-replicating microbial consortium catalyzes it. No stoichiometric reagent is needed, so catalyst manufacture and disposal are eliminated. The process runs at ambient temperature and atmospheric pressure with no external heating after the initial infusion, and water is the only solvent. Atom economy is favorable: one inexpensive feedstock yields both a beverage and a structural biopolymer, and the pellicle that is conventionally discarded as waste is a recoverable bacterial-cellulose material with documented uses in packaging films, wound dressings and pharmaceutical excipients[31,32,49]. Process-mass-intensity thinking offers a useful yardstick. Raising the sucrose charge from 15 to 20% w/v increases pellicle thickness by 34% while leaving titratable acidity unchanged, which suggests that the incremental sugar is directed preferentially into pellicle biomass (pellicle mass and recoverability were not measured) instead of being lost as waste. The material efficiency of the whole process may therefore improve rather than degrade[32,39].

From a fermentation-biotechnology standpoint, the study shows that a classical pharmaceutical development framework transfers cleanly to an artisanal bioprocess. Quality by design, as codified in ICH Q8(R2), has four core elements: the definition of quality attributes, the identification of the process parameter that governs them, the modeling of the response surface, and the reporting of a design space instead of a single point. All four were addressed here, within the limits of a single-factor, four-level design. The result is a usable specification. A small enterprise can set the sugar dose with a kitchen scale (150–200 g of sugar per liter of tea, on the volume basis to be confirmed in Section 2.3), although ethanol should still be verified analytically (Section 4.7).

4.7. Indonesian pharmaceutical and regulatory context

For Indonesian producers, the ethanol result matters most in operational terms. Fatwa No. 10 of 2018 of the Indonesian Council of Ulama classifies a beverage containing 0.5% ethanol or more as khamr and therefore haram, and holds a fermented beverage with less than 0.5% ethanol to be permissible provided it is medically harmless[29]. The National Agency of Drug and Food Control lists kombucha, as a fermented tea beverage, among the non-alcoholic beverages of its food-category regulation[50], which states no ethanol limit for it. The two instruments are often conflated, so the division of regulatory labor deserves precision. The 0.50% figure is the halal threshold set by the Council's fatwa. The Agency's Regulation No. 5 of 2021 sets safety and quality standards for beverages that are already classified as alcoholic. A kombucha held below 0.50% therefore satisfies the fatwa and falls outside the scope of the alcoholic-beverage standard. Independent surveys of Indonesian vinegars, by gas chromatography, and of commercial kombucha show that naturally formed ethanol in acidic beverages can approach or exceed such thresholds when the process is uncontrolled[51–54], whereas stored fruit juices stayed below 0.1% ethanol[55]. Instrumental verification is thus required, not assumption. All four sucrose charges met the requirement. Upper confidence bounds were no higher than 0.354% w/v, so halal compliance in this system is governed less by the sugar dose than by the aeration and harvest-time discipline that keep acetification going. Recommending 15–20% w/v sucrose creates no compliance risk, provided producers retain the 14-day harvest and open-vessel aeration used here. Ethanol should be verified by gas chromatography. Hydrometry overestimates alcohol in sugar-rich media.

4.8. Strengths and limitations

This work has four main strengths. The analytical panel is complete. Four orthogonal chemical measurements and one biomaterial measurement were made on the same samples, and an accredited third-party laboratory, blind to the study hypothesis, performed the analyses. Every process variable other than sucrose was held constant. The statistical treatment reports assumption diagnostics, effect sizes, confidence intervals and explicit lack-of-fit tests, and does not rely on p-values alone. Acetic acid and titratable acidity were reported separately, and the acetification index and non-acetic acid fraction were derived from them, which gave mechanistic insight that neither measurement alone would have provided.

The limitations are equally clear and should temper the interpretation. The dominant one is the composite sampling design. Because the three vessels of each level were pooled before analysis, the replicates entering the ANOVA are analytical, not biological. The dispersion terms therefore describe measurement repeatability. The reported F ratios and effect sizes are inflated relative to what a fully replicated biological design would yield, although the direction and rank order of the treatment effects are probably reliable, because they are large relative to the inter-vessel variance one would typically expect (not measured here) and agree with the correlation and regression structure. The precision statements should still be read as analytical, not biological. Second, the design has only four levels of a single factor. The cubic models are saturated and cannot be validated against an independent point, whereas a five- to seven-level design, or a central composite design crossing sucrose with fermentation time and temperature, would allow genuine model validation. Third, microbiological status was recorded observationally and not by plate counts or sequencing. The microbiological-integrity desirability term thus relies on visual assessment and on the osmotic-protection mechanism, not on enumeration. Fourth, only one time point was sampled. This precludes kinetic

modeling of the ethanol maximum. Fifth, no sensory panel was run and gluconic and glucuronic acids were not measured, so the composition of the non-acetic pool is inferred and not demonstrated.

4.9. Future directions

Four experiments follow directly. Chromatographic quantification of gluconic, glucuronic, lactic and citric acids would convert the inferred non-acetic pool into a measured one and test the shunt hypothesis explicitly. A fully replicated, non-composited time-course design sampling at days 3, 7, 10, 14 and 21 would resolve the ethanol maximum kinetically. It would also give honest biological variance estimates. Amplicon sequencing of the consortium across the sucrose gradient would show whether osmotolerant yeasts and cellulose-forming *Komagataeibacter* are genuinely enriched at 20% w/v, as proposed here[12,20,56]. Last, the pellicle obtained at each level should be characterized for crystallinity, water-holding capacity and tensile behavior, which would establish whether the extra biomass generated at 20% w/v is of pharmaceutically useful quality and would strengthen the case for treating kombucha as a two-product biorefinery rather than a single-product fermentation[49,57].

5. Conclusion

Initial sucrose charge is a genuine critical process parameter for black-tea kombucha. It acts on residual ethanol, acetic acid, pH, titratable acidity and biocellulose yield in different and partly opposing directions ($p \leq 0.021$; $\eta^2 = 0.685–0.998$). Residual ethanol was biphasic. It peaked at 0.341 ± 0.007% w/v at 10% w/v sucrose and stayed below the 0.50% w/v Indonesian ceiling at every level, while acetic acid fell and the acid budget shifted towards non-acetic organic acids that accompanied a linear increase in pellicle thickness over the tested range. Multi-response desirability optimization identified 19.1% w/v as the predicted optimum within a practical design space of 15–20% w/v. Within that space, 15% is preferred when regulatory headroom matters most and 20% when biocellulose recovery is the objective. These findings give Indonesian producers a defensible, simple setpoint, with ethanol verified analytically for halal-compliant kombucha and support the treatment of kombucha fermentation as a green, two-product biotechnological process.

6. Declarations

Statement on the Use of Artificial Intelligence

The authors declare that no generative artificial intelligence tools were used in the design, analysis, or writing of this manuscript.

Author Contribution Statement

E.N.I.: Conceptualization, Methodology, Investigation, Formal analysis, Data curation, Visualization, Writing – original draft. S.H.Y.: Conceptualization, Methodology, Validation, Resources, Supervision, Writing – review & editing. Both authors read and approved the final manuscript.

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Conflict of Interest / Competing Interests

The authors declare that they have no competing interests.

Data Availability Statement

The de-identified dataset and the analysis scripts supporting the findings are available from the corresponding author on reasonable request.

Ethics Approval and Consent to Participate

Not applicable. This study used in vitro fermentation and instrumental analytical methods and did not involve human subjects or animal experiments; ethics committee approval was therefore not required. No sensory panel was convened and no human material was collected at any stage.

Consent for Publication

Not applicable.

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