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

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A B S T R A C T

Kombucha is a functional beverage produced by a symbiotic consortium of acetic acid bacteria and osmotolerant yeasts, and its commercial acceptability depends on a narrow compliance window in which residual ethanol must remain below 0.50% w/v while organic acidity is sufficient for flavour and microbial safety. The initial sucrose charge is the single manipulable process parameter that governs both outcomes, yet it is rarely treated as a formally optimised critical process parameter. Four sucrose charges (5, 10, 15 and 20% w/v) were evaluated in black-tea kombucha fermented statically for 14 days at 25–28 °C with a standardised 50 g pellicle and 10% v/v starter, three vessels per level. Composites were assayed in analytical triplicate by GC-FID (ethanol), ion-exclusion HPLC (acetic acid), potentiometry (pH) and alkalimetry (titratable acidity), with calliper measurement of pellicle thickness. Data were analysed by one-way ANOVA with Shapiro–Wilk, Levene and lack-of-fit diagnostics, Tukey's HSD, polynomial response modelling and Derringer–Suich desirability optimisation. Sucrose charge affected every attribute (all $p < 0.001$; $\eta^2 = 0.685$–0.998). Ethanol was biphasic, peaking at 0.341 ± 0.007% w/v (95% CI 0.324–0.359) at 10% sucrose — only 68% of the compliance threshold — whereas acetic acid fell monotonically and the acetification index collapsed 9.9-fold from 5% to 20% sucrose. Pellicle thickness increased strictly linearly ($R^2 = 0.974$). Composite desirability identified 19.1% w/v as the predicted optimum within a 15–20% w/v design space, with 15% preferred when ethanol headroom is prioritised. Sucrose charge is a tractable, instrument-free control lever for halal-compliant kombucha manufacture.

1. Introduction

Kombucha is a lightly effervescent, acidic beverage obtained by fermenting sweetened tea with a symbiotic culture of bacteria and yeast (SCOBY), and it has become one of the fastest-growing categories in the global functional-beverage market over the past decade[1,2]. Consumer interest is driven by the reported antioxidant, antimicrobial and metabolic activities of the fermented matrix, and by the presence of organic acids, polyphenol transformation products and live microorganisms[3,4,5]. 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 and without in-process analytical control, so batch-to-batch variability in acidity and residual alcohol is substantial[6,7,8]. This variability is not merely a sensory problem: it is a regulatory and religious-compliance problem, because a beverage that drifts above the permitted ethanol threshold cannot lawfully be marketed as a non-alcoholic product.

The biochemistry that produces this risk is well described. Yeasts of the genera *Saccharomyces*, *Zygosaccharomyces*, *Brettanomyces* and *Pichia* secrete extracellular invertase that hydrolyses sucrose to glucose and fructose, and ferment the released hexoses through the Embden–Meyerhof–Parnas pathway to ethanol and carbon dioxide[9,10]. Acetic acid bacteria, dominated in most consortia by *Komagataeibacter* and *Acetobacter*, then oxidise that ethanol through membrane-bound pyrroloquinoline-

quinone-dependent alcohol dehydrogenase and aldehyde dehydrogenase to acetic acid, which acidifies the medium and confers antimicrobial protection[11,12]. The same organisms also oxidise free glucose directly through membrane-bound glucose dehydrogenase to gluconic and glucuronic acids, and polymerise glucose into the extracellular cellulose pellicle that floats at the air–liquid interface[13,14]. Ethanol is therefore an obligate intermediate rather than an end product, and 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 simultaneously 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[18,19,20], and several Indonesian studies have varied sugar concentration and fermentation duration together while reporting total acidity or sensory acceptance[6,21,22]; recent studies optimised green-tea/pineapple kombucha for chemical and bioactive profiles without treating residual ethanol as a constrained response[16,23,24]. What remains under-addressed is the treatment of the sucrose charge as a formally optimised critical process parameter, evaluated against several conflicting quality attributes at once. Studies that maximise acidity alone will drive the process towards conditions that also raise ethanol; studies that minimise ethanol alone will sacrifice 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 routinely used interchangeably in kombucha quality reports, yet they measure different things: the former is the total neutralisable acid capacity, the latter a single analyte. When the two are measured on the same sample, the difference between them quantifies the non-acetic organic-acid pool generated by the direct glucose-oxidation shunt[13,20]. That difference carries mechanistic information about how the consortium partitions carbon, and 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 exceptional biological and fermentation-culture diversity provides a strong resource base for applied fermentation biotechnology[25,26]. Applying the design-space logic of pharmaceutical quality by design — the critical-quality-attribute, critical-process-parameter and design-space vocabulary codified in ICH guideline Q8(R2) — to an artisanal food fermentation is an appropriate translational contribution from a pharmacy graduate programme, 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, which 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[27,28].

This study therefore aimed to (i) 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; (ii) derive and model carbon-partitioning indices — the acetification index and the non-acetic acid fraction — that expose the underlying metabolic mechanism; and (iii) identify, by multi-response Derringer–Suich desirability analysis, the sucrose charge and the surrounding design space that

jointly maximise acidity, biocellulose yield and microbiological protection while holding residual ethanol below the Indonesian halal compliance threshold. The novelty lies in converting a descriptive artisanal variable into 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 rather than a chemical catalyst, requires no organic solvent, operates at ambient temperature and pressure, and converts an inexpensive agricultural sugar into an acidified beverage and a recoverable bacterial-cellulose biomaterial in a single step[29,30].

2. Methods

2.1. Materials

Commercial black tea (*Camellia sinensis*, Tjatoet brand, Indonesia) was purchased from a local retailer in Yogyakarta. Refined commercial cane sucrose (food grade, ≥99.8% sucrose) and bottled steam-sterilised mineral water were used as received. The starter culture consisted of a mature SCOBY pellicle and its liquor obtained from a single mother culture maintained in the laboratory, so that all vessels received genetically and physiologically equivalent inoculum. Ethanol 70% v/v (technical grade) was used for surface sanitation. Sodium hydroxide volumetric standard solution and phenolphthalein indicator (analytical grade) were supplied to the contract laboratory. All fermentation glassware was borosilicate.

2.2. Study design and ethical statement

A single-factor, four-level, completely randomised experimental design was used, with the initial sucrose charge as the sole manipulated factor at 5, 10, 15 and 20% w/v (coded S5, S10, S15 and S20). Every level was prepared in three independent fermentation vessels (n = 3 biological units, 12 vessels in total), and all other process variables — tea concentration, water source, working volume, inoculum mass, starter fraction, temperature, light exposure and harvest day — were held constant. The complete five-phase protocol, including the point at which composite pooling occurs, is illustrated in Figure 1. The study was carried out at the Faculty of Pharmacy, Universitas Sanata Dharma, Yogyakarta, Indonesia, with analytical determinations subcontracted to an accredited third-party testing laboratory (PT Saraswanti Indo Genetech, Bogor, Indonesia).

Ethical statement. This study was conducted using *in vitro* fermentation and instrumental analytical methods and did not involve human subjects or animal experiments; therefore, ethics committee approval was not required. No sensory panel was convened and no human material was collected at any stage.

2.3. Preparation of the fermentation medium

All jars, measuring cylinders and stirring rods were washed with detergent, rinsed with potable water, sprayed internally with 70% v/v ethanol and air-dried before use; the working bench was sanitised identically. Mineral water was boiled to 100 °C and black tea was infused at 10 g L^{-1} for 15 min to extract polyphenols and assimilable nitrogen, then clarified through filter paper and a fine mesh sieve. Nine hundred millilitres of hot infusion (≥70 °C, to guarantee complete dissolution) was transferred to each jar 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 final 900 mL of sweetened tea. Jars were then covered loosely and left to cool to 25–28 °C before inoculation, because 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) transferred with sanitised forceps. Jars were closed with a double layer of clean, porous cotton cloth secured with a rubber band, which permits oxygen transfer for the obligately aerobic

acetic acid bacteria while excluding insects and coarse particulates. Fermentation proceeded statically and without agitation for 14 days in a dark cabinet held at 25–28 °C. At harvest the parent and newly formed pellicles were removed with sterile forceps, the liquid phase was filtered through clean cloth, and samples were bottled immediately for dispatch to the contract laboratory under chilled conditions.

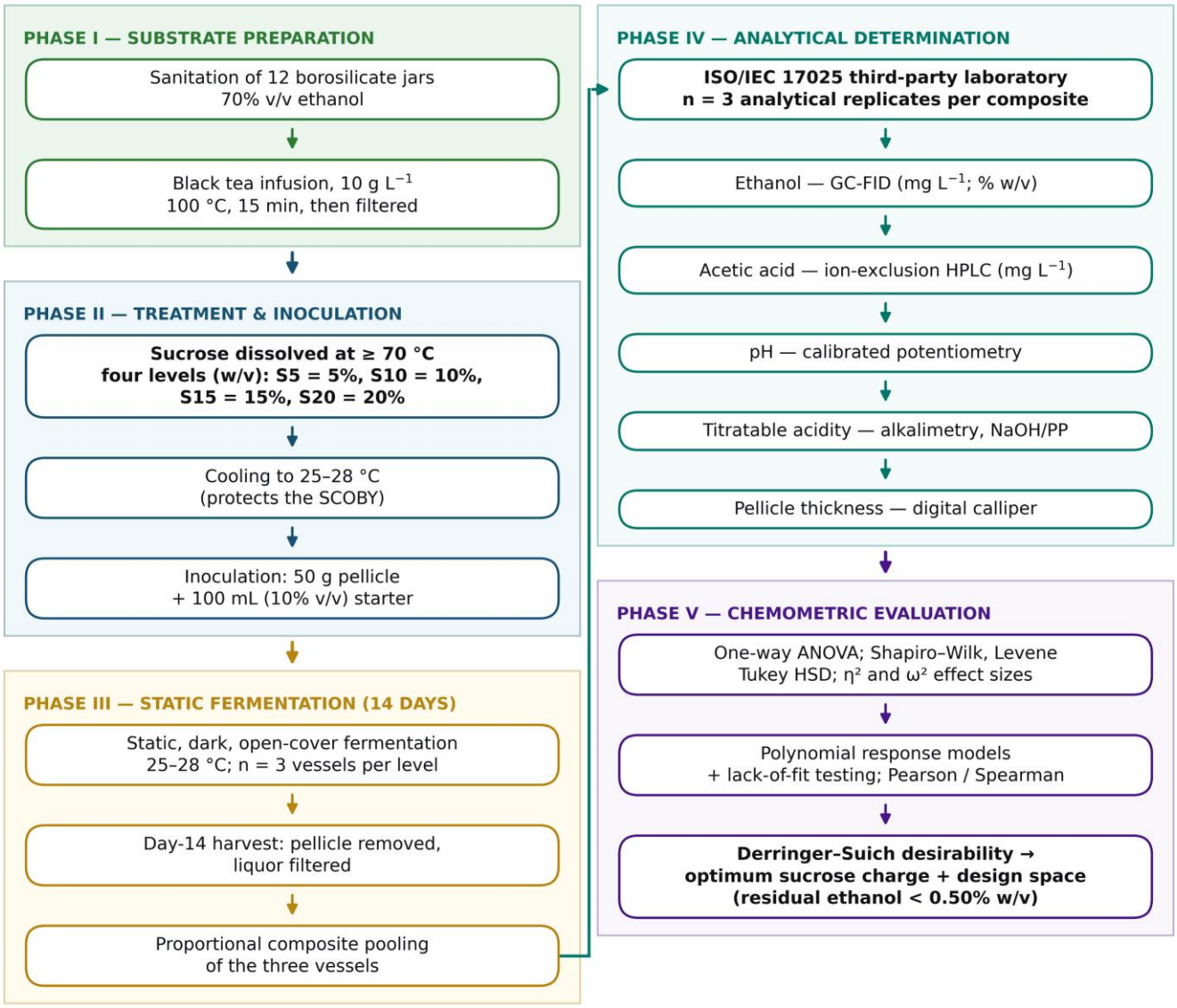

Figure 1. Experimental workflow for the multi-response optimisation of sucrose charge in black-tea kombucha, from substrate preparation through static fermentation, third-party analytical determination, and chemometric quality-control evaluation.

2.5. Sampling strategy

Proportional composite sampling was applied within each treatment level: equal volumes were withdrawn from each of the three independent vessels of a level and combined into a single homogeneous composite before instrumental analysis, in accordance with the Codex Alimentarius general guidelines on sampling (CAC/GL 50-2004). Physical pooling acts as a mechanical averaging step that 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), so that all dispersion statistics reported here describe analytical repeatability rather than biological variance.

The consequence of this design choice for inference is addressed explicitly in Section 4.8.

2.6. Analytical determinations

Ethanol. Residual ethanol was determined by gas chromatography with flame-ionisation detection after direct injection of the filtered beverage, using an internal-standard calibration bracketing 0.01–1.00% w/v. Gas chromatography is the reference technique 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[31,32]. Results were expressed both as mg L^{-1} and as % w/v.

Acetic acid. Acetic acid was quantified by high-performance liquid chromatography on an ion-exclusion column with ultraviolet detection at 210 nm, which separates acetic acid from gluconic, glucuronic, lactic, malic and citric acids that co-occur in kombucha and would otherwise be counted together[13]. Quantification was against an external calibration curve of acetic acid reference standard and results were expressed in mg L^{-1}.

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

Total titratable acidity. Titratable acidity was determined by alkalimetry: a measured aliquot of beverage was titrated with standardised sodium hydroxide to a stable faint pink phenolphthalein endpoint, and the result was expressed as % w/v acetic acid equivalents according to $CH_3COOH + NaOH \rightarrow CH_3COONa + H_2O$. Because titratable acidity measures the total neutralisable acid capacity while pH measures only the instantaneous free hydrogen-ion activity, the two parameters are complementary rather than redundant[18,26].

Pellicle thickness. The newly synthesised cellulose pellicle of each vessel was blotted free of adhering liquor and measured with a digital calliper at five evenly distributed points; 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 expresses how completely the ethanol pool has been oxidised 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, and 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. Metabolite yields were additionally normalised to the sucrose charged (mg metabolite per g sucrose).

2.8. Statistical analysis

All data are reported as mean ± standard deviation of $n = 3$ analytical replicates with two-sided 95% confidence intervals. 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 and homogeneity of variance by Levene's test (mean-centred) with Bartlett's test as a supporting check. Treatment effects were assessed by one-way analysis of variance; 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 robustness checks. Pairwise comparisons used Tukey's honestly significant difference procedure at $\alpha = 0.05$, and the resulting compact letter display 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 behaviour was modelled by ordinary least squares polynomial regression of degree one to three; 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 confirmed by Spearman ρ. Exact p-values are reported to three decimal places and values below 0.001 are shown as $p < 0.001$.

Multi-response optimisation used the Derringer–Suich desirability approach. Six individual desirability functions were defined on the interpolated sucrose domain: acetic acid (larger-the-better), titratable acidity (larger-the-better), residual ethanol (smaller-the-better, with a hard constraint setting desirability to zero above 0.50% w/v), pH (target-the-best, fully desirable between 2.80 and 3.20 and zero outside 2.50–4.20), pellicle thickness (larger-the-better) and microbiological integrity, modelled as a logistic function of sucrose centred 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, $D = (d_1^{w1} \times d_2^{w2} \times \ldots \times d_6^{w6})^{1/W}$, 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

Residuals were consistent with normality for 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 $p > 0.05$), and variances were homogeneous (Levene $p = 0.131$, 0.439, 0.996, 0.999 and 0.154, respectively). The untransformed acetification index violated homoscedasticity (Levene $p = 0.037$) because its dispersion scales with its magnitude; $\log_{10}$ transformation restored both normality (Shapiro–Wilk $p = 0.376$) and variance homogeneity (Levene $p = 0.486$), and all inference on this index is reported on the transformed scale with back-transformed means 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. The complete descriptive and inferential results are detailed in Table 1 and the four primary critical quality attributes are illustrated in Figure 2. Residual ethanol followed a pronounced biphasic profile rather than a monotone rise: it was lowest at S5 (0.056 ± 0.001% w/v; 95% CI 0.053–0.059), rose sharply to a maximum at S10 (0.341 ± 0.007% w/v; 95% CI 0.324–0.359), fell significantly at S15 (0.215 ± 0.005% w/v) and rose again at S20 (0.308 ± 0.006% w/v). The overall effect was very 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 shown by the four different letters in the ethanol rows of Table 1 and above the bars of Figure 2A. Critically, as illustrated by the dashed regulatory line in Figure 2A, every level remained below the 0.50% w/v compliance threshold: the least favourable level, S10, reached only 68.3% of the ceiling with a 95% upper confidence bound of 0.354% w/v, leaving a margin of 31.7%.

Acetic acid behaved in the opposite direction, decreasing monotonically from 664.87 ± 10.64 mg L^{-1} at S5 to 344.17 ± 5.51 mg L^{-1} at S15 before a small but statistically detectable recovery to 369.64 ± 5.91 mg L^{-1} at S20, as detailed in the third row of Table 1 and illustrated in Figure 2B ($F(3,8) = 1089.6$, $p < 0.001$, $\eta^2 = 0.998$). All pairwise contrasts were significant, including S15 versus S20 ($p = 0.015$). pH declined from 3.080 ± 0.011 at S5 to 2.880 ± 0.010 at S20 ($F(3,8) = 223.1$, $p < 0.001$, $\eta^2 = 0.988$), with S10 and S15 statistically indistinguishable from one another (they share the letter b in Table 1); as illustrated by the shaded band in Figure 2C, all values fell inside the 2.50–4.20 window recommended for safe kombucha. Total titratable acidity, illustrated in 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 ($p = 0.349$), which 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 ($F(3,8) = 103.8$, $p < 0.001$, $\eta^2 = 0.975$), with all four levels significantly different from each other, as detailed in the final row of Table 1.

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).
Different superscript letters within a row indicate means that differ significantly by Tukey's HSD test (α = 0.05).

Quality attribute	S5 (5% w/v)	S10 (10% w/v)	S15 (15% w/v)	S20 (20% w/v)	F(3,8)	p	η²
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

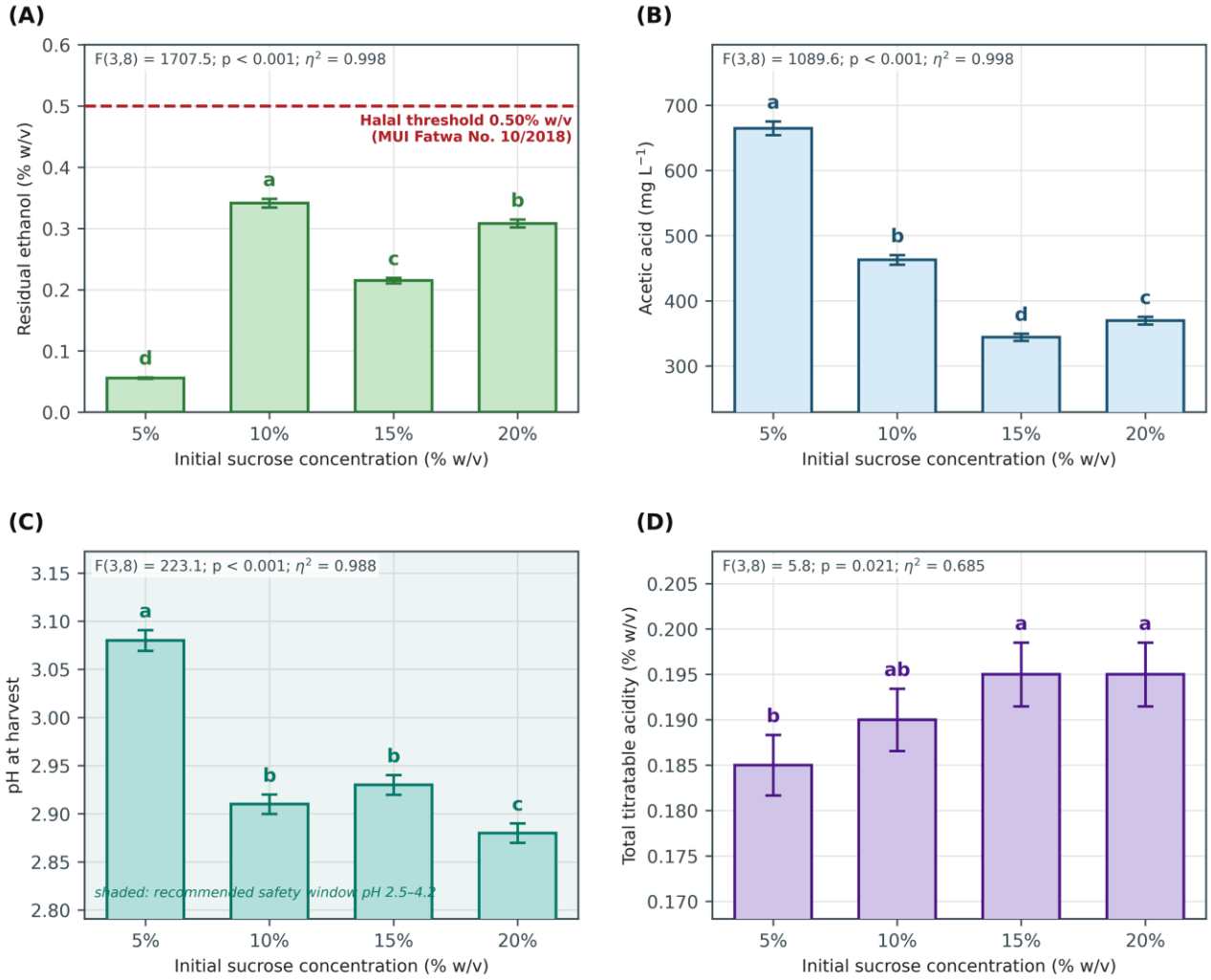

Figure 2. Primary critical quality attributes of black-tea kombucha after 14 days of static fermentation at four sucrose charges: (A) residual ethanol, (B) acetic acid, (C) pH at harvest, and (D) total titratable acidity. Bars are mean ± SD of three analytical replicates; different lowercase letters 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.

3.3. Carbon partitioning and derived indices

The three derived carbon-partitioning indices, together with the substrate-normalised yields and the regulatory headroom, are detailed in Table 2; they clarify what the primary attributes only imply. The acetification index — the molar ratio of acetate produced to ethanol remaining — was close to unity at S5 (0.915 ± 0.019 mol mol^{-1}) but collapsed by nearly an order of magnitude at every higher charge, 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, while 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} ($F(3,8) = 96.0$, $p < 0.001$, $\eta^2 = 0.973$). In other words, higher sucrose charges did not reduce total acid output; they redirected it away from acetic acid and towards secondary organic acids. The same redirection was mirrored in the biomaterial phase, where the non-acetic fraction and pellicle thickness were strongly correlated ($r = 0.880$, 95% CI 0.618–0.966, $p < 0.001$). The metabolic logic behind this redistribution — the coupling of the ethanologenic and oxidative fluxes through the shared ethanol pool and the competing PQQ-dependent glucose-oxidation shunt — is illustrated in Figure 3, and the structures of the substrate and of every metabolite named in this section are illustrated in Figure 4.

Yield normalisation, detailed in the fourth and fifth rows of Table 2, reinforced the point. Ethanol yield per gram of sucrose charged peaked at S10 (34.14 ± 0.72 mg g^{-1}) and was two- to three-fold lower at every other level, 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 ($F(3,8) = 6431$, $p < 0.001$). Sucrose added beyond about 15% w/v therefore bought progressively less acetic acid per unit of substrate while continuing to buy pellicle.

Table 2. Derived bioprocess and carbon-partitioning indices (mean ± SD, n = 3).
Superscript letters denote Tukey groupings (α = 0.05); the acetification index and the acetic-acid share were tested on the $\log_{10}$ scale.

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

3.4. Response modelling and correlation structure

The polynomial models fitted to the replicate-level data are illustrated panel by panel in Figure 5 and their goodness-of-fit statistics are detailed in Table 3. Pellicle thickness, in 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 likewise adequately linear (lack-of-fit $p = 0.427$) but with a modest coefficient of determination ($R^2 = 0.610$), consistent with its early plateau. By contrast, acetic acid (Figure 5B), pH (Figure 5E), ethanol (Figure 5A) and the acetification index (Figure 5F) all 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 required the saturated cubic form, which reproduces the four treatment means exactly and is therefore used for interpolation only, never for extrapolation beyond 20% w/v. The non-acetic acid fraction in Figure 5C was the one intermediate case, adequately described by a quadratic model with no detectable lack of fit ($p = 0.243$).

The full correlation structure, detailed in Table 4, was internally consistent with the mechanism. Sucrose charge correlated almost perfectly with pellicle thickness ($r = 0.987$, $p < 0.001$), strongly and negatively with acetic acid ($r = -0.890$, $p < 0.001$) and with pH ($r = -0.836$, $p < 0.001$), and positively with the non-acetic acid fraction ($r = 0.894$, $p < 0.001$). Ethanol and acetic acid were inversely related ($r = -0.727$, $p = 0.007$), which is the expected signature of a consortium in which incomplete acetification leaves ethanol unconsumed, and ethanol tracked pH inversely and very closely ($r = -0.939$, $p < 0.001$).

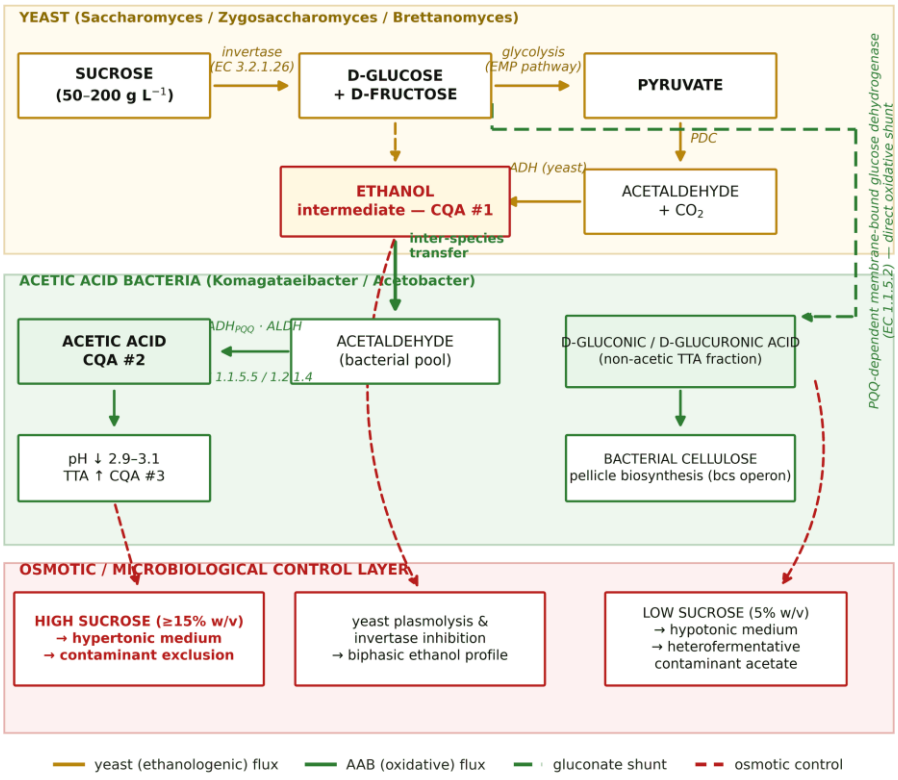

Figure 3. Proposed mechanism by which the initial sucrose charge governs carbon partitioning in the kombucha consortium. The ethanologenic (yeast) flux and the oxidative (acetic acid bacteria) flux are coupled through the ethanol pool, while the PQQ-dependent membrane-bound glucose dehydrogenase shunt diverts free glucose towards gluconic and glucuronic acids and biocellulose. The osmotic control layer summarises how the sucrose charge simultaneously modulates contaminant exclusion and yeast stress.

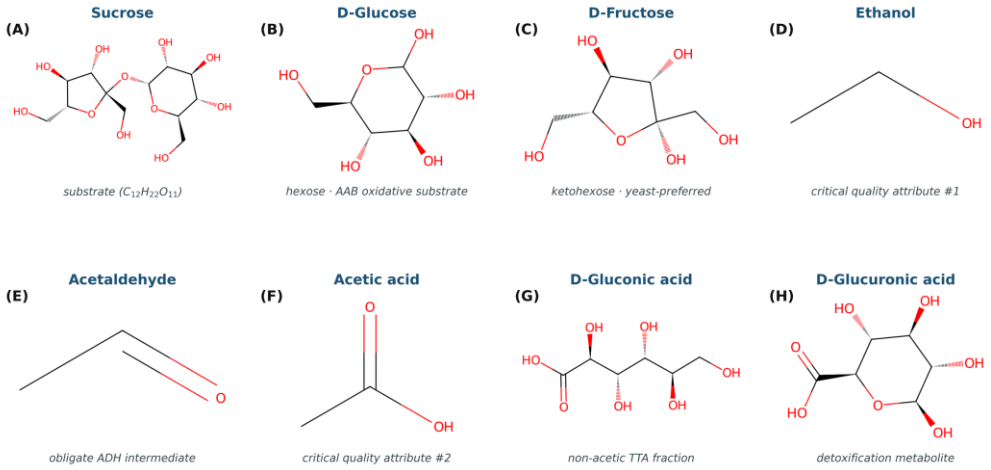

Figure 4. Chemical structures of the substrate and the principal metabolites quantified or inferred in this study: (A) sucrose, (B) D-glucose, (C) D-fructose, (D) ethanol, (E) acetaldehyde, (F) acetic acid, (G) D-gluconic acid and (H) D-glucuronic acid.

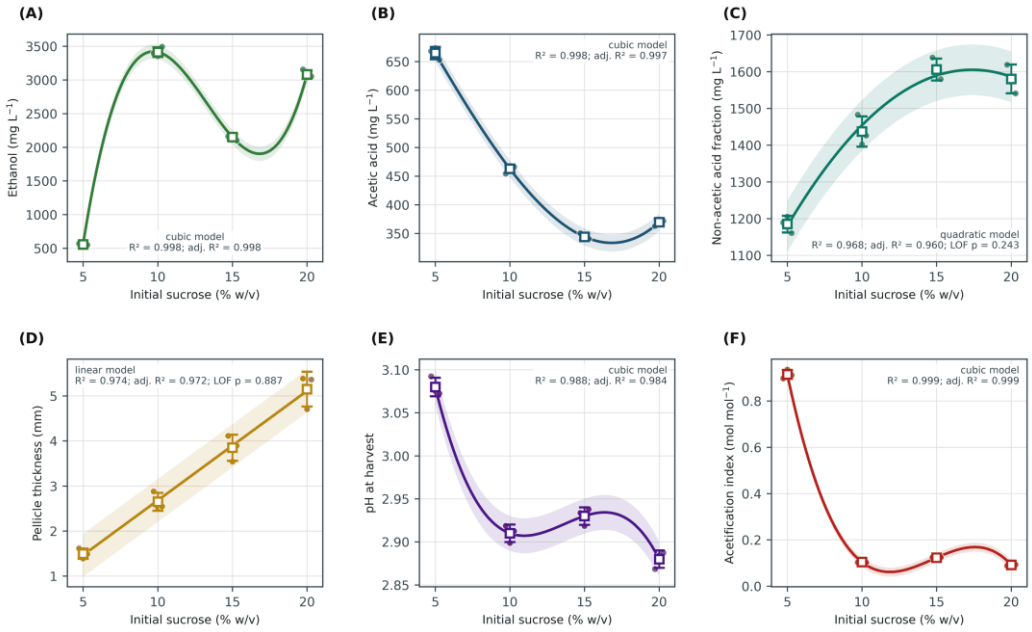

Figure 5. Polynomial response models relating the initial sucrose charge to (A) ethanol, (B) acetic acid, (C) the non-acetic titratable acid fraction, (D) pellicle thickness, (E) pH and (F) the acetification index. Open squares are treatment means ± SD, filled circles are individual analytical replicates, 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.
The selected model is shown in bold in the first column.

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	—

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

3.5. Multi-response optimisation and regulatory margin

The individual and composite desirability functions are illustrated across the whole sucrose domain in Figure 6A and their values at the four tested levels are illustrated in Figure 6B and detailed in Table 5. Composite desirability was negligible at S5 (D = 0.002), because although this level maximised acetic acid and minimised ethanol, it failed the microbiological-integrity and pellicle criteria outright (d_{micro} = 0.000, $d_{pellicle}$ = 0.000). S10 also scored poorly (D = 0.093) because 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, as 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 substantially more regulatory headroom ($d_{ethanol}$ = 0.444 versus 0.119), whereas S20 maximises biocellulose yield ($d_{pellicle}$ = 1.000 versus 0.644).

Across the interpolated domain, composite desirability was maximised at 19.1% w/v sucrose (D_{max} = 0.538), at which 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 contiguous interval over which D remained within 90% of its maximum spanned 18.1–20.0% w/v — the shaded band in Figure 6A — and the practical design space supported by the tested levels is 15–20% w/v. Every level satisfied the regulatory constraint with a wide margin: 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.

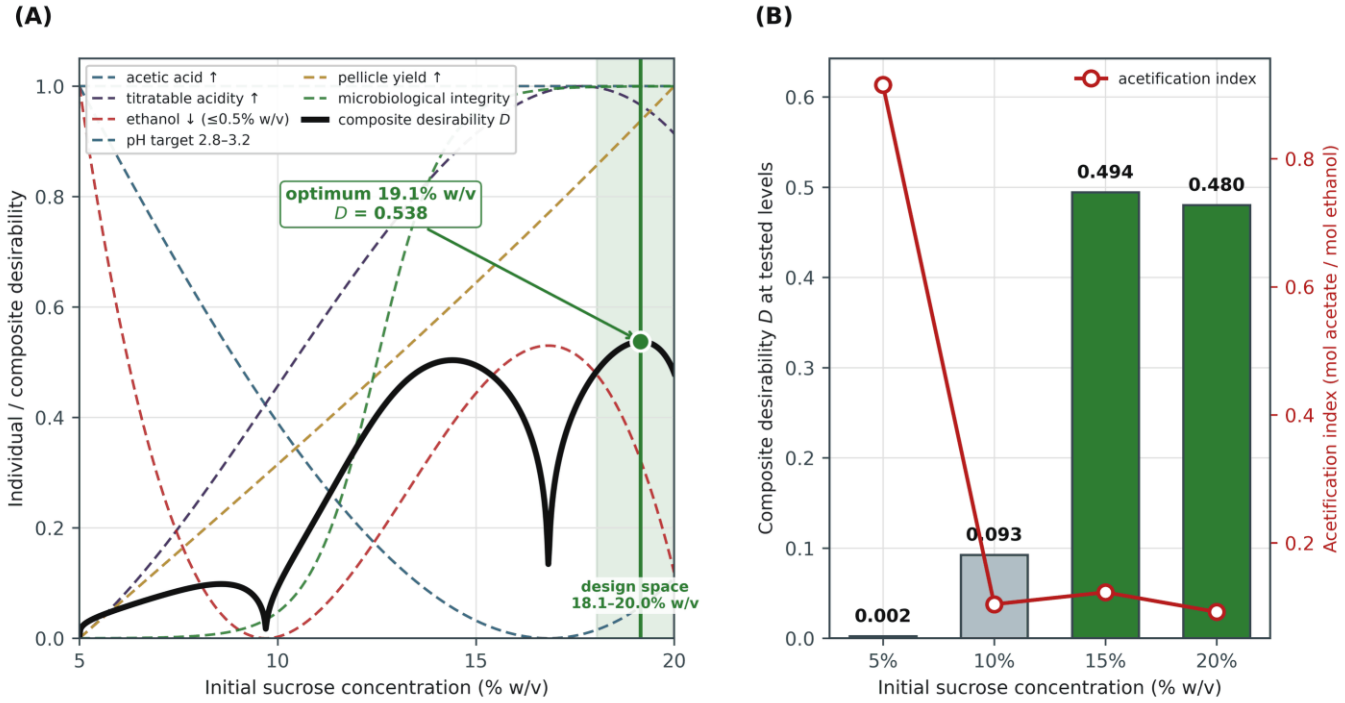

Figure 6. Multi-response optimisation of the sucrose charge. (A) Individual desirability functions (dashed) and the composite Derringer–Suich desirability D (solid black) across the interpolated sucrose domain; the vertical line marks the predicted optimum and the shaded band the design space within 90% of D_{max}. (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.
Individual desirabilities range from 0 (unacceptable) to 1 (fully desirable).

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

Because desirability results depend on the weights chosen, a sensitivity analysis was run over five alternative weighting schemes, detailed in 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 robust. What the weights do change is the ordering of the two acceptable levels, and they change it in the direction the mechanism predicts: emphasising the ethanol constraint (weight 3.0) makes S15 clearly superior to S20 (D = 0.485 versus 0.380), whereas relaxing it (weight 1.0) or emphasising acetic acid or pellicle yield reverses the ranking in favour of S20. Removing the microbiological-integrity term altogether left the optimum unchanged at 19.1% w/v and did not rehabilitate S5 (D = 0.007), because that level also fails the titratable-acidity and pellicle criteria; the exclusion of low sucrose charges is therefore over-determined rather than an artefact of the contamination term. The practical reading is that the acceptable region is stable and the choice within it is a legitimate manufacturing decision rather than a statistical one.

Table 6. Sensitivity of the multi-response optimisation to the weighting scheme.
The interpolated optimum and the 90%-of-maximum region are stable; only the ranking of the two acceptable levels responds to the weights.

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
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

4. Discussion

4.1. Principal findings

Three findings emerge. First, the relationship 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 in the opposite direction to sucrose while total titratable acidity rose and then plateaued, so that the acetic-acid share of total acidity fell from roughly one third to less than one fifth as sucrose increased — the acid budget was redistributed rather than reduced. Third, and most usefully for practice, when these conflicting responses were reconciled by desirability analysis the optimum was not a knife edge but a broad plateau between approximately 15 and 20% w/v, within which the choice of setpoint depends on whether the manufacturer prioritises regulatory headroom or biocellulose yield.

4.2. Why ethanol is biphasic

The biphasic ethanol profile is best explained by two opposing osmotic effects acting on different members of the consortium. Increasing sucrose from 5 to 10% w/v relieves carbon limitation and allows yeast glycolytic flux to approach its maximum, which is the classical substrate-saturation response reported for kombucha and other tea ferments[17,22]. Beyond roughly 12% w/v, however, the medium becomes sufficiently hypertonic to impose water stress on the yeast population, reducing invertase activity and intracellular glycolytic flux and depressing ethanol output — the effect observed at 15% w/v. Direct evidence for this mechanism comes from high-gravity fermentations, in which raising wort osmolality measurably depresses yeast viability and ethanol productivity unless osmoprotectants are supplied[33]. At 20% w/v ethanol rose again, which is most parsimoniously explained by the 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 both osmotically and oxygen limited at the air–liquid interface[11,15]. Consistent with that reading, the acetification index at 20% w/v was the lowest of all four levels, as detailed in the first row of Table 2 and illustrated in the red trace of Figure 6B, indicating that ethanol accumulated not because more was produced per unit substrate — 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 oxidised away.

This interpretation is important for quality control because it means that the ethanol risk in a high-sugar kombucha is a consumption problem rather than a production problem. Interventions that improve oxygen transfer at the surface — 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 would do so without sacrificing acidity or pellicle yield[15,34].

4.3. The redistribution of the acid budget

The decoupling of acetic acid from total titratable acidity is the analytically most informative 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 dominated by gluconic and glucuronic acids, 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, and it also feeds the UDP-glucose pool used for cellulose synthesis[14,35]. The strong correlation detailed in Table 4 between the non-acetic acid fraction and pellicle thickness ($r = 0.880$) is exactly what that shared origin predicts. The practical corollary is that titratable acidity alone is an inadequate quality specification for kombucha: 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[22,36]. 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[7,23,37]. That every one of our levels stayed below 0.5% at day 14, even at 20% w/v sucrose, 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 frequently repeated

advice that sugar must be restricted to 5–10% w/v to keep kombucha non-alcoholic.

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 rather than acetic acid by chromatography[6,21,26]. Our data reconcile the apparent conflict: measured by titration our results agree with theirs, since titratable acidity did increase with sucrose; 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 at high sugar loadings[13,20].

The pellicle response — a strictly linear 0.243 mm increase per percentage point of sucrose with no detectable curvature up to 20% w/v, as detailed in Table 3 and illustrated in Figure 5D — extends observations that carbon availability is the principal determinant of bacterial cellulose productivity in kombucha and related *Komagataeibacter* cultures[14,38,39]. The absence of substrate inhibition across this range is notable, since it implies that the cellulose-forming population tolerates osmotic conditions that measurably stress the ethanologenic yeasts. Physicochemical values reported here — pH 2.88–3.08, titratable acidity 0.185–0.195% — are consistent with the ranges given for black-tea kombucha at comparable fermentation times[3,7,18,25], which supports the external validity of the dataset despite its composite sampling design.

4.5. Mechanistic synthesis

The control scheme illustrated in Figure 3 integrates these observations. The sucrose charge acts simultaneously on four levers. It sets the total carbon available, which drives all downstream fluxes. It sets the water activity of the medium, which selectively suppresses contaminating microorganisms at high concentrations while stressing the resident yeasts. 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. And, through 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 optimisation can identify a sound setpoint — which is precisely the argument for the multi-response approach used here.

The behaviour at 5% w/v deserves particular comment because 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 was hypotonic enough to offer no osmotic barrier to airborne contaminants in an open vessel. An acetification index near one in a system where ethanol is barely detectable is equally consistent with heterofermentative organisms generating acetate directly from glucose without an ethanol intermediate, a route open to lactic acid bacteria and to several contaminant genera[9,19]. Ratio indices computed on very small denominators must therefore be interpreted alongside absolute concentrations, and the desirability framework handles this correctly by weighting microbiological integrity most heavily.

4.6. Green chemistry and biotechnology significance

The process examined here embodies several green-chemistry principles in a form directly relevant to the scope of this journal. It is catalysed by a self-replicating microbial consortium rather than a stoichiometric reagent, so catalyst manufacture and disposal are eliminated. It runs at ambient temperature and atmospheric pressure with no external heating after the initial infusion, and the only solvent is water. Atom economy is favourable in the sense that a single 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[29,30,40]. Assessed against modern process-mass-intensity thinking, the fact that raising the sucrose charge from 15 to 20% w/v increases pellicle thickness by 34% while leaving titratable acidity unchanged means the incremental sugar is converted preferentially into recoverable biomaterial rather than lost as waste, which improves rather than degrades the material efficiency of the overall process[30,35].

From a fermentation-biotechnology standpoint, the study also demonstrates that a classical pharmaceutical development framework transfers cleanly to an artisanal bioprocess. Defining critical quality attributes, identifying a critical process parameter, modelling the response surface and reporting a design space rather than a single point are the core elements of quality by design as codified in ICH Q8(R2), and they yield here a specification that a small enterprise can implement with a kitchen scale and no analytical instruments: weigh 150–200 g of sugar per litre of tea.

4.7. Indonesian pharmaceutical and regulatory context

For Indonesian producers the ethanol result is the operationally decisive one. Fatwa No. 10 of 2018 of the Indonesian Council of Ulama holds that a fermented beverage not produced with the intent of making an intoxicant is permissible provided its natural ethanol content remains below 0.5% and it is not harmful, and the National Agency of Drug and Food Control applies a corresponding quality standard to non-alcoholic fermented beverages[27,28]. It is worth being precise about the division of regulatory labour here, because the two instruments are often conflated: the 0.50% figure is the halal threshold established by the Council's fatwa, whereas 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 simultaneously stays outside the alcoholic-beverage category altogether. Independent gas-chromatographic surveys of Indonesian vinegars and of stored fruit juices show that naturally formed ethanol in acidic beverages can approach or exceed such thresholds when the process is uncontrolled, which is why instrumental verification rather than assumption is required[41,42]. All four sucrose charges satisfied this requirement with upper confidence bounds no higher than 0.354% w/v, which means that 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 therefore does not create a compliance risk, provided producers retain the 14-day harvest and open-vessel aeration used here and verify ethanol by gas chromatography rather than by hydrometry, which overestimates alcohol in sugar-rich media.

The Master's Program in Pharmacy at Universitas Sanata Dharma is well positioned to carry this translational agenda forward. Pharmacists are trained in the analytical validation, specification setting and stability thinking that Indonesian food and beverage small enterprises most conspicuously lack, and kombucha sits at the intersection of natural product chemistry, microbiology and consumer safety where that training is directly applicable[4,5].

4.8. Strengths and limitations

The main strengths of this work are the completeness of the analytical panel — four orthogonal chemical measurements plus a biomaterial measurement on the same samples — the use of an accredited third-party laboratory blind to the study hypothesis, the rigorous control of every process variable other than sucrose, and the statistical treatment, which reports assumption diagnostics, effect sizes, confidence intervals and explicit lack-of-fit tests rather than p-values alone. Reporting acetic acid and titratable acidity separately, and deriving the acetification index and non-acetic acid fraction from them, provided mechanistic insight that neither measurement alone would have given.

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 that enter the ANOVA are analytical rather than biological, so the dispersion terms describe measurement repeatability and the reported F ratios and effect sizes are inflated relative to what a fully replicated biological design would yield. The direction and rank order of the treatment effects are robust — they are large relative to any plausible inter-vessel variance and are corroborated by the correlation and regression structure — but the precision statements should be read as analytical, not biological. Second, the design has only four levels of a single factor, so the cubic models are saturated and cannot be validated against an independent point; 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 rather than by plate counts or sequencing, so the microbiological-integrity desirability term rests on visual assessment and on the osmotic-protection mechanism rather than on enumeration. Fourth, only a single time point was sampled, which precludes kinetic modelling of the ethanol maximum; and fifth, no sensory panel and no measurement of gluconic and glucuronic acids were performed, so the composition of the non-acetic pool is inferred rather than demonstrated.

4.9. Future directions

Four experiments follow directly. Quantifying gluconic, glucuronic, lactic and citric acids chromatographically would convert the inferred non-acetic pool into a measured one and test the shunt hypothesis explicitly. A time-course design sampling at days 3, 7, 10, 14 and 21 with a fully replicated, non-composited layout would resolve the ethanol maximum kinetically and provide honest biological variance estimates. Amplicon sequencing of the consortium across the sucrose gradient would test whether osmotolerant yeasts and cellulose-forming *Komagataeibacter* are genuinely enriched at 20% w/v as proposed here[12,20,34]. Finally, characterising the pellicle obtained at each level for crystallinity, water-holding capacity and tensile behaviour would establish whether the extra biomass generated at 20% w/v is of pharmaceutically useful quality, which would strengthen the case for treating kombucha as a dual-product biorefinery rather than a single-product fermentation[40,43].

5. Conclusion

Initial sucrose charge is a genuine critical process parameter for black-tea kombucha and acts on residual ethanol, acetic acid, pH, titratable acidity and biocellulose yield in different and partly opposing directions (all $p < 0.001$; $\eta^2 = 0.685–0.998$). Residual ethanol was biphasic, peaking at 0.341 ± 0.007% w/v at 10% w/v sucrose and remaining 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 strictly linear increase in pellicle thickness. Multi-response desirability optimisation identified 19.1% w/v as the predicted optimum within a practical design space of 15–20% w/v, with 15% preferred when regulatory headroom matters most and 20% when biocellulose recovery is the objective. These findings give Indonesian producers a defensible, instrument-free setpoint for halal-compliant kombucha and support the treatment of kombucha fermentation as a green, dual-product biotechnological process.

Declarations

Ethical statement

This study was conducted using *in vitro* fermentation and instrumental analytical methods and did not involve human subjects or animal experiments; therefore, ethics committee approval was not required.

Author contributions

ENI: conceptualisation, methodology, investigation, formal analysis, data curation, visualisation, writing — original draft. SHY: conceptualisation, methodology, validation, supervision, resources, writing — review and editing. Both authors read and approved the final manuscript.

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Conflict of interest

The authors declare that they have no competing interests.

Data availability

The datasets generated and analysed during the current study, including the replicate-level analytical values and the complete statistical output, are available from the corresponding author on reasonable request.

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