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Modulation of Acidic and Sweet Taste in Reconstituted Tobacco Sheets through Microbial Co-Cultivation and Enzymatic Hydrolysis

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Abstract

Traditional reconstituted tobacco production is often limited by a monotonous flavor profile and insufficient utilization of natural components. Biotechnology, known for its green, efficient processes and capability for targeted metabolic regulation, has emerged as a promising strategy to improve product quality. In this study, a synergistic system combining microbial co-fermentation and enzymatic hydrolysis was developed to modulate the acidic and sweet taste attributes of reconstituted tobacco sheets. A co-culture of *Lactiplantibacillus plantarum* and *Saccharomyces cerevisiae* was applied alongside compound enzymatic treatment using cellulase and pectinase to process various tobacco sheets. In single-strain fermentations, *L. plantarum* significantly enhanced acidity (acid value (a measure of free fatty acid content indicating the level of acidity) increased from −25.56 to −21.36), while *S. cerevisiae* improved sweetness and aroma. However, neither strain alone achieved a well-balanced acid-sweet profile. Enzymatic hydrolysis using a cellulase to pectinase ratio of 2:1 effectively disrupted cell wall structure, promoting the release of soluble sugars and intensifying both acidic and sweet sensory perceptions. The combined application of microbial co-cultivation and enzymatic hydrolysis yielded the most desirable results, driven by mechanisms such as sequential carbon source utilization (*S. cerevisiae* consuming monosaccharides to produce ethanol, while *L. plantarum* metabolized polysaccharides to generate organic acids), enzyme-microbe interactions (system pH aligned with enzyme activity optima), and the co-production of flavor compounds. This approach lowered the acid value to approximately −21.05, increased the sweetness index to 0.19, and enhanced acid-sweet balance, aroma richness, and overall sensory acceptability by 10 - 15 units compared to the untreated controls. The method demonstrated broad applicability across diverse tobacco sheets. These findings highlight the metabolic interplay within microbial consortia and the catalytic role of enzymatic hydrolysis in converting flavor precursors, providing a theoretical foundation for the precise integration of biotechnology into tobacco processing. For industrial implementation, strategies such as sequential dual-strain inoculation, gradient enzymatic hydrolysis, online metabolic monitoring, and immobilized microbial agents are recommended to achieve standardized flavor regulation in reconstituted tobacco products.

Keywords

Microbial Co-Cultivation, Cellulase, Pectinase, Acidic and Sweet Taste Perception, Acid-Sweet Balance, Sensory Acceptability

1. Introduction

Reconstituted tobacco, as a core component in the circular economy of the tobacco industry, enables the reutilization of tobacco waste to improve resource efficiency and reduce tar content. However, traditional manufacturing processes often exhibit limited flavor complexity and insufficient exploitation of natural components, hindering further enhancement of product quality [1]. In recent years, biotechnology, including microbial fermentation and enzymatic treatment, has emerged as a key approach to improving the quality of reconstituted tobacco due to its environmentally friendly nature, high efficiency, and capacity for precise metabolic regulation [2] [3].

Studies on the application of microbial fermentation in cigarette production have shown that lactic acid bacteria can reduce the pH of reconstituted tobacco, thereby enhancing the perception and balance of acidic taste. Meanwhile, yeasts can generate ester aroma compounds (such as ethyl acetate) through sugar metabolism [4] [5]. Both domestic and international researchers have successfully increased lactic acid content in conventional tobacco shreds by 30% - 50% through the selection of high acid-producing strains. However, the synergistic effects and underlying mechanisms of mixed-strain fermentation in reconstituted tobacco remain largely unexplored [4] [6] [7].

In terms of enzymatic modification of tobacco materials, combined treatment with cellulase and pectinase has been reported to disrupt plant cell walls, facilitating the release of soluble sugars and phenolic compounds [8] [9]. Previous studies have shown that enzymatic hydrolysis can increase the soluble sugar content of tobacco extracts by 20% - 40%. However, the application of enzymatic treatment in reconstituted tobacco remains at an early stage, and further investigation is required to optimize enzyme dosages and substrate concentrations for improved efficacy [10].

In the sensory quality of reconstituted tobacco, a balanced acid-sweet profile plays a vital role in masking harshness, improving smoothness, and enhancing consumer acceptance. Therefore, modulating this balance has become a key target in flavor improvement strategies. To address these challenges, this study established a synergistic “microbial fermentation-enzymatic treatment” system aimed at targeted enhancement of acidic and sweet taste perception in reconstituted tobacco. By investigating the effects of strain combinations and enzyme ratios, this work seeks to clarify the metabolic interactions within microbial co-cultures and the catalytic pathways responsible for flavor compound transformation. The findings are expected to provide a theoretical foundation for the precise and standardized application of biotechnology in tobacco processing.

2. Materials and Methods

2.1. Major Reagents and Instruments

All reconstituted tobacco sheet samples (CTS0201T, CTS0231T, CTS0207, CTS0304T, CTS0308, and CTS0205) were supplied by Yunnan Tobacco Industrial Co., Ltd.

Two microbial strains were used: *Lactiplantibacillus plantarum* (JNC003.001) and *Saccharomyces cerevisiae* (JNC001.002), both preserved at the China Center of Industrial Culture Collection and originally isolated by our laboratory. *L. plantarum* was activated in MRS medium at 37˚C for 24 h, and *S. cerevisiae* in YPD medium at 30˚C for 48 h. Analytical-grade reagents were purchased from Sinopharm Chemical Reagent Co., Ltd., including glucose, yeast extract, agar, 3,5-dinitrosalicylic acid, glycerol, metal salts (e.g., copper sulfate, potassium sulfate), chloramphenicol, peptone, hydrochloric acid, copper hydroxide, and anthraquinone.

The following culture media were prepared: Luria-Bertani (LB) liquid (10 g/L NaCl, 5 g/L yeast extract, 10 g/L tryptone) and LB solid (plus 15 - 20 g/L agar), both autoclaved at 121˚C for 20 min. Ampicillin-supplemented LB (50 mg/L for liquid, 100 mg/L for solid) added post-sterilization at 45˚C. Super-Rich (SR) medium (10 g/L tryptone, 3 g/L K_2HPO_4, 30 g/L glucose, 5 g/L yeast extract), sterilized at 115˚C for 30 min. YPD medium (10 g yeast extract, 20 g peptone, 20 g agar in 900 mL water), autoclaved at 121˚C for 20 min and supplemented with 100 mL of sterile 20 g/L glucose. Cellulase (10,000 U/g) and pectinase (5000 U/g) were sourced from Novozymes.

Key instruments included an Insent SA402B electronic tongue (Insent Inc., Japan), a ZQZY-88AN incubator (Shanghai Zhichu Instrument Co., China), an AB 265-S analytical balance (Mettler Toledo, Switzerland), and a KQ 2200B ultrasonic cleaner (Kunshan Ultrasonic Instrument Co., China).

2.2. Microbial Fermentation Regulation of Reconstituted Tobacco

Co-culture system construction: Reconstituted tobacco was inoculated with either

Lactiplantibacillus plantarum, *Saccharomyces cerevisiae*, or a 1:1 (v/v) mixed culture of both strains. The total inoculum volume was 5% (v/v) across all treatments. Cultivation was conducted at 30˚C with shaking at 150 rpm for 48 h.

Preparation of reconstituted tobacco extracts: Samples were ground, passed through a 40-mesh sieve, and extracted with distilled water at a solid-to-liquid ratio of 1:10. The mixture was incubated at 80˚C for 2 h, then centrifuged and filtered to obtain the crude extract.

2.3. Enzymatic Treatment Regulation of Reconstituted Tobacco

Reconstituted tobacco sheets were subjected to enzymatic treatment using either cellulase (10,000 U/g), pectinase (5000 U/g), or a composite mixture of cellulase and pectinase at a mass ratio of 2:1. Enzyme dosages were 50 U/g for cellulase and 30 U/g for pectinase, based on substrate weight. Prior to treatment, tobacco samples were equilibrated at 22 ± 1 ˚C and 60 ± 2% relative humidity for 48 h. Then, 5 g of reconstituted tobacco was evenly distributed on trays, and the appropriately diluted enzyme solutions were uniformly applied using an atomizing spray nozzle. The control group received an equal volume of deionized water. Treated samples were sealed and incubated in a humidity-controlled chamber at 50 ˚C and 60% relative humidity. After 4 h of enzymatic hydrolysis, the reaction was terminated by drying the samples at 80˚C for 30 min. Control treatments included single-enzyme applications (cellulase at 50 U/g or pectinase at 30 U/g), and a blank control with no enzyme added.

2.4. Extraction of Reconstituted Tobacco Taste Analysis Solution and Sensory Measurement

Accurately weighed samples of reconstituted tobacco (including CTS0201T, CTS0231T, CTS0207, CTS0304T, CTS0308, CTS0205 sheets, and their corresponding biotechnologically modified counterparts) were homogenized with deionized water at a ratio of 5 g to 150 mL. The resulting slurry was transferred to centrifuge tubes and centrifuged at 10,000 rpm for 15 min. The supernatant was subsequently filtered twice through Whatman filter paper. A total of 80 mL of the final filtrate was collected for taste analysis and chemical composition determination.

Taste profiling was performed using the Insent SA402B taste analysis system equipped with multiple sensor electrodes, including AAE, CT0, CA0, C00, and Ael. These sensors are designed to detect the five basic taste qualities (umami, salty, sour, bitter, and astringent), as well as aftertaste and richness. Prior to measurement, the system was calibrated and the sensor status was verified to ensure response stability and reproducibility, thereby confirming the validity of the data.

Sample measurements were conducted in accordance with the predefined protocol, with the testing temperature maintained at 20˚C using a circulating water system. Prior to each measurement, the electronic tongue system underwent a cleaning procedure consisting of 90 s each for anode and cathode cleaning, followed by two rinses of 120 s each using the reference solution. The anode cleaning solution contained water, KCl, ethanol, and KOH, while the cathode cleaning solution comprised water, ethanol, and hydrochloric acid. The reference solution, a mixture of KCl and tartaric acid, was formulated to simulate artificial saliva. Following the cleaning cycle, each sample was measured for 30 s, with data recorded at one-second intervals. The sensor reading at the 30th s was taken as the final measurement value. Each sample was analyzed in four consecutive cycles; the first was discarded, and the remaining three were averaged for subsequent analysis. Using the reference solution as a baseline, the system's built-in software converted the electrode potential values into corresponding taste values for further profiling and comparison.

2.5. Data Analysis

Principal component analysis (PCA) was conducted using the Astree system's built-in statistical software to distinguish differences among the samples. Sensory evaluation results and taste response data (expressed as mean ± standard deviation) were analyzed using SAS 8.2 and OriginPro 9.0. Partial least squares (PLS) correlation analysis between taste response values and sensory evaluation scores was performed using Unscrambler version 9.7.

Since the taste values of the test samples were measured relative to a reference solution, which served as the neutral (no-taste) baseline, specific taste thresholds were established accordingly. The reference solution, composed of KCl and tartaric acid, was used to calibrate the baselines for sourness and saltiness. Based on system calibration, the taste threshold was defined as −13 for sourness and −6 for saltiness. In other words, if the measured taste value for sourness or saltiness was below −13 or −6, respectively, the corresponding taste was considered imperceptible; values above these thresholds indicated a perceptible taste. For all other taste attributes, a value of 0 or below was defined as the no-taste point.

3. Results and Discussion

3.1. Effect of Microbial Fermentation Treatment on Reconstituted Tobacco Base

A panel of ten sensory evaluation experts, randomly selected and certified in accordance with ISO 8586:2012, was assembled to assess the reconstituted tobacco samples across five key sensory attributes: sourness intensity, sweetness intensity, acid-sweet balance, aroma richness, and overall acceptability. Evaluations were performed using a 9-point hedonic scale, and the mean values were visualized using radar plots. As shown in Table 1, four types of reconstituted tobacco sheets (CTS0201T, CTS0231T, CTS0207, and CTS0304T) were evaluated under six conditions: untreated (control), treatment with *Lactiplantibacillus plantarum* (LP), *Saccharomyces cerevisiae* (SC), or a combination of both (co-fermentation), in addition to baseline "no-taste" threshold values for comparison. The five sensory dimensions were used to differentiate the sensory responses among the various treatment groups. The control samples across all sheet types exhibited nearly identical, with approximate values of −25 for sourness, −30 for sweetness, −30 for balance, −30 for aroma, and −25 for overall acceptability. These results highlight the extremely poor flavor characteristics of the untreated sheets, indicating minimal sensory impact and a lack of distinctive taste or aroma features.

Table 1. Effects of microbial fermentation treatment on acid-sweet taste perception and overall acceptability of reconstituted tobacco sheets.

Types	Treatments	Sourness	Sweetness	Acid-sweet balance	Aroma richness	Overall acceptability
CTS0201T	control	−25.56	0.07	3.52	3.44	3.51
	LP	−21.36	0.08	3.95	3.98	4.01
	SC	−22.04	0.09	4.02	4.10	4.09
	co-fermentation	−21.10	0.10	4.18	4.21	4.32
CTS0231T	control	−23.73	0.03	3.40	3.42	3.49
	LP	−21.38	0.08	3.19	3.95	4.01
	SC	−21.99	0.09	3.89	3.34	4.05
	co-fermentation	−21.16	0.10	4.02	4.18	4.08
CTS0207	control	−23.51	0.04	3.30	3.41	3.46
	LP	−21.53	0.07	3.76	3.87	3.89
	SC	−22.16	0.08	3.82	3.98	3.96
	co-fermentation	−21.19	0.09	3.97	4.09	4.15
CTS0304T	control	−25.32	0.05	3.2	3.38	3.45
	LP	−23.06	0.08	3.65	3.75	3.85
	SC	−23.80	0.09	3.71	3.86	3.91
	co-fermentation	−22.78	0.11	3.85	3.97	4.13
no-taste		−13	0	0	0	0

In the single-strain microbial treatment groups, LAB significantly enhanced the sourness perception of all reconstituted tobacco sheets, increasing the sourness scores to approximately −15 to −20. This represents a 5 to 10 unit improvement compared to the untreated controls. This effect is attributed to the accumulation of lactic acid, the primary metabolite of LP, which directly contributes to sourness. However, the sweetness and aroma scores remained low, ranging from −25 to −30, indicating that LAB exerted a unidirectional effect primarily on sourness enhancement. In contrast, SC increased the sweetness scores to −20 to −25, also reflecting a relative improvement of 5 to 10 units. A modest enhancement in aroma perception was also observed, with scores rising from −25 to −30 in the control group to approximately −20 to −25. These effects are primarily associated with the formation of ethyl acetate and other esters derived from sugar metabolism by SC, which contribute to fruity and sweet aroma notes. However, the sourness scores remained largely unchanged at around −25 to −30.

Following co-fermentation with both LP and SC, all sensory attributes of the reconstituted tobacco sheets showed marked improvements. Sourness scores increased to approximately −10 to −15, representing a relative enhancement of 10 to 15 units. Similarly, sweetness, acid-sweet balance, aroma richness, and overall acceptability all improved to the range of −15 to −20, each showing an increase of 10 to 15 units compared to the untreated controls. These results suggest that co-fermentation facilitates the concurrent accumulation of lactic acid and ester compounds such as ethyl acetate and ethyl lactate, achieving a balanced and layered flavor profile. Moreover, the sensory profiles of the four tobacco sheet types were highly consistent under co-fermentation treatment, indicating that the synergistic interaction between the two strains produces a robust and broadly applicable optimization effect across different raw materials.

When LP (a lactic acid bacterium, anaerobic/facultative anaerobic) is co-cultured with SC (a yeast, aerobic/facultative anaerobic), three synergistic metabolic interactions can be observed. One of the primary mechanisms involves microenvironmental regulation. At the onset of fermentation, SC rapidly consumes dissolved oxygen, creating a mildly anaerobic environment that favors the growth of LP, resulting in a twofold increase in viable LP cell counts [5] [11]. Simultaneously, the organic acids produced by LP, primarily lactic and acetic acids, lower the pH, thereby inhibiting the growth of undesirable microorganisms such as molds, while also activating esterification pathways in SC that contribute to aroma development. Another important interaction is the complementary utilization of carbon sources [11]. SC preferentially degrades complex carbohydrates such as starch and pectin into ethanol, whereas LC metabolizes simple sugars like glucose and fructose to produce organic acids. During the later stages of fermentation, SC can further utilize these organic acids to synthesize higher-order esters such as ethyl lactate, facilitating a sequential conversion of carbon substrates into key flavor compounds [6]. In addition, co-culture enables the co-activation of enzymatic systems. Proteases secreted by LB hydrolyze proteins into amino acids, which are then utilized by SC for the biosynthesis of aromatic compounds including phenylacetaldehyde and furfural. Meanwhile, amylases secreted by the yeast break down polysaccharides into fermentable sugars, supporting continued acid production by the LP. These interactions form a closed-loop metabolic network that integrates protein degradation, sugar metabolism, and flavor compound formation [7]. Together, these metabolic interactions not only enhance individual flavor attributes but also contribute to an integrated improvement in the acid-sweet balance and aroma complexity of reconstituted tobacco.

3.2. Effect of Enzymatic Treatment on Reconstituted Tobacco Sheets

The effects of enzymatic treatments on acid-sweet taste perception and overall acceptability of reconstituted tobacco sheets are shown in **Table 2**. Four types of tobacco sheets (CTS0201T, CTS0231T, CTS0207, and CTS0304T) were subjected to enzymatic treatment using either cellulase, pectinase, or a composite enzyme mixture (cellulase : pectinase at a mass ratio of 2:1). Untreated controls and established no-taste threshold values were included for comparative analysis. Sensory evaluation was conducted across five dimensions: sourness, sweetness, acid-sweet balance, aroma richness, and overall acceptability, providing a comprehensive assessment of the treatment effects.

Table 2. Effects of enzymatic treatment on acid-sweet taste perception and overall acceptability of reconstituted tobacco sheets.

Types	Treatments	Sourness	Sweetness	Acid-sweet balance	Aroma richness	Overall acceptability
CTS0201T	control	−25.56	0.07	3.52	3.44	3.51
	cellulase	−20.84	0.12	3.97	4.12	4.15
	pectinase	−20.96	0.14	4.03	4.21	4.24
	cellulase:pectinase = 2:1	−20.56	0.15	4.21	4.38	4.37
CTS0231T	control	−23.73	0.03	3.40	3.42	3.49
	cellulase	−20.87	0.13	4.34	4.09	4.12
	pectinase	−20.98	0.14	4.26	4.33	4.40
	cellulase:pectinase = 2:1	−21.11	0.15	4.09	4.31	4.33
CTS0207	control	−23.51	0.04	3.30	3.41	3.46
	cellulase	−21.09	0.11	3.78	4.00	4.02
	pectinase	−21.16	0.13	3.83	4.09	4.11
	cellulase:pectinase = 2:1	−20.79	0.14	4.08	4.26	4.21
CTS0304T	control	−25.32	0.05	3.2	3.38	3.45
	cellulase	−22.50	0.13	3.65	3.88	3.97
	pectinase	−22.63	0.14	3.71	3.97	4.14
	cellulase:pectinase = 2:1	−22.20	0.14	3.87	4.13	4.18
no-taste		−13	0	0	0	0

As shown in Table 2, the sensory values of the untreated controls for all four sheets were highly overlapping, indicating an extremely weak baseline flavor profile with minimal sensory contribution. In the single-enzyme treatment groups, cellulase significantly enhanced sweetness perception, increasing sweetness scores to −20 to −25 (a 5 - 10 unit improvement compared to the control), and slightly improved aroma intensity (from −25 to −30 in the control to −20 to −25). However, it had no notable effect on sourness, which remained in the −25 to −30 range. This outcome is consistent with cellulase's primary function of degrading plant cell walls to release sugars, while exerting limited influence on acid precursors. In contrast, pectinase increased sourness scores to approximately −20 to −25 (a 5 - 10 units improvement), and produced a comparable enhancement in aroma to that observed with cellulase. However, its effect on sweetness was relatively modest. These results align with the enzymatic specificity of pectinase, which mainly targets pectin substrates to release acidic compounds such as galacturonic acid, but has a limited capacity to liberate fermentable sugars.

Following composite enzymatic treatment with cellulase and pectinase at a mass ratio of 2:1, all reconstituted tobacco sheets exhibited notable improvements across all sensory attributes. Moreover, the sensory profiles of the four sheet types in the composite enzymatic group were highly overlapping, further indicating that the synergistic enzyme system exerted a universally applicable optimization effect across diverse raw material substrates.

The composite enzymatic hydrolysis of reconstituted tobacco resulted in the simultaneous enhancement of sourness, sweetness, and aroma through three synergistic mechanisms: (1) coordinated cell wall deconstruction, (2) complementary release of flavor precursors, and (3) enzyme activity compatibility within the substrate environment. Collectively, these mechanisms effectively compensated for the sensory deficiencies of the control sheets [12] [13]. Owing to its broad-spectrum efficacy, this composite enzymatic strategy holds strong potential as a standardized pretreatment approach for reconstituted tobacco processing. Specifically, in terms of cell wall deconstruction, cellulase hydrolyzes the cellulose-hemicellulose matrix, thereby facilitating deeper penetration and action of pectinase on the pectin layer [14]. Subsequent pectin degradation by pectinase exposes cellulose microfibrils, resulting in a layer-by-layer breakdown effect. This synergistic action elevated the cell wall disruption rate from 40% - 50% (observed under single-enzyme treatment) to 70% - 80% with the composite enzyme system, thereby significantly enhancing the release efficiency of intracellular compounds such as sugars, acids, and aroma precursors [15] [16]. Regarding the complementary release of compounds, cellulase primarily liberates reducing sugars (e.g., glucose, fructose) and neutral aroma precursors such as terpene glycosides, while pectinase releases acidic compounds (e.g., galacturonic acid, quinic acid) and acidic aroma precursors including chlorogenic acid. The composite enzymatic treatment led to a 50% - 80% increase in the release of these key compounds, thereby promoting the co-accumulation of sweet, sour, and aromatic components in a balanced manner [17] [18]. In terms of enzymatic compatibility with the reaction environment, cellulase exhibits optimal activity at pH 4.5 - 5.5, while pectinase shows at pH 3.5 - 4.5. The natural pH of tobacco extracts (approximately 5.0 - 5.5) is conducive to the simultaneous function of both enzymes, sustaining over 80% of their maximal activity. This compatibility prevents the enzymatic efficiency loss commonly encountered in single-enzyme systems operating under suboptimal pH conditions [8].

Raw material adaptability tests revealed that compositional differences among the four sheets (CTS0201T, CTS0231T, CTS0207, and CTS0304T), notably in cellulose, pectin, and total sugar content, had minimal impact on the efficacy of the composite enzymatic treatment. These results suggest that the cellulase-pectinase enzymatic system possesses robust capability to degrade a broad spectrum of tobacco cell wall polymers and is highly compatible with diverse structural features of tobacco raw materials. The flavor-deficiency compensation effect was consistently observed across all sheet types, supporting its potential as a standardized pretreatment technology for reconstituted tobacco production.

3.3. Effect of Combined Microbial Co-culture and Composite Enzymatic Treatment on Reconstituted Tobacco Sheets

The effects of combined microbial co-culture and composite enzymatic treatment on reconstituted tobacco sheets are showed in Figure 1. Sensory evaluation was performed for five attributes: sourness, sweetness, acid and sweet balance, aroma richness, and overall acceptability. Four types of base sheets (CTS0201T, CTS0231T, CTS0207, and CTS0304T) were tested under four processing conditions, including an untreated control (UC), enzymatic treatment followed by microbial co-culture (EFM), microbial co-culture followed by enzymatic treatment (MFE), and simultaneous microbial co-culture with enzymatic hydrolysis (SME).

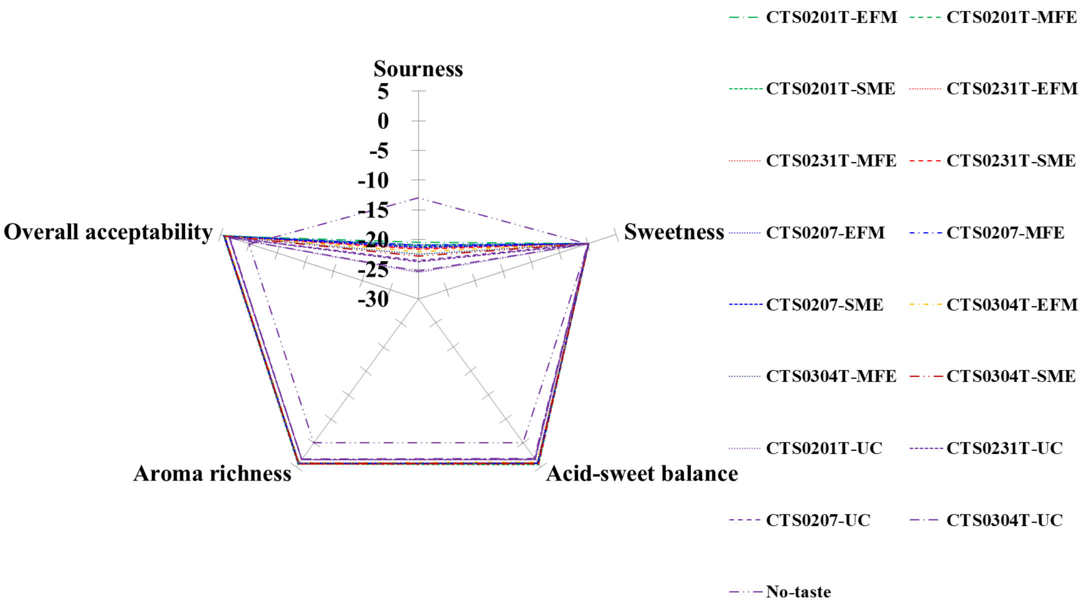

Figure 1. Effects of Microbial Co-culture and Composite Enzymatic Treatment on Taste Attributes of Reconstituted Tobacco.

The results revealed consistent trends across all materials. Sourness restoration was evident. The UC groups exhibited extremely low sourness scores, ranging from −23.51 to −25.56. All three combined processing methods significantly enhanced sourness perception by reducing its absolute value, with the SME treatment showing the most pronounced effect. For example, in CTS0201T, the sourness score improved from −25.56 to −21.05. This was followed by the MFE treatment, while the EFM produced comparatively weaker improvement. These differences can be attributed to the enhanced synergy between microbial acid production and enzyme-promoted release of organic acids during the SME treatment, which promotes more efficient accumulation of sour compounds such as lactic acid and quinic acid. Sweetness perception, initially negligible in the UC samples

(0.03 - 0.07), also increased under all treatment conditions. The SME process yielded the highest sweetness values, with an average of 0.19, followed by MFE (0.17), and EFM (0.16). The increase in sweetness resulted from the release of reducing sugars such as glucose and fructose during enzymatic degradation. The SME process facilitated better preservation and utilization of sweetness-related compounds [17] [19]. Similar improvement patterns were observed for acid-sweet balance, aroma richness, and overall acceptability, all of which reached their highest levels under the SME treatment strategy. These findings suggest that the coordinated microbial and enzymatic synergy provides a comprehensive sensory enhancement effect that is consistently effective across different types of tobacco raw materials.

The values of acid-sweet balance, aroma richness, and overall acceptability in the SME treatment group, ranging from 4.21 to 4.51, were consistently higher than those observed in the stepwise treatment groups. This finding suggests that the SME treatment allows for better coordination among sensory attributes. As a result, a more balanced acid-sweet profile, enhanced aroma richness, and improved overall acceptability were achieved. The synchronization of microbial and enzymatic metabolism appears to be the key factor in optimizing sensory balance [10] [20] [21].

As shown in **Table 3**, the sequence of microbial co-culture and composite enzymatic treatment led to notable differences in metabolic pathways among the three integrated processes, which in turn significantly influenced the sensory outcomes [10]. In the simultaneous treatment group, enzymatic hydrolysis and microbial fermentation occurred concurrently. Microorganisms were able to utilize hydrolysis products such as monosaccharides, polysaccharides and pectins in real time to produce organic acids and esters. This real-time metabolic coupling effectively directed metabolic fluxes toward flavor synthesis, leading to the most pronounced improvements in sourness, sweetness, and aroma. Consequently, this group exhibited the best acid-sweet balance and overall acceptability. In contrast, the MFE treatment group involved initial microbial fermentation, during which readily available carbon sources were consumed to generate acids. However, enzymatic hydrolysis in the later phase primarily acted on more recalcitrant substrates such as polysaccharides. By this time, microbial activity had declined due to substrate depletion and environmental changes, which limited the effectiveness of further flavor compound formation. As a result, although sourness was moderately improved, the enhancement in sweetness and aroma was less pronounced than that observed in the SME treatment group. In the EFM group, hydrolysis initially released large amounts of polysaccharides and pectic substances [22]. During the subsequent microbial fermentation stage, microorganisms were exposed to a high-substrate environment, requiring time to adapt before initiating active metabolism. This process led to moderate improvements in sweetness and aroma but relatively weaker enhancement in sourness compared to the SME treatment [17] [21].

Through process parameter optimization, the SME treatment was identified as the most effective approach for enhancing overall sensory quality. The optimal conditions are as follows: a cellulase-to-pectinase ratio of 2:1, which balances the release rates of sugars and acidic compounds, thereby aligning with microbial metabolic dynamics to maintain acid-sweet balance; an inoculation ratio of Lactobacillus plantarum to Saccharomyces cerevisiae of 1:1, which harmonizes acid production with ester biosynthesis to synergistically enhance sourness and aroma; a treatment temperature of 30˚C, which preserves approximately 50% of enzymatic activity while supporting vigorous microbial growth and metabolism; and a treatment duration of 48 h, allowing sufficient time for both the release of flavor precursors and the subsequent microbial conversion into key flavor compounds, ultimately achieving optimal sensory performance [17] [20] [23].

Table 3. Comparison of metabolic characteristics and sensory performance under different sequences of microbial co-culture and composite enzymatic treatment.

Processing Strategy	Metabolic Characteristics	Sensory Impact
MFE	Microorganisms first consume readily available carbon sources, such as monosaccharides, to produce organic acids. Subsequent enzymatic hydrolysis acts on recalcitrant substrates like polysaccharides, but limited substrate availability at this stage leads to dispersed metabolic fluxes	Moderate enhancement of sourness, but weaker improvements in aroma and sweetness compared with the simultaneous process
EFM	Enzymatic hydrolysis initially releases large amounts of polysaccharides and pectic substances. During the subsequent microbial fermentation, microorganisms require an adaptation period to the high-substrate environment before active metabolism begins	Improvement in sweetness and aroma, but relatively weaker enhancement of sourness compared with the simultaneous process
SME	Enzymatic hydrolysis and fermentation occur concurrently. Microorganisms utilize hydrolysis products, including monosaccharides, polysaccharides, and pectins, in real time to produce organic acids and esters. Metabolic fluxes are concentrated toward flavor synthesis	Simultaneous enhancement of sourness, sweetness, and aroma, with optimal acid-sweet balance and overall acceptability

The SME treatment strategy achieved coordinated enhancement of sourness, sweetness, and aroma through staged carbon source utilization, enzyme-microbe interactions, and the co-synthesis of key flavor compounds. This integrated approach showed clear advantages over stepwise treatments, whether applying MFE or EFM, in terms of achieving a balanced acid-sweet profile, enhancing aroma complexity, and improving overall sensory acceptability. In addition, this process demonstrated strong adaptability to reconstituted tobacco sheets with diverse chemical compositions, providing a robust and reliable method for sensory quality improvement. The strong adaptability of the proposed SME treatment to different tobacco base sheets can be attributed to the widespread occurrence of common substrates like cellulose and pectin across various plant-derived materials. These universal components provide consistent targets for enzymatic hydrolysis and microbial fermentation, thereby enabling robust flavor modulation despite compositional differences among raw materials.

In addition to its sensory advantages, the SME strategy offers notable practical benefits for industrial-scale applications. By integrating microbial fermentation and enzymatic hydrolysis into a single unified process, SME eliminates the need for sequential treatment steps, intermediate transfers, and environmental adjustments. This streamlined workflow reduces total processing time, minimizes energy consumption, and lowers operational costs. Compared with stepwise approaches, SME facilitates more efficient, scalable production and supports consistent flavor modulation, making it a compelling solution for modern tobacco processing.

4. Conclusions

This study demonstrated the effectiveness of integrating microbial co-culture with composite enzymatic treatment to improve the sensory quality of reconstituted tobacco base sheets. The co-culture of *L. plantarum* and *S. cerevisiae* enhanced specific sensory attributes, with *L. plantarum* primarily increasing sourness and *S. cerevisiae* contributing to improvements in sweetness and aroma. Composite enzymatic treatment, using a cellulase-to-pectinase ratio of 2:1, effectively disrupted the cell wall structure of the tobacco matrix. This process increased the release of soluble sugars, which in turn improved both sourness and sweetness.

Among all tested approaches, the simultaneous application of microbial co-culture and composite enzymatic treatment produced the most favorable results. The sourness value decreased to approximately −21.05, and the sweetness value increased to 0.19. Furthermore, acid-sweet balance, aroma richness, and overall acceptability improved by 10 - 15 units compared to the untreated control. This process also exhibited strong adaptability to different types of tobacco base sheets, demonstrating its potential as a robust and practical pretreatment technology for the tobacco industry. Although this study comprehensively evaluated flavor changes using sensory panels and electronic tongue analysis, it did not include quantitative chemical characterization of key flavor compounds. Future research incorporating targeted chemical analyses, such as GC-MS or HPLC, will be essential to deepen the understanding of flavor formation mechanisms and to corroborate the sensory data.

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Conflicts of Interest

The authors declare no conflicts of interest regarding the publication of this paper.

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