JEE Journal of Ecological Engineering

Journal of Ecological Engineering, 2026, 27(1), 165–175
https://doi.org/10.12911/22998993/208795
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Received: 2025.07.24
Accepted: 2025.09.20
Published: 2025.11.25

Integrated microbial co-culture engineering for sustainable valorization of tobacco waste

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ABSTRACT

In this study, we developed an integrated microbial co-culture system involving *Saccharomyces cerevisiae*, *Arthrobacter nicotinovorans*, and the engineered *Escherichia coli* for the multi-metabolic conversion of tobacco waste biomass and simultaneous degradation of nicotine. The co-culture system aims to achieve efficient ethanol synthesis, nicotine degradation, and linalool production. *A. nicotinovorans* exhibited strong nicotine degradation capability, removing nearly all nicotine from 50 g/L tobacco hydrolysate within 96 h, while *S. cerevisiae* achieved up to 13.8 g/L ethanol production in the presence of 50 g/L tobacco hydrolysate with exogenous glucose. Besides, the engineered *E. coli* demonstrated the capacity to produce linalool, yielding a titer of 0.65 g/L in 50 g/L tobacco hydrolysate with exogenous glycerol. Co-fermentation of these three strains at a 1:3:1 ratio enabled efficient multi-pathway conversion, achieving 0.17 g/L linalool production, 8.3 g/L ethanol, and near-complete nicotine degradation at 10 g/L tobacco hydrolysate, highlighting the impact of inoculation ratio and substrate concentration on metabolic performance. The co-culture system enabled efficient sugar utilization, effective nicotine detoxification, and the production of valuable chemicals, presenting a sustainable strategy for the comprehensive valorization of tobacco waste.

Keywords: tobacco waste valorization, co-culture fermentation, nicotine degradation, ethanol, linalool.

INTRODUCTION

Tobacco (*Nicotiana tabacum*) is a globally cultivated economic crop, generating substantial waste during cultivation and processing, including agricultural residues (e.g., stalks, low-grade leaves) and processing by-products like tobacco dust. In China, over 1 million tons of tobacco leaf and stalk waste are produced annually (Lin et al., 2024). Improper disposal, such as landfilling or incineration, leads to resource wastage and environmental pollution, impacting soil structure, water quality, and air quality through the release of nicotine and other harmful compounds (Beutel et al., 2021). Current valorization strategies, such as chemical extraction or microbial nicotine degradation, often target single components, neglecting the comprehensive utilization of tobacco biomass. Ethanol fermentation using microbes like *Saccharomyces cerevisiae* is feasible (Martín et al., 2002), but yields low economic returns compared to established feedstocks like corn stover and generates secondary waste streams (e.g., wastewater, exhaust gases) that require further treatment. Thus, there is a pressing need for innovative, integrated bioprocessing technologies to achieve sustainable, low-carbon valorization of tobacco waste, aligning with global circular bioeconomy objectives.

A major challenge in tobacco waste valorization is nicotine, a stable and toxic alkaloid that inhibits microbial activity. Studies show that nicotine concentrations could significantly affect the microbial activity and function (Ruan and Liu, 2015). The U.S. Environmental Protection Agency classifies pure nicotine as a hazardous waste (EPA Code P075), necessitating stringent disposal measures. This toxicity hinders enzymatic

hydrolysis and fermentation efficiency, posing a critical bottleneck for biological resource recovery. Traditional pre-treatments, such as physical or chemical methods, often fail to simultaneously address nicotine degradation and comprehensive biomass utilization, resulting in suboptimal resource recovery and persistent environmental risks. Recent research emphasizes the hazards of nicotine contamination and the potential of nicotine-degrading microbes for remediation, the use of microbes to reduce nicotine content and improve tobacco quality has become a new thought (Li et al., 2024).

Microbial co-culture systems or enzymatic–microbial one-pot strategies offer a promising solution by harnessing the synergistic metabolic capabilities of multiple strains or biotransformation steps to process complex substrates, such as tobacco waste. These approaches have been demonstrated as efficient methods for upcycling lignocellulosic-derived waste (Shahab et al., 2020) as well as chitin-containing biomass (Ma et al., 2020). Unlike single-strain systems, co-cultures enable division of labor, where different strains handle specific tasks (e.g., detoxification, substrate utilization, or product synthesis), enhancing overall process efficiency (Jones and Wang, 2018). For instance, *S. cerevisiae* is well-established for ethanol production from lignocellulosic hydrolysates but is sensitive to nicotine toxicity (Ye et al., 2021). In contrast, *Arthrobacter nicotinovorans* efficiently degrades nicotine into non-toxic intermediates like 2,6-dihydroxypyridine, mitigating its inhibitory effects (Brandsch, 2006). Recent advances in microbial engineering have further enabled the development of microbial consortia to produce high-value compounds, such as terpenoids, from waste biomass (Cao and Xian, 2024). Co-culture systems have been successfully applied in lignocellulosic biomass conversion, with studies demonstrating improved sugar utilization and product yields through optimized strain ratios and substrate concentrations (Unrean and Khajeeram, 2015). However, their application to tobacco waste, which combines high sugar content with toxic alkaloids, remains underexplored, highlighting the need for tailored co-culture designs.

The bioproduction of high-value aroma compounds from agricultural residues is gaining momentum as part of the bio-based economy (Ma et al., 2022b). Linalool, a monoterpene alcohol, is a high-value compound widely used in the fragrance, flavor, and pharmaceutical industries due to its pleasant floral aroma and bioactive properties (Liu et al., 2021). Traditional linalool production relies on either plant extraction or chemical synthesis, both of which are costly and environmentally intensive. Microbial synthesis of linalool, particularly through engineered *Escherichia coli* strains, offers a sustainable alternative (Ma et al., 2022a). By introducing plant-derived terpene synthase genes (e.g., linalool synthase) and optimizing the mevalonate (MVA) or methylerythritol phosphate (MEP) pathways, engineered *E. coli* can produce monoterpene alcohol (e.g., linalool) from simple sugars (Yang et al., 2024). Integrating linalool-producing *E. coli* into a co-culture system with nicotine-degrading and ethanol-producing strains enables simultaneous detoxification and valorization of tobacco waste, maximizing resource recovery. However, challenges such as metabolic burden, strain compatibility, and byproduct inhibition must be addressed to optimize co-culture performance.

This study develops an integrated microbial co-culture system combining *S. cerevisiae* (ethanol production), *A. nicotinovorans* (nicotine degradation), and an engineered *E. coli* (tMTScLS, linalool production) to achieve multi-pathway valorization of tobacco waste (Figure 1). Under optimized conditions, 100 g/L acid-hydrolyzed tobacco leaf waste yielded 20 g/L total sugars including glucose plus xylose and 0.86 g/L initial nicotine. Inoculation ratios (e.g., 1:3:1 and 3:1:1) significantly influenced performance, with the 1:3:1 ratio enabling near-complete nicotine degradation and 0.17 g/L linalool at 10 g/L hydrolysate, while the 3:1:1 ratio achieved 52% nicotine degradation at 25 g/L hydrolysate. This work provides a sustainable microbial platform for the comprehensive valorization of tobacco waste, contributing to the advancement of integrated biorefinery and circular bioeconomy practices.

MATERIALS AND METHODS

Chemicals and strains

Tobacco dust samples were provided by the Technology Center of Shanghai Tobacco Group Co., Ltd. L-(−)-nicotine (N3876) was purchased from Sigma-Aldrich, USA. LB broth medium (A507002) was purchased from Sangon Biotech (Shanghai) Co., Ltd. Glucose (G116303), xylose

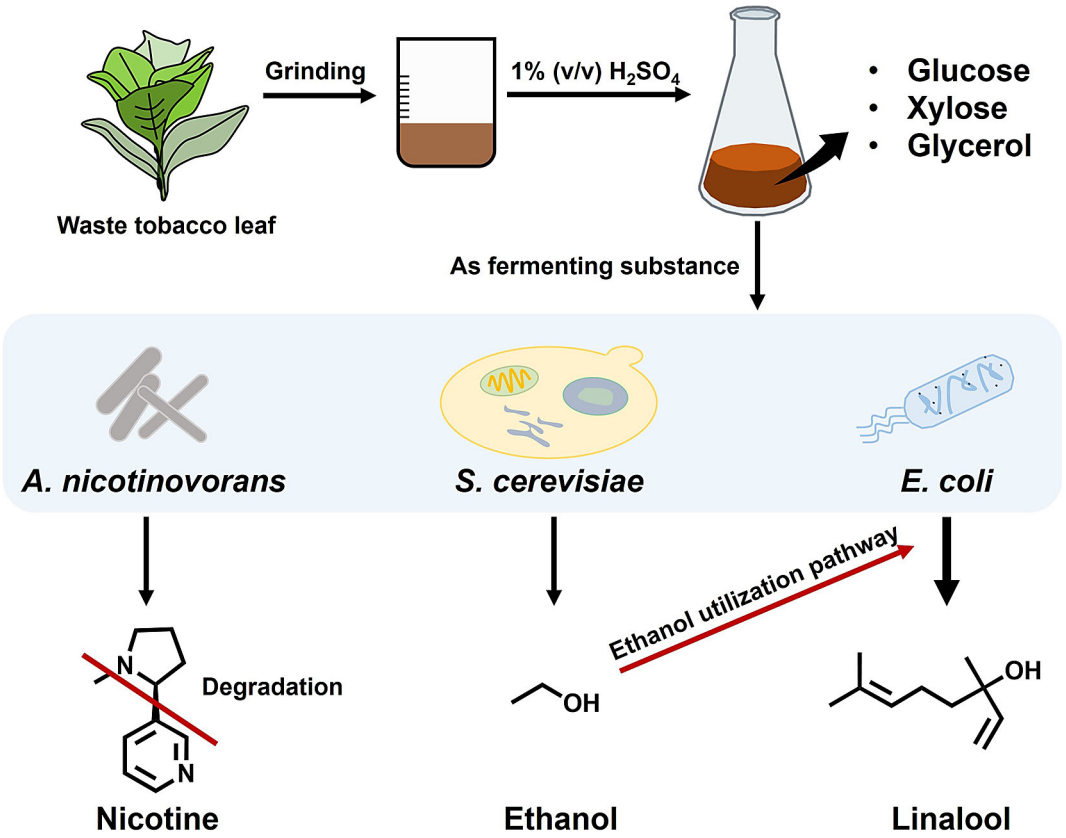

Figure 1. Design of the multi-functional microbial co-culture platform for converting tobacco waste into value-added products. Tobacco leaves were subjected to acid hydrolysis to produce a sugar- and nicotine-rich hydrolysate. The co-culture system integrates three specialized strains: *A. nicotinovorans* for nicotine degradation, *S. cerevisiae* for ethanol production, and engineered *E. coli* for linalool biosynthesis. This system enables simultaneous detoxification and biosynthesis of functional metabolites from tobacco biomass

(X101009), sodium chloride (C111547), ethanol (E111994), hexadecane (H6703) and chloroform (C434286) were purchased from Aladdin (Shanghai Aladdin Biochemical Technology Co., Ltd., China). Agarose (A8201) was purchased from Solarbio Technology Co., Ltd., Beijing. Sulfuric acid (95–98%, 10021608) was from Sinopharm Chemical Reagent Co., Ltd., China. Yeast extract (LP0021B) and tryptone (LP0042B) were purchased from Oxoid (Thermo Fisher Scientific Inc., USA). *E. coli* DH5α (TSC-C14, Beijing Tsingke Biotechnology Co., Ltd., China) was used to propagate recombinant plasmids. *A. nicotinovorans* BMZ014532 was purchased from Mingzhou Biotechnology Co., Ltd., China.

Tobacco waste pre-treatment and hydrolysate preparation

Tobacco leaf waste samples were air-dried, pulverized, and stored in a desiccator. For acid hydrolysis, 10 g of tobacco leaf waste was mixed with 100 mL of ultra-pure water to a final concentration of 100 g/L. Concentrated H_2SO_4 (1%, v/v) was added, followed by autoclaving at 121°C for 20 min. After cooling, the pH was adjusted to 7.0 with concentrated NaOH (400 g/L). The mixture was then centrifuged at 9000 rpm, and the supernatant was collected as Tobacco Acid Hydrolysate for medium preparation. Utilizing acid-hydrolyzed tobacco leaf waste processed under the optimized conditions yielded a maximum total sugar content of 20 g/L (including glucose and xylose) from 100 g/L hydrolysates and an initial nicotine concentration of 0.86 g/L.

Culture media preparation

YPD Liquid Medium: 10 g/L yeast extract, 20 g/L tryptone, 20 g/L glucose. YPD Solid Medium: YPD liquid medium with 20 g/L agar powder. LB Liquid Medium: 5 g/L yeast extract, 10 g/L tryptone, 10 g/L sodium chloride. LB Solid Medium: LB liquid medium with 20 g/L agar powder.

Tobacco acid hydrolysate medium: 10 g/L yeast extract, 20 g/L tryptone, 50 g/L acid hydrolysate supernatant. Tobacco acid hydrolysate glucose medium: Tobacco acid hydrolysate medium with 40 g/L glucose. Tobacco acid hydrolysate medium for tMTScLS: 10 g/L tryptone, 5 g/L yeast extract, 13.3 g/L KH_2PO_4, 4 g/L $(NH_4)_2HPO_4$, 10 g/L or 25 g/L acid hydrolysate supernatant with varying addition of carbon source (no exogenous carbon source addition, exogenous 10 g/L glucose addition and exogenous 10 g/L glycerol addition). Optimized Co-culture Fermentation Medium: 10 g/L yeast extract, 20 g/L tryptone, 10 g/L glycerol, 40 g/L glucose, 13.3 g/L KH_2PO_4 and 4 g/L $(NH_4)_2HPO_4$ with varying acid hydrolysate concentrations (10 g/L or 25 g/L) based on optimization. All media for fermentation experiments were prepared in 2 mL culture volume, with sterile water added to reach the final volume.

Plasmid and strain construction

The linalool-producing strain tMTScLS was constructed by co-transforming two plasmids, pMVA1 and pScLS, into *E. coli* MG1655(DE3) *ΔrecA ΔendA ΔtnaA* (Yang et al., 2024). Plasmid pMVA1 (SpecR+pAC) contains genes for the mevalonate (MVA) pathway enzymes, including *EcatoB*, *Schmgs*, *Schmgr*, *ScMK*, *ScPMK*, *ScPMD*, and *EcIDI*, controlled by T7 and tac promoters. Plasmid pScLS (AmpR+pMB1) carries the genes coding the enzymes for linalool biosynthesis, including *trAgGPPS*, *ScLS*, *DpADA*, and *ScADH2*, controlled by T7, pLuxB, and pGyrA promoters, respectively. Target genes were amplified via PCR, verified by agarose gel electrophoresis, and assembled into the backbone plasmids. The construction of the plasmids in this study was based on the GT DNA assembly standard (Ma et al., 2019). The standard electroporation protocol was used to transform the plasmid into *E. coli* DH5α. The recombinant plasmids were transformed into *E. coli* by electroporation. All plasmid, strain, and gene information is summarized in Tables 1–2.

Fermentation experiments

For single-strain fermentations, individual colonies of *S. cerevisiae* and *A. nicotinovorans* were inoculated from solid media into 1 mL liquid media (YPD for *S. cerevisiae*, LB for *A. nicotinovorans*) and cultured overnight at 30 °C, 250 rpm to prepare seed cultures. For *S. cerevisiae* ethanol production, 40 µL of seed culture was transferred to 2 mL tobacco acid hydrolysate medium or tobacco acid hydrolysate glucose medium, fermented at 30 °C, 250 rpm for 96 h. For *A. nicotinovorans* nicotine degradation, 40 µL of seed culture was transferred to 2 mL tobacco acid hydrolysate medium, fermented in a shaker at 30 °C for 48 h, 72 h, and 96 h, respectively. For linalool biosynthesis, seed cultures were inoculated at 2% (v/v) into 2 mL tobacco acid hydrolysate medium for tMTScLS with different carbon sources (no exogenous carbon source, 10 g/L glucose, 10 g/L glycerol) and tobacco hydrolysate concentrations (10 g/L, 25 g/L). Fermentation was conducted at 37 °C, 250 rpm for 3–4 h, when the OD_{600} reached about 1.0, the inducer (0.05 mM of isopropyl-β-D-thiopyran galactoside (IPTG) and 0.01 µM N-3-oxy-hexanoyl hyperserine lactone (3OC6-HSL) were added, and 20% (v/v) hexadecane was added to capture the produced linalool. Finally,

Table 1. Information of the plasmids used in this study

Name	Genotype of plasmid	Purpose in this study
pMVA1	SpecR-pAC-LacI-pT7_RBS1_EcatoB_RBS2stop_Sahmgs_RBS4stop_Sahmgr-rrnBT-ptrc_RBS1_Scmk_RBS2stop_Scpmk_RBS4stop_Scpmd_RBS3stop_Ecidi-T7T	IPP and DMAPP production
pScLS	AmpR-pMB1-LacI-pT7_RBS1_trAgGGPS2-T7T-pLuxB_RBS1_ScLs-L3S2P21T-LuxR-pGyrA_RBS1_DpADA_RBS2stop_ScADH2-T7T	Linalool production

Table 2. Information of the strains used in this study

Name	Strain genotype	Plasmid 1	Plasmid 2	Purpose
S. cerevisiae	*S. cerevisiae* BY4741, *MATa his3Δ1 leu2Δ0 met15Δ0 ura3Δ0*	n.a.	n.a.	Ethanol production
A. nicotinovorans	*A. nicotinovorans* BMZ014532	n.a.	n.a.	Nicotine degradation
tMTScLS	*E. coli* MG1655 *ΔrecA ΔendA ΔtnaA*	pMVA1	pScLS	Linalool production

the test tube containing the 2 mL fermentation broth was placed on a shaker at 30 °C and 250 rpm for 48 h during two-phase fermentation.

For the Co-culture system, seed cultures of *S. cerevisiae*, *A. nicotinovorans*, and tMTScLS were prepared, respectively. The inoculation ratios of *A. nicotinovorans*, *S. cerevisiae* and tMTScLS were set at 1:1:1, 1:1:2, 1:1:3, 1:2:1, 1:3:1, 2:1:1 and 3:1:1, to investigate the effects of different inoculation ratios on the fermentation results. The overnight culture seeds of the them were respectively inoculated into the optimized co-culture fermentation medium. The total inoculation volume of the three strains was 40 μL (2%, v/v) according to the designed ratio. They were cultured on a shaker at 30 °C until the OD_{600} reached 1.0. Then 0.05 mM IPTG and 0.01 μM 3OC6-HSL were simultaneously added as inducers, and 20% (v/v) hexadecane was added. Then it was transferred to a shaker at 30 °C, 250 rpm for two-phase fermentation for 96 h.

Analytical methods

The high performance liquid chromatography (HPLC) analysis was performed using a system of LC-20A (Shimadzu) equipped with an Aminex HPX-87H Column (300 × 7.8 mm, Bio-Rad) and Refractive Index Detector. One milliliter of the supernatant obtained at sampling time points was used for glycerol and other byproducts concentration quantification. The mobile phase is 5 mM H_2SO_4, the flow rate is 0.7 mL/min, and the column temperature is set at 40 °C. Commercially available glycerol, glucose, xylose, ethanol and acetate were used as standards.

The gas chromatography-mass spectrometry (GC-MS) analysis was processed by 7890B-5977B from Agilent Technologies, Santa Clara, CA, USA, with the chromatographic column was Agilent HP-5MS (30 m × 0.25 mm × 0.25 μm). After fermentation, 500 μL of the fermentation broth was mixed 500 μL of chloroform to detect nicotine. Then, it was placed in a heated constant-temperature mixer and shake at 800 rpm and 30 °C to extract nicotine (for the detection of linalool, the upper organic phase was collected directly). After centrifugation at 13.000 rpm for 5 min, one microliter of supernatant was injected into the GC in split 1:10 mode (for linalool, 10 μL supernatant was mixed with 90 μL ethyl acetate). The heating program as followed: Initial temperature was 50 °C, maintained for 1 min; Then increased the temperature to 100 °C at 5 °C/min and held for 1 min; increased to 200 °C at 5 °C/min and held for 1 min. Finally Raised to 300 °C at 5 °C/min and held for 1 min. The mass spectrometry scanning range is 10–500 m/z.

The GC analysis was performed using GC-9720Plus (Zhejiang Fuli Analytical Instruments Co., Ltd, China), and the column was HP-5MS Capillary Column (30 m × 0.25 mm × 0.25 μm, Agilent Technologies, CA, USA). The method for sample preparation is the same as that of GC-MS. When detecting the samples, two microliters of supernatant were injected into the GC. The oven temperature was programmed to be maintained at the initial temperature of 50 °C for 2 min and then increased to 250 °C at a rate of 15 °C/min, held for 7 min.

RESULTS AND DISCUSSION

Ethanol production by *Saccharomyces cerevisiae*

The ethanol production capacity of *S. cerevisiae* was assessed using 50 g/L acid-hydrolyzed waste tobacco leaf supernatant, with and without the addition of 40 g/L exogenous glucose. The results revealed a clear dependence on substrate composition. In the absence of added glucose, the ethanol yield was limited to 1.4 g/L (Figure 2a), reflecting *S. cerevisiae*'s ability to metabolize endogenous sugars such as glucose and xylose released during tobacco biomass pretreatment. However, the low yield also suggests that inhibitory compounds—particularly residual nicotine and phenolics—may impair sugar uptake and metabolic efficiency. By contrast, supplementation with 40 g/L glucose significantly increased ethanol production to 13.8 g/L (Figure 2a), indicating that *S. cerevisiae* preferentially utilizes readily fermentable glucose instead of xylose (Figure 2b) and that an external carbon supply effectively overcomes bottlenecks imposed by the complex composition of the hydrolysate. Although ethanol can be produced directly from tobacco hydrolysate without supplementation, the limited yield suggests the challenges associated with valorizing lignocellulosic waste containing fermentation inhibitors. These findings highlight the crucial role of carbon source availability in *S. cerevisiae*'s ability to produce ethanol in nicotine-containing medium, thereby facilitating the utilization of tobacco-derived biomass.

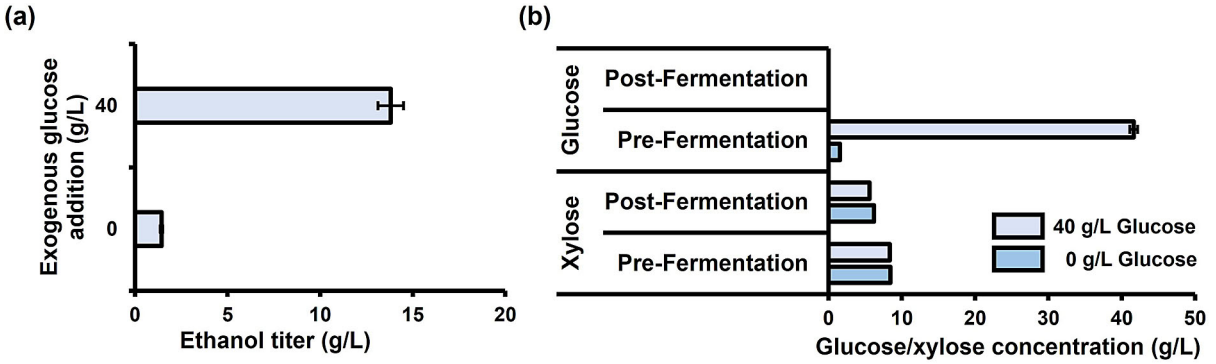

Figure 2. Impact of exogenous glucose addition on ethanol fermentation by *S. cerevisiae* in tobacco acid hydrolysate. (a) Ethanol titers under two conditions: without glucose and with 40 g/L glucose supplementation in 50 g/L hydrolysate medium. (b) Composition of residual glucose and xylose, alongside ethanol titers, after 96 h fermentation. Glucose addition significantly enhanced ethanol production by *S. cerevisiae*, indicating preferential utilization of hexose sugars in the hydrolysate matrix. Error bars represent standard deviations from biological triplicates

Nicotine degradation by *Arthrobacter nicotinovorans*

A. nicotinovorans exhibited highly efficient nicotine degradation capabilities, as evidenced by the time-course analysis in the fermentation experiments. When inoculated into tobacco acid hydrolysate medium containing an initial nicotine concentration of approximately 430 mg/L in 50 g/L waste tobacco leaf acid hydrolysis supernatant (8.5 g/L xylose and 1.5 g/L glucose), the nicotine content displayed a significant decreasing trend over time, with levels dropping progressively from 48 h to undetectable (<0.01 mg/L) after a 96-h fermentation period (Figure 3a, b). This rapid detoxification demonstrates the robust metabolic potential of *A. nicotinovorans* to degrade nicotine present in tobacco waste hydrolysate through the pyridine pathway, converting it into non-toxic intermediates such as 2, 6-dihydroxypyridine and succinic acid, which can be further assimilated or excreted without inhibiting downstream processes. This step is crucial for enabling subsequent microbial valorization, as residual nicotine could otherwise suppress the activity of sensitive strains like *S. cerevisiae* and engineered *E. coli*. Recent studies have advanced nicotine degradation strategies, and achieved up to 97.56% efficiency in tobacco waste fermentation (Yuan et al., 2005). For instance, insights into microbial pathways in tobacco-associated bacteria reveal hybrid pyridine-pyrrolidine routes that enhance degradation rates under aerobic conditions (Fitzpatrick, 2018). Additionally, biochar-immobilized nicotine-degrading strains have shown improved performance in soil remediation (Zhu et al., 2025). Fungal approaches, such as *Trametes versicolor*, have also demonstrated degradation in diverse media, including natural tobacco extracts, emphasizing the versatility of microbial systems for waste detoxification (Su et al., 2016). Overall, the efficient degradation obtained by *A. nicotinovorans* would be core strain to mitigate nicotine's environmental impact, paving the way for integrated co-culture processes.

Linalool production by engineered *E. coli*

The engineered *E. coli* strain tMTScLS, harboring the genes coding the MVA pathway enzymes (Yang et al., 2024), linalool biosynthetic enzyme, and ethanol utilization enzymes (Liang et al., 2021), demonstrated robust linalool production under optimized conditions. Specifically, upon induction with 0.05 mM IPTG and 0.01 μM 3OC6-HSL in an optimized fermentation medium, the strain achieved a maximum titer of approximately 0.65 g/L (Figure 4a), with GC-MS analysis confirming product identity through comparison with an authentic standard (Figure 4b, c). When cultured in acid-hydrolyzed waste tobacco leaf medium, linalool biosynthesis was significantly affected by both the concentration of hydrolysate and the availability of external carbon sources. At 10 g/L hydrolysate containing endogenous sugars (0.3 g/L glucose and 2.7 g/L xylose), the addition of 10 g/L glycerol enabled production of up to 0.65 g/L linalool (Figure 4a). In contrast, cultures adding with glucose or without exogenous carbon source produced very low

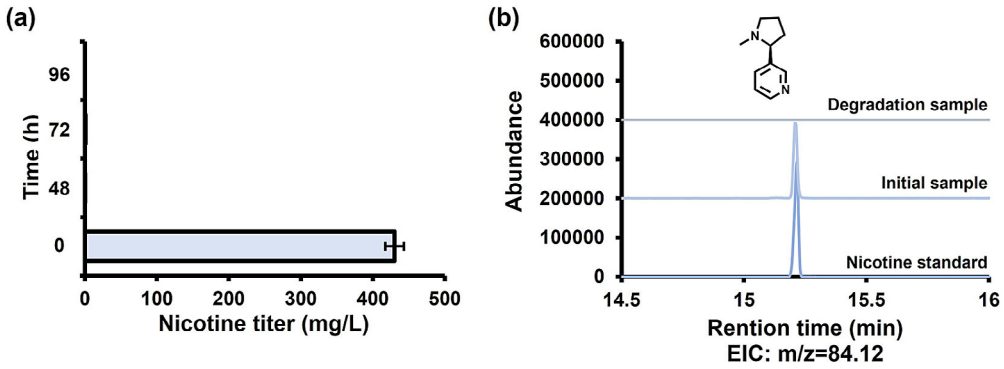

Figure 3. Nicotine degradation profile of *A. nicotinovorans* in tobacco hydrolysate. (a) Time-dependent nicotine removal in 50 g/L acid hydrolysate medium over a 96-hour fermentation period. The initial nicotine concentration (~430 mg/L) decreased to undetectable levels (<0.01 mg/L) by 96 h. (b) GC-MS analysis of fermentation supernatants at various time points. The nicotine peak (retention time ~8.9 min) was progressively diminished, confirming biological degradation. The chromatogram at 0 h corresponds to the untreated control, while later time points represent post-fermentation samples. Error bars represent the standard deviation of biological triplicates

concentration of linalool, indicating the critical role of readily metabolizable carbon sources like glycerol in supporting monoterpene biosynthesis as demonstrated in previous study (Yang et al., 2024). However, increasing the hydrolysate concentration to 25 g/L led to uniformly low linalool titers regardless of carbon supplementation, likely due to elevated nicotine levels (~0.22 g/L), which are known to impair microbial growth and reduce pathway flux. This observation highlights the necessity of both feedstock optimization and detoxification to enable efficient valorization of tobacco waste.

Comparable studies have reported higher linalool titers (e.g., 4.16 g/L in 2-L fed-batch bioreactor cultivation) using biodiesel-derived glycerol as substrate via a balanced MVA pathway, which mitigates metabolic burden and improves flux distribution (Wang et al., 2023). Moreover, although linalool production was not the primary focus, prior research demonstrated its antimicrobial activity against *E. coli* biofilms, with concentrations was 4 μL/mL, no viable *E. coli* cells were observed (Wang et al., 2025), providing useful insights into potential toxicity thresholds during strain engineering. Comparative host studies have shown that alternative platforms such as *Rhodotorula toruloides* can achieve titers of 2.59 g/L in bioreactors (Zheng et al., 2025), suggesting that further enhancement of tMTScLS—through strategies such as CRISPR-mediated engineering for nicotine tolerance and redox cofactor regeneration – could substantially improve performance. Collectively, these findings support the feasibility of using engineered *E. coli* as a sustainable microbial chassis for monoterpene biosynthesis from tobacco-derived feedstocks, providing the basis for setting up a co-culture system to upcycle waste biomass into value-added chemicals.

Integrated multi-metabolic conversion in co-culture system

Following the functional evaluation of each module strain in acid-hydrolyzed tobacco waste medium, an integrated co-culture system comprising *S. cerevisiae*, *A. nicotinovorans*, and engineered *E. coli* tMTScLS was established to enable simultaneous ethanol fermentation, nicotine degradation, and linalool biosynthesis. The system's performance was strongly influenced by inoculation ratios and substrate concentrations. At a tobacco hydrolysate concentration of 10 g/L, the inoculation ratio of 1:3:1 (*A. nicotinovorans: S. cerevisiae: E. coli*) exhibited optimal synergy, achieving 8.3 g/L ethanol, 0.17 g/L linalool, and over 69% nicotine degradation (Figure 5a). This demonstrates a well-coordinated division of metabolic labor, wherein *A. nicotinovorans* efficiently detoxified the medium – primarily by degrading nicotine – thereby alleviating the inhibitory effects on both *S. cerevisiae* and tMTScLS. As a result, both ethanol and linalool production were significantly enhanced. However, when the hydrolysate concentration increased to 25 g/L, the same co-culture system with a 3:1:1 inoculation ratio exhibited a marked performance drop. Nicotine degradation was reduced to 52%, and both ethanol and linalool titers declined sharply (Figure 5b). This reduction is attributed to the elevated

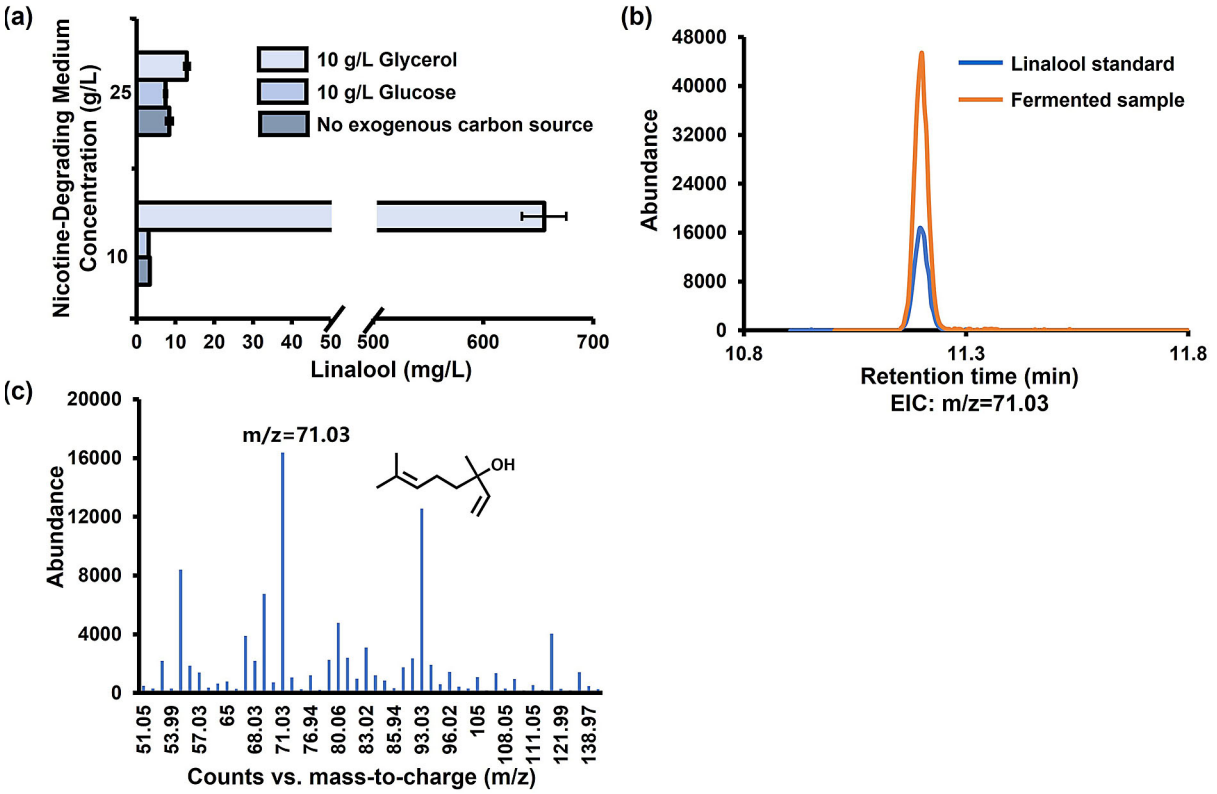

Figure 4. Linalool production by engineered *E. coli* tMTScLS in tobacco hydrolysate-based media under different carbon sources. (a) Linalool titers at varying hydrolysate concentrations (10 g/L and 25 g/L) and carbon supplementation (no addition, 10 g/L glucose, or 10 g/L glycerol). (b) GC-MS chromatograms of fermentation extract compared with authentic linalool standard. (c) GC-MS fragmentation spectrum confirming linalool identity based on NIST database matching. Error bars represent the standard deviation of biological triplicates

levels of inhibitors – such as nicotine and phenolic compounds – that exceeded the detoxification capacity of *A. nicotinovorans*, highlighting the system's sensitivity to substrate toxicity at higher concentrations. Across various tested inoculation ratios (e.g., 1:1:1 to 3:1:1), increasing the proportion of *A. nicotinovorans* enhanced nicotine removal but introduced carbon source competition, which adversely affected ethanol and linalool yields. In contrast, more balanced ratios, particularly 1:3:1, supported coordinated multi-functionality, enabling efficient carbon utilization, robust detoxification, and high-value metabolite production. The enhanced performance of the co-culture system likely stems from synergistic interactions, including cross-feeding and mutual detoxification, which help overcome the limitations observed in monoculture fermentations.

These findings are consistent with recent advances in microbial co-culture strategies for waste valorization, such as converting food residues into biofuels and organic acids (Dharmalingam et al., 2023). For example, the co-culture of *Trichoderma reesei* and *Corynebacterium glutamicum* achieved a 95% cellulose-to-glucose conversion, providing a model for lignocellulosic bioprocessing (Singh et al., 2022). Similarly, engineered consortia have been developed for producing flavonoids, terpenoids, and other high-value compounds from agro-industrial residues (Gulsunoglu-Konuskan and Kilic-Akyilmaz, 2022), and more recently for the microbial upcycling of plastic waste into biodegradable polymers (Cao et al., 2022). By enabling the concurrent synthesis of multiple value-added products from a complex and inhibitory feedstock, this co-culture platform offers a blueprint for tobacco waste biorefinery. It represents a meaningful advancement toward synthetic biology-enabled circular economy models, addressing both environmental pollution and sustainable chemical production. Future efforts should focus on integrating dynamic control strategies and real-time monitoring systems to maintain optimal strain ratios and metabolic balance, particularly in large-scale bioreactor operations. In this system, *A. nicotinovorans* initiates detoxification by degrading nicotine, thereby alleviating the inhibitory effect of nicotine on both *S. cerevisiae* and engineered *E. coli*.

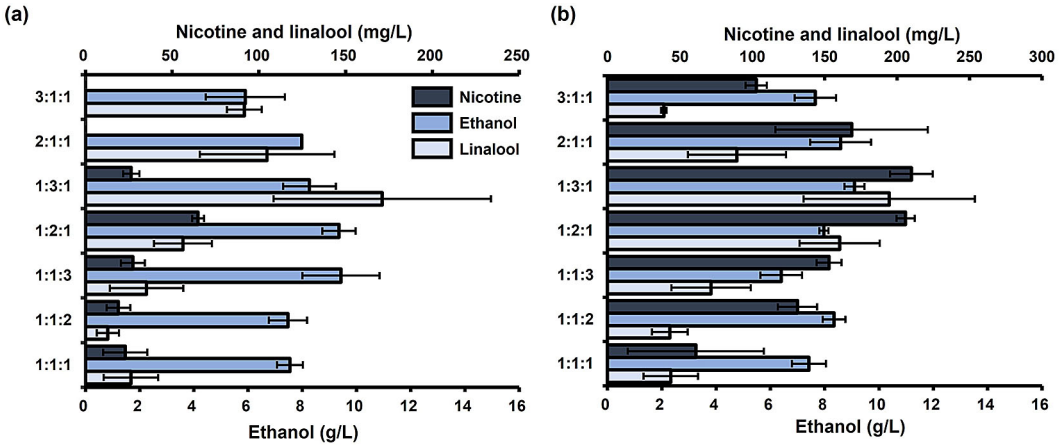

Figure 5. Co-culture fermentation performance under different inoculation ratios (*A. nicotinovorans: S. cerevisiae: E. coli*) at (a) 10 g/L and (b) 25 g/L tobacco hydrolysate concentrations. The titers of ethanol and linalool, along with the residual nicotine content, were measured after 96 h fermentation. Error bars represent the standard deviation of biological triplicates

S. cerevisiae ferments available sugars to produce ethanol, a potential intermediate substrate for monoterpene biosynthesis, while the engineered *E. coli* (tMTScLS) utilizes glycerol and ethanol to synthesize linalool. This coordinated metabolic division of labor allows for cross-feeding and mutual support, where detoxification by *A. nicotinovorans* facilitates sugar metabolism and monoterpene synthesis, and the presence of *S. cerevisiae* enhances carbon flow efficiency. The co-culture system thus mimics a synthetic symbiotic relationship where each strain contributes a distinct metabolic function toward comprehensive tobacco waste valorization. Although the co-culture system enabled the high-production of ethanol, the utilization rate of ethanol by engineered *E. coli* remains limited due to possible bottlenecks in ethanol uptake and metabolic flux toward monoterpene biosynthesis. To address this, future studies may focus on enhancing *E. coli* ethanol assimilation efficiency by overexpressing or engineering key genes involved in ethanol uptake and conversion (e.g., *adhE*, or ethanol-utilizing modules from *Zymomonas mobilis* (Cardona and Sánchez, 2007). Additionally, adaptive laboratory evolution (ALE) or CRISPR-based regulatory tuning could be applied to improve ethanol tolerance and redox balance, enabling more efficient channeling of ethanol-derived carbon into the mevalonate pathways for terpenoid production. Dynamic control strategies, such as quorum sensing-based regulation or metabolite-responsive promoters, may also help to optimize timing and flux distribution between growth and production phases in future scaled-up systems.

CONCLUSIONS

In this study, an integrated microbial co-culture system was successfully developed to achieve the sustainable valorization of tobacco waste biomass through the simultaneous ethanol production, nicotine degradation, and linalool biosynthesis. *S. cerevisiae* exhibited efficient ethanol production, particularly under glucose-supplemented conditions. The engineered *E. coli* tMTScLS demonstrated the ability to produce linalool from tobacco hydrolysate, while *A. nicotinovorans* effectively degraded nicotine, alleviating its inhibitory effects on microbial metabolism. The co-culture platform enabled coordinated metabolic interactions among the three strains, enhancing substrate utilization and mitigating toxicity. The optimized system achieved near-complete nicotine removal and high titers of ethanol and linalool under defined fermentation conditions. Although linalool was chosen as a proof-of-concept monoterpene, the modular design of the engineered *E. coli* chassis allows for facile substitution of biosynthetic genes to produce a wide range of terpenoids or flavor compounds, including ionone, geraniol, and methyl salicylate—many of which are widely used in the tobacco and fragrance industries. Moreover, the established detoxification and sugar fermentation modules (*A. nicotinovorans* and *S. cerevisiae*) are compatible with other synthetic biology pathways, enabling the production of polyphenols, organic acids, or even nicotine analogs for pharmaceutical research. This flexible, plug-and-play co-culture strategy provides a versatile platform

for value-added biomanufacturing from tobacco biomass. And this work presents a versatile and modular strategy for the bioconversion of complex and inhibitory waste streams, offering a new route for tobacco waste biorefinery. Further scale-up and process control strategies may expand its applicability in industrial biomanufacturing and contribute to the development of a circular bioeconomy.

Acknowledgments

This work was supported by the Open Research Project of Shanghai Tobacco Group (2023) and Project of China Tobacco Corporation (110202202037). We sincerely thank Dr. Xiaoqiang Ma from the Department of Food Science and Technology, Shanghai Jiao Tong University, Shanghai, for his generous assistance, funding support, and provision of experimental resources, as well as his constructive insights during the project. We also thank Prof. Fengqing Wang from East China University of Science and Technology for kindly providing the strain of *S. cerevisiae* BY4741.

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