Microbial Valorization of Lignin to Malic Acid by *Aspergillus niger*

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To be submitted to Bioresource Technology for special issues on Lignin Valorization

Keywords

Lignin valorization; *Aspergillus niger*; Malic acid; Aromatic acids; Transporter engineering

Highlights

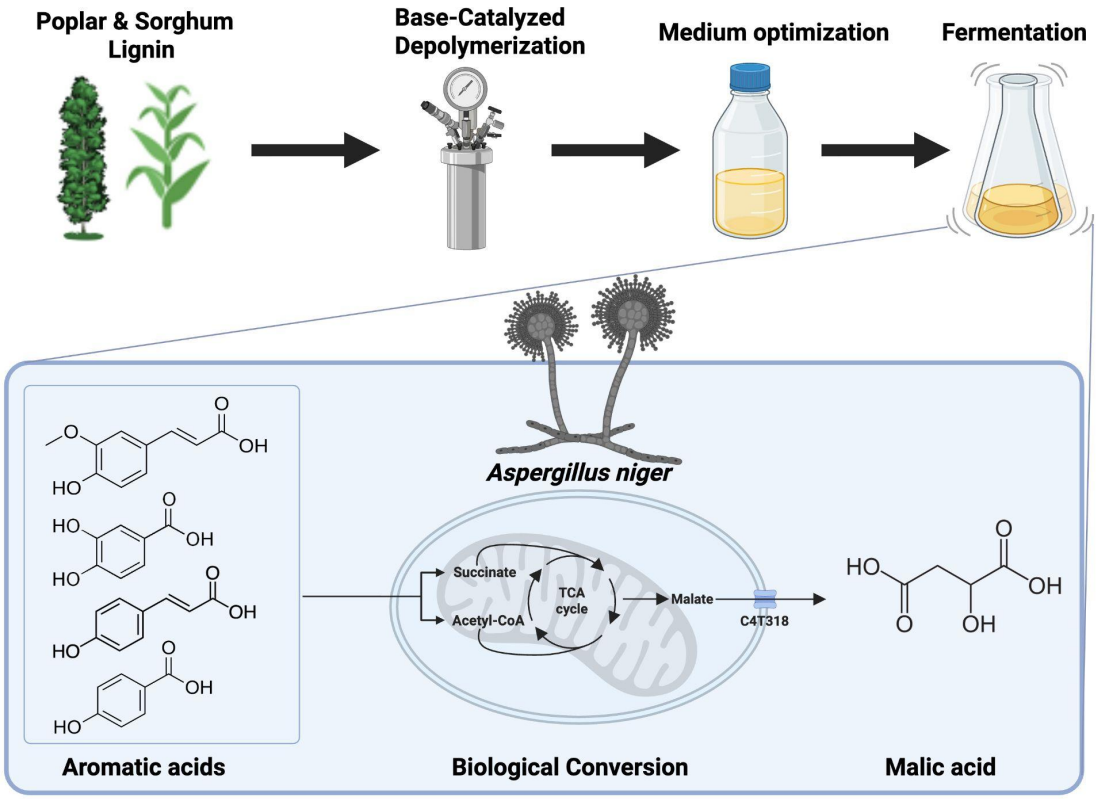
Engineered *Aspergillus niger* strains converts lignin-derived aromatics to malic acid

Overexpression of C4 dicarboxylate transporter enables efficient malic acid secretion

Medium optimization enhances fungal conversion and production stability

Demonstrates first direct fungal conversion of real lignin streams to malic acid

Graphical abstract



Abstract

Lignin represents the largest renewable source of aromatic carbon but remains underutilized due to its structural heterogeneity and recalcitrance. In this study, *Aspergillus niger* was engineered to enable the bioconversion of lignin-derived aromatic acids and base-catalyzed depolymerized (BCD) lignin streams into malic acid, a value added C4 dicarboxylic acid with broad industrial relevance. Overexpression of the C4 dicarboxylate transporter *C4T318* facilitated efficient malic acid secretion, while medium optimization further enhanced production under buffered conditions. The engineered strain exhibited robust assimilation of representative lignin-derived aromatics, including 4-hydroxybenzoic acid and *p*-coumaric acid, and maintained consistent malic acid synthesis across both individual and mixed substrate fermentations. Conversion of BCD lignin liquors from poplar and sorghum confirmed effective utilization of heterogeneous aromatic mixtures and stable fermentation performance. Comparable carbon conversion efficiencies were observed between hardwood and grass lignin streams, indicating the strain's adaptability to diverse feedstocks. This work represents the first demonstration of direct fungal conversion of real lignin streams into malic acid and establishes *A. niger* as a robust microbial platform for sustainable lignin valorization. The integration of transporter engineering with process optimization provides a promising strategy for advancing biological lignin conversion and expanding the role of filamentous fungi in renewable bioproduct synthesis within future biorefineries.

1. Introduction

Lignin is an abundant aromatic biopolymer that constitutes 15–40% of lignocellulosic biomass and represents the largest renewable source of aromatic carbon on Earth (Chu and Majumdar, 2012; Ragauskas et al., 2014; Zhang, 2020). It is estimated that there are 360 billion tons of lignin resources in nature currently (Becker and Wittmann, 2019). Each year, approximately 50–70 million tons of lignin are generated as byproducts of the pulp and paper industry, yet the majority is underutilized and commonly burned for low-value heat and power (Beaucamp et al., 2022; R.-Y. Liu et al., 2024). The intrinsic recalcitrance and structural heterogeneity of lignin have posed major barriers to its valorization, limiting its integration into biorefinery schemes (Rinaldi et al., 2016; Sun et al., 2018; Zakzeski et al., 2010). Developing efficient and economically viable routes for lignin conversion is therefore essential not only to enhance the profitability of lignocellulosic biorefineries but also to realize the sustainable production of value-added chemicals from renewable aromatic feedstocks (Chen et al., 2023; Li et al., 2024).

Biological conversion represents a promising approach to lignin valorization because of its selectivity, mild operating conditions, and ability to funnel diverse aromatic compounds into common intermediates (Becker and Wittmann, 2015; Kamimura et al., 2017; Linger et al., 2014; Liu et al., 2022). Considerable progress has been made in bacterial hosts: for example, *Pseudomonas putida* has been engineered to produce β-ketoadipic acid (Werner et al., 2023), cis, cis-muconic acid (H. Liu et al., 2024), and itaconic acid (Elmore et al., 2021) from depolymerized lignin; *Corynebacterium glutamicum* has been used to generate pterostilbene, kaempferol, quercetin (Kallscheuer et al., 2017) and cis,cis-muconic acid (Becker et al., 2018); and

Rhodococcus opacus has been employed to convert lignin-derived aromatics into lipids (Zhao et al., 2024). These studies demonstrate the feasibility of microbial funneling, but most efforts have focused on bacterial systems, which often have limited acid tolerance and secretion capacity (Lee et al., 2019; Zhou et al., 2025).

Filamentous fungi offer complementary advantages for lignin valorization, including robustness, inhibitor tolerance, and a long industrial track record in organic acid production (Mäkelä et al., 2020; Umashankar and Nygård, 2024; Upton et al., 2017). Notably, *Aspergillus* exhibits several advantages as a microbial cell factory for the production of organic acids compared to other microorganisms. Its high biotransformation capability and metabolic flexibility enable it to undergo various bioconversion processes using multiple carbon sources for fermentation, thereby enhancing its diversity and versatility in biotechnological application (Parshikov et al., 2015). Additionally, *Aspergillus* demonstrates strong environmental adaptability, with a broad tolerance to pH and temperature variations, allowing it to thrive in diverse conditions and facilitating effective organic acid production under varying environmental circumstances (Upton et al., 2017; Wu et al., 2025). *Aspergillus niger* in particular is widely used for citric acid fermentation at million-ton scales and is also capable of producing 3-hydroxypropionic, oxalic, gluconic, itaconic, and malic acids (Adakawa et al., 2025; Dai et al., 2023; Papagianni, 2007).

Malic acid is a four-carbon dicarboxylic acid that has broad application in the food, pharmaceutical, and household chemical industries (Chi et al., 2016; Kövilein et al., 2020; Wu et al., 2022). It serves multiple roles including acidulant, flavor enhancer, antioxidant, detergent, and deodorizer (Marques et al., 2020; Mondala, 2015). Owing to

its versatility, malic acid has been listed by the U.S. Department of Energy as one of the twelve top value-added platform chemicals derived from biomass, with global demand projected to exceed 200,000 tons annually (Werpy and Petersen, 2004). At present, malic acid is primarily produced by chemical synthesis or enzymatic conversion of petroleum derived fumaric acid, yielding either racemic mixtures or enantiopure malic acid (Kövilein et al., 2020; Liu et al., 2017). These processes, however, suffer from high operating costs, harsh reaction conditions, and environmental concerns, which constrain their industrial scalability (Liu et al., 2017; Quitmann et al., 2014). Microbial fermentation offers a renewable and environmentally responsible alternative, enabling the production of malic acid from sustainable feedstocks such as lignocellulose (Wu et al., 2022; Xi et al., 2023). Although most microbial routes rely on glucose as the carbon source, expanding substrate utilization to lignin-derived aromatics can improve process integration and enhance the economic feasibility of lignocellulosic biomass conversion by incorporating lignin valorization into the production framework.

In this study, *A. niger* was genetically engineered to produce malic acid from lignin-derived substrates by eliminating oxalic acid formation through deletion of *oahA* and enabling efficient secretion via overexpression of the C4-dicarboxylate transporter C4T318, in combination with medium optimization. The engineered strain was tested on model lignin-derived aromatics, 4-hydroxybenzoic acid (4HBA) and p-coumaric acid (p-CA), as well as on base-catalyzed depolymerized (BCD) lignin stream from poplar and sorghum, and malic acid production was demonstrated. To our knowledge, this represents the first demonstration of a fungal platform directly converting real lignin

streams into malic acid, thereby advancing strategies for integrating lignin utilization into sustainable biorefineries.

2. Materials and Methods

2.1 Chemicals, strains and media

All chemicals were purchased from Sigma-Aldrich if not otherwise stated (Sigma-Aldrich, St Louis, MO, USA). The complete medium (CM) contained (per litter): 10 g glucose, 2 g trypticase peptone, 5 g yeast extract, 1 g casamino acid, 50 mL 20 × NO_3 salts, 1 mL of 1000 × trace elements, and 1 mL of 1000 × vitamin stock. The pH was adjusted to 6.5 with 1 M NaOH. The 20 × NO_3 salts solution contained (per litter): 120 g Na_2NO_3, 10.4 g KCl, 10.4 g $MgSO_4.7H_2O$, and 30.4 g KH_2PO_4. The 1000 × vitamin stock solution contained (per 100 mL): 0.01 g biotin, 0.01 g pyridoxine-HCl, 0.01 g thiamine-HCl, 0.01 g riboflavin, 0.01 g para-aminobenzoic acid, and 0.01 g nicotinic acid. The vitamin stock solution was filtered and stored at 4 °C. The 1000 × trace element solution contained (per 100 mL): 2.2 g $ZnSO_4{\cdot}7H_2O$, 1.1 g H_3BO_3, 0.5 g $MnCl_2{\cdot}4H_2O$, 0.5 g $FeSO_4{\cdot}7H_2O$, 0.17 g $CoCl_2{\cdot}6H_2O$, 0.16 g $CuSO_4{\cdot}5H_2O$, 0.15 g $Na_2MoO_4{\cdot}2H_2O$, and 5 g Na_2EDTA. The trace element constituents were added in the listed order and mixed. Then the pH was adjusted to 6.5 with KOH and brought to 100 mL. The trace elements stock solution was filtered and stored at 4 °C. The minimal medium (MM) contained (per litter) of 50 mL 20 × NO_3 salts, 1 mL 1000 × trace elements, and 1 mL 1% thiamine. The pH was adjusted to 6.5 with 1 M NaOH.

Escherichia coli strain DH5α was employed for routine plasmid DNA preparation. *Aspergillus niger* (ATCC 11414), obtained from the American Type Culture Collection

(Rockville, MD, USA), was cultivated on CM agar plates at 30 °C for strain maintenance and spore generation. Spores were collected by rinsing the plates with 5–10 mL of sterile 0.4% (v/v) Tween 80 solution (polyoxyethylene sorbitan monooleate).

Phenotypic assays were conducted on MM supplemented with glucose or individual aromatic compounds as the sole carbon source. Each plate was inoculated with approximately 10^3 spores in a 2 µL suspension. Because of differences in compound toxicity, distinct substrate concentrations were used for growth profiling: 2 mM for ferulic acid, 3 mM for benzoic acid, and 5 mM for all other aromatic compounds.

The toxicity of aromatic acids was evaluated by examining their effects on *A. niger* growth. Approximately 1×10^8 spores were inoculated into CM in 250 mL Erlenmeyer flasks and cultivated overnight at 30 °C with shaking at 200 rpm. The resulting mycelia were collected by filtration through Miracloth and washed thoroughly with sterile water. The washed mycelia were then transferred into MM supplemented with 1.25 - 20 g/L of the respective aromatic acid in 250 mL Erlenmeyer flasks and incubated at 30 °C and 200 rpm for 48 h to assess growth inhibition.

2.2 Genetic engineering

The CRISPR/Cas9 plasmid pGY47, designed for targeted disruption of the *oahA* gene, was constructed from the Cas9 backbone pGY18 (Yuan et al., 2024). The empty vector pGY18 (Addgene plasmid #221708) was first linearized with BsaI, and pGY47 was assembled by ligating the linearized backbone with three gBlocks (gBlock_65, gBlock_66, and gBlock_56) using BsaI-based Golden Gate Assembly. Assembly was performed according to the manufacturer’s instructions for the NEBridge® Golden Gate

Assembly Kit (BsaI-HF®v2) (New England BioLabs, Ipswich, MA, USA). Two Guide RNAs (gRNAs) targeting *oahA* were designed using the online tool CHOPCHOP (Labun et al., 2019). All gRNAs and gBlocks used in this study were provided in Table S1 and S2.

Overexpression constructs were generated using Gibson Assembly following the manufacturer's protocol (Gibson Assembly Master Mix, New England BioLabs, Ipswich, MA, USA). For overexpression, the *gpdA* promoter (*gpdAp*), *C4T318* (C4 dicarboxylate transporter gene), hygromycin B phosphotransferase (*hph*) selection marker, and *trpC* terminator (*trpCt*) were assembled into a pBluescript SK(−) backbone linearized with HindIII and PstI. Synthetic oligos used for construct are described in Table S3. The resulting plasmid was linearized with BssHII and introduced into *A. niger* through chemical mediated protoplast transformation as previously described (Dai et al., 2013). All gBlocks Gene Fragments and synthetic oligonucleotides used for construct assembly were synthesized by Integrated DNA Technologies (IDT, Coralville, IA, USA). Cloning PCR reactions were performed using Q5 High Fidelity DNA Polymerase Master Mix (New England BioLabs, Ipswich, MA, USA) according to the manufacturer's instructions. Transformants were isolated, purified by single spore plating, and verified by PCR analysis.

2.3 Fermentation for malic acid production and process optimization

Two media were employed for different experimental objectives. The optimization experiments for malic acid production were conducted using a defined fermentation medium (FM) prepared according to the formulation reported by Brown et al. (Brown et al., 2013), with modifications to the carbon source and neutralizing agent. The FM

contained 10 g/L aromatic acids, 5 g/L neutralizing agent, 6 g/L Bacto Peptone, 150 mg/L KH_2PO_4, 150 mg/L K_2HPO_4, 100 mg/L $MgSO_4 \cdot 7H_2O$, 100 mg/L $CaCl_2 \cdot 2H_2O$, 5 mg/L $FeSO_4 \cdot 7H_2O$, and 5 mg/L NaCl. The effects of different neutralizing agents (MES, $CaCO_3$, $NaHCO_3$, and K_2CO_3), initial pH (3.0 – 8.0), and neutralizing-agent concentration (5 – 90 g/L) were systematically evaluated to determine their influence on malic acid production. In parallel, the same experimental design was applied to fermentations containing 1 g/L $CaCO_3$ in both FM and MM to compare substrate utilization behaviors under lignin stream. Approximately 1×10^8 spores of *A. niger* were inoculated into 250 mL Erlenmeyer flasks containing CM and cultured overnight at 30 °C with shaking at 200 rpm. Mycelia were harvested by filtration through Miracloth, rinsed with sterile water, and transferred into the respective fermentation media (FM or MM + $CaCO_3$) under identical incubation conditions for 72 h.

2.4 BCD of lignin

Base-catalyzed depolymerization (BCD) reactions were conducted using a 1 L Parr batch reactor system. The lignin samples (hybrid poplar lignin and sorghum lignin) were pretreated using an ionic liquid protocol previously published (Choudhary et al., 2023). For each BCD experiment, 250 mL of a 5 wt.% NaOH aqueous solution (prepared with deionized water) and 15 g of lignin substrate were introduced into a 1 L type 316 stainless steel reactor vessel. The reactor was sealed and stirred at 200 rpm using a paddle stirrer with flat blades mounted on a rotating shaft. The system was then heated to 120 °C over a period of 45 minutes, at which point the reaction time was considered to begin (t = 0 min). The reaction was maintained at 120 °C for 30 minutes before being cooled to room temperature using the reactor's internal cooling coil system. Once

cooled, the reactor was opened and the reaction mixture was transferred into a 1 L centrifuge tube. The sample was centrifuged at 4000 rpm and 4 °C for 30 minutes. The supernatant was then collected, the pH was adjusted to 6.5, and it was stored as the BCD liquor.

2.5 Flux balance analysis

The theoretical maximum yield of malic acid from lignin-derived aromatic compounds was calculated using Flux Balance Analysis (FBA). The genome-scale metabolic model of *A. niger* iJB1325 (Brandl et al., 2018) was used for the analysis. The maximum malic acid production from 4HBA or p-CA as a sole carbon source and from a 1:1 mixture by weight was predicted by FBA. In addition, the genome-scale metabolic model was used to predict the metabolic flux distribution in *A. niger* producing malic acid from a 1:1 mixture of 4HBA and p-CA. The parsimonious FBA from cobrapy (Ebrahim et al., 2013) was used to generate flux values and Escher (King et al., 2015) was used to build a metabolic map for flux visualization.

2.6 Analytical methods

Aromatic compounds were analyzed using the method followed. The samples were analyzed on a 1260 Infinity II (Agilent, Santa Clara, CA, USA) equipped with an Eclipse Plus Phenyl-Hexyl column (250 mm length, 2.6 mm diameter, 5 µm particle, Agilent Technologies, 95990-912), and a UV detector. Two mobile phases were used, 10 mM ammonium acetate with 0.07% formic acid in water (Solvent A) and 10 mM ammonium acetate with 0.07% formic acid in 90% acetonitrile (Solvent B). The profile mixture was as follows: 30% Solvent B, 0.5 mL min^{-1} for 12 min, 80% Solvent B, 0.5 mL min^{-1} for 0.1

min, 100% Solvent B, 0.5 mL min^{-1} for 0.5 min, 100% Solvent B, 1.0 mL min^{-1} for 0.2 min, and 30% Solvent B, 1.0 mL min^{-1} for 2.8 min. The column temperature was kept at 50 °C. Aromatic acids concentrations are compared against analytical standards using 254, 280, and 310 nm spectral profiles.

Malic acid titers were quantified with an Agilent Technologies 1200 series HPLC system equipped with an Aminex HPX-87H column (BioRad Laboratories, Hercules, CA, USA), with Waters 2489 UV/Visible detector collecting signal at 210 nm, kept at 50 °C during analysis. 4 mM sulfuric acid was used as a mobile phase with a flow rate of 0.55 mL min^{-1}. Prior to analysis, samples were filtered through 0.45 µm polypropylene filter plate (Agilent, Santa Clara, CA, USA) and 5 µL sample injection volumes were used. Calibration curves were built for the quantified compounds of interest using linear regression and used to determine the concentration in the analyzed samples.

3. Results and Discussion

3.1 Establishing *A. niger* for aromatic acid utilization

To identify suitable carbon sources for supporting bioprocess applications in *A. niger*, the growth potential on different lignin-derived aromatic acids was first examined. These compounds represent potential monomers present in depolymerized lignin streams and were assessed as sole carbon sources in minimal medium (MM). Growth assays on MM plates demonstrated that *A. niger* can utilize a variety of lignin-derived aromatic acids as sole carbon sources (Fig. 1). Robust colony formation was observed on 4-hydroxybenzoic acid (4-HBA), p-coumaric acid (p-CA), and protocatechuic acid (PCA), indicating efficient assimilation of these substrates. In contrast, relatively weak growth

was detected on ferulic acid (FA), syringic acid (SA), vanillic acid (VA), and benzoic acid (BA). Although growth on these substrates exceeded the MM control, their performance was markedly inferior to that on 4-HBA, p-CA, or PCA, suggesting restricted metabolic accessibility.

These growth patterns align with previous studies showing that *A. niger* preferentially metabolizes non-methoxylated aromatic monomers, which are readily funneled through the protocatechuate and β-ketoadipate pathways (Lubbers, 2025; Martínková et al., 2023). Weak utilization of syringic acid is consistent with its recalcitrance, as double methoxylation hinders demethylation and ring cleavage in fungi lacking specialized O-demethylases (Margesin et al., 2022; Perez et al., 2021). Similarly, limited growth on vanillic and benzoic acids reflects known metabolic bottlenecks, requiring either additional enzymatic activation or co-metabolism with more accessible substrates.

Substrate tolerance was further examined in liquid cultures with increasing concentrations of 4-HBA and p-CA (Fig. S1). *A. niger* efficiently consumed both substrates at concentrations up to 10 g/L, supporting sustained growth and complete aromatic acid depletion within 48 h. However, at 20 g/L, both 4-HBA and p-CA strongly inhibited growth, and substrate utilization was negligible. The inhibition mirrors prior observations across fungi and bacteria where excessive aromatic loads impair membrane integrity and trigger oxidative stress (Perez et al., 2021; Zhu et al., 2023). Notably, *A. niger* tolerated higher aromatic concentrations than other model organisms such as *Saccharomyces cerevisiae* (Yaguchi et al., 2021), which require engineering to resist aromatic toxicity. Such robustness enhances the suitability of *A. niger* as a host for lignin valorization.

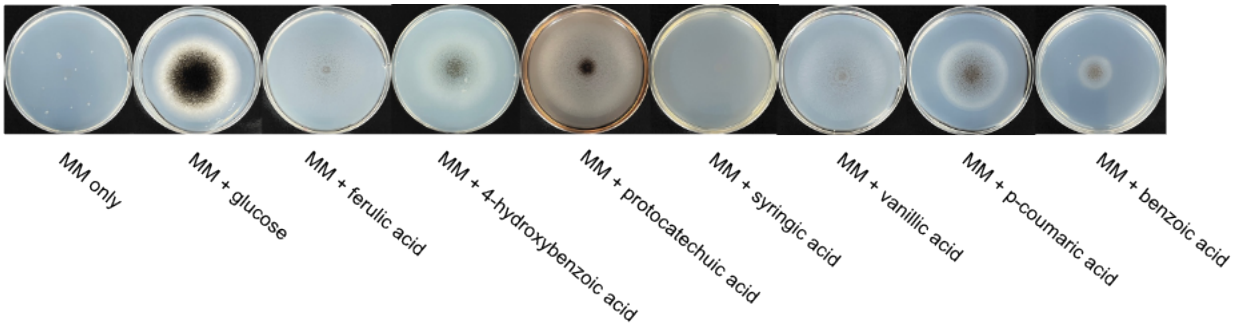


Fig. 1. Growth profile of the *A. niger* wild-type strain on minimal medium (MM) agar plates supplemented with selected aromatic compounds. “MM only” refers to a minimal medium without any carbon sources. Agar plates were incubated at 30 °C for 7 days.

3.2 Redirecting carbon flux and enabling malic acid secretion

A. niger possesses versatile aromatic catabolic pathways that allow conversion of lignin-derived monomers into central metabolic intermediates. A wide range of substrates are funneled into protocatechuate, which serves as a hub metabolite in aromatic degradation (Lubbers, 2025). p-CA are degraded through a CoA-dependent β-oxidative pathway, whereas 4-HBA is more directly converted into PCA. Once funneled into central metabolism, these intermediates connect to the tricarboxylic acid (TCA) cycle via acetyl-CoA and succinyl-CoA, where oxaloacetate emerges as a key branching point for organic acid biosynthesis (Fig. 2). As illustrated in Fig. 2, oxalate can be reduced to malate by malate dehydrogenase (*mdh*) or hydrolyzed to oxalate via oxaloacetate acetylhydrolase (*OahA*). *A. niger* predominantly produces citric acid under acidic conditions (pH < 2.5), whereas at near-neutral pH (6.0), oxalic acid and malic acid become the main organic acids (Niu et al., 2015). Because both pathways share oxaloacetate as a common precursor, disruption of oxalate formation is predicted to

redirect carbon flux toward malate. The genome of *A. niger* contains a single copy of the *oahA* gene, which therefore represents a key engineering target for eliminating oxalate as a competing byproduct (Andersen et al., 2011).

Cultivation of wild-type *A. niger* with 4-HBA or p-CA confirmed that oxalic acid was the predominant extracellular metabolite (Fig. 3A). Cultivation with 4-HBA and p-CA resulted in oxalic acid accumulation of 1.26 g/L and 0.57 g/L, respectively, establishing oxalic acid as the primary product from aromatic acid utilization. Deletion of *OahA* in strain JJ47 completely abolished oxalic acid secretion (Fig. 3B). However, in the absence of *OahA*, malic acid did not accumulate extracellularly, indicating that although flux into oxalate was preserved, secretion of malic acid was strongly limited.

Transport efficiency has previously been recognized as a critical bottleneck in organic acid production by filamentous fungi (Li et al., 2025). Overexpression of *C4T318* encoding a C4-dicarboxylic acid transporter in *A. oryzae* led to a twofold increase in malic acid secretion (Brown et al., 2013), highlighting the importance of efflux mechanisms. To test whether transport capacity limits malate secretion, the C4 dicarboxylate transporter gene *C4T318* was overexpressed in both the *ΔoahA* background (JJ147) and the wild-type background (JJ1). In minimal medium cultures both engineered strains secreted detectable but low levels of malic acid, 0.33 g/L for JJ147 and 0.35 g/L for JJ1 (Fig. 3C). These results demonstrate that transporter expression enables malate export, while *oahA* deletion eliminates oxalate formation but does not enhance secretion efficiency. The low titers under minimal medium conditions suggest that environmental factors such as pH buffering and nutrient composition may play a major role in determining malic acid productivity.

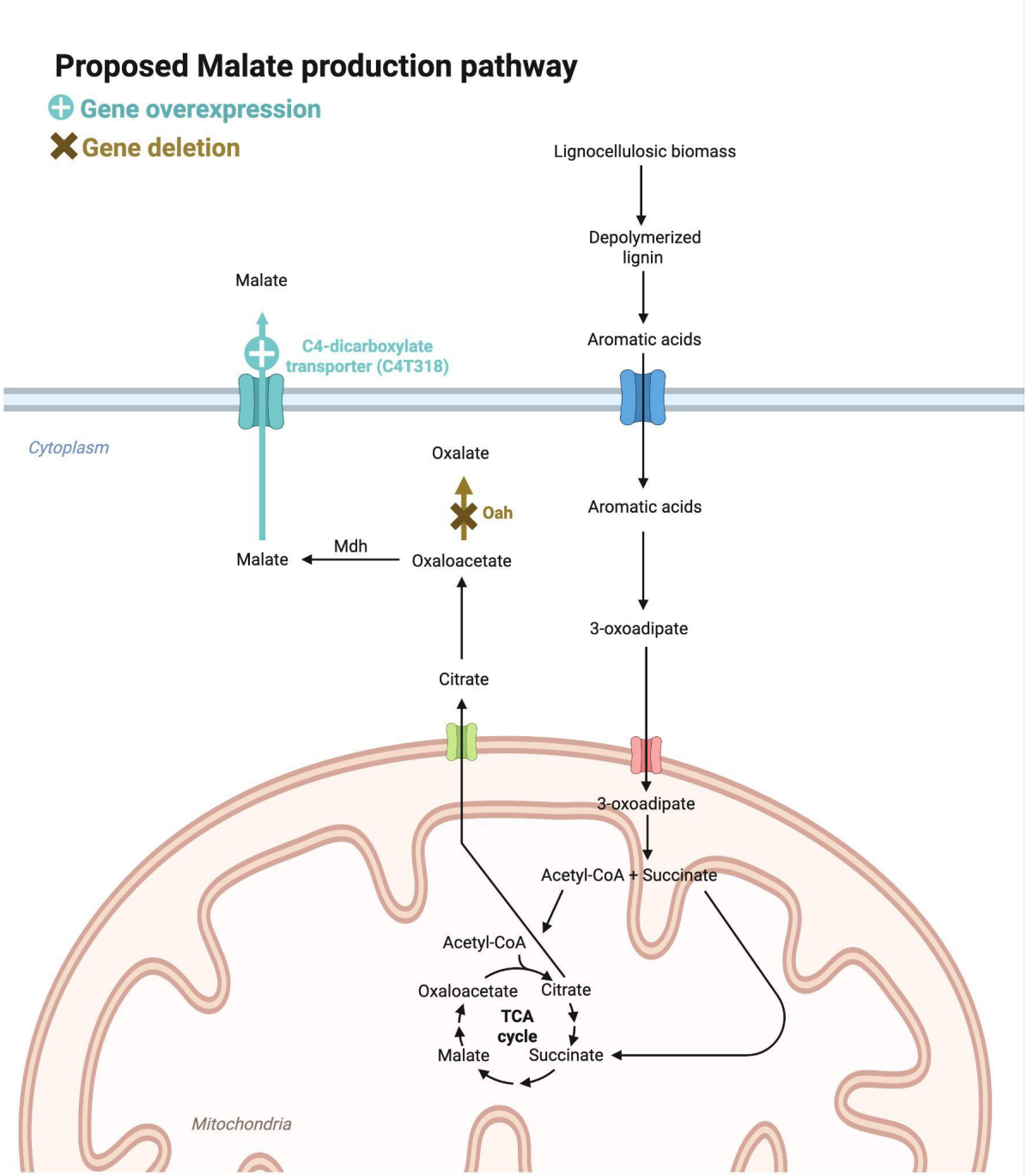


Fig. 2. Proposed metabolic pathway for the biological conversion of aromatic acids to malic acid. The genetic modifications applied to *A. niger* are depicted with an “X” or a filled circle with a “+” for gene deletion or gene overexpression, respectively.

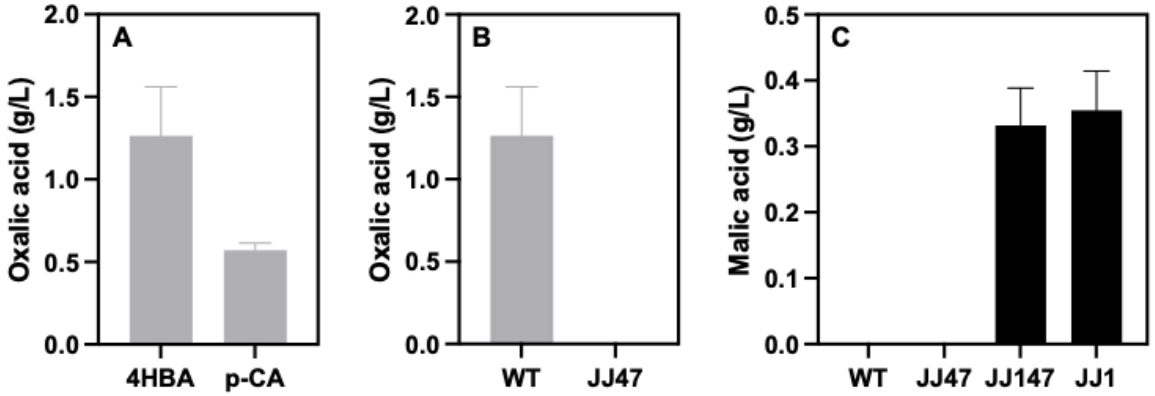


Fig. 3. Redirecting carbon flux toward malic acid production in engineered *A. niger*. (A) Oxalic acid titers produced by the wild-type (WT) strain cultivated on 4-hydroxybenzoic acid (4-HBA) and p-coumaric acid (p-CA) as sole carbon sources. (B) Comparison of oxalic acid titers between the WT and strain JJ47 cultivated on 4-HBA. (C) Malic acid titer produced by WT, JJ47, JJ147, and JJ1 with 4-HBA as the carbon source. Cultivations were performed in a minimal medium containing 10 g/L aromatic substrate in shake flasks for 72 h at 30 °C.

3.3 Medium optimization for malic acid production

Achieving high malic acid titers depends not only on the availability of suitable carbon and nitrogen sources but also on how the fermentation environment is managed (Kövilein et al., 2021; Wu et al., 2022). Among the most decisive factors are the type of neutralizing agent employed, the initial and dynamic pH of the medium, and the concentration of neutralizing agent used for buffering (Gopaliya et al., 2023; Iyyappan et al., 2018). These variables have been identified as critical constraints for organic acid fermentations in filamentous fungi. Building on this knowledge, systematic optimization was carried out to determine the roles of neutralizing agent, pH, and $CaCO_3$

concentration in the engineered strain. A defined fermentation medium was adopted instead of the minimal medium used in earlier sections. The formulation was based on that reported for *A. oryzae* by Brown et al., which originally used glucose as the carbon source and has been applied for malic acid fermentations (Brown et al., 2013). Based on the comparable malic acid titers of JJ147 and JJ1 observed in minimal medium (Fig. 3C), JJ1 was selected as the representative production strain for subsequent medium optimization experiments.

Neutralizing agents are indispensable in organic acid fermentations because the accumulation of acidic products can quickly drop the pH, imposing stress on fungal physiology and inhibiting metabolic activity. Without adequate buffering, titers are severely restricted. Different buffers, including MES, $CaCO_3$, $NaHCO_3$, and K_2CO_3, have been explored. $CaCO_3$ is generally preferred due to its gradual dissolution and stable pH control, while $NaHCO_3$ and K_2CO_3 are more soluble and less effective over time (Czajka et al., 2025; Liu et al., 2013; Zambanini et al., 2016). MES has also been applied as a pH-stabilizing buffer in malic acid fermentations (Czajka et al., 2025; Liu et al., 2013; Zambanini et al., 2016). In the present study, fermentations supplemented with MES, $CaCO_3$, $NaHCO_3$, or K_2CO_3 displayed significant differences in malic acid production. Among these agents, only $CaCO_3$ supported substantial accumulation, yielding 3.54 g/L malic acid, whereas negligible titers were observed with the other neutralizers (Fig. 4A). The superiority of $CaCO_3$ is attributed not only to its buffering properties but also to the release of Ca^{2+} ions, which play essential roles in fungal physiology. Calcium signaling regulates a broad spectrum of processes, including cell wall growth, morphology, stress tolerance, and membrane stability. Elevated Ca^{2+}

availability has been associated with activation of transcription factors controlling cell wall organization and stress response pathways, thereby reinforcing fungal robustness under acidic conditions (de Castro et al., 2019). Such effects likely contribute to the higher malic acid titers observed in $CaCO_3$ supplemented fermentations, compared with sodium or potassium based salts, which do not provide equivalent physiological benefits.

The importance of medium pH was further evaluated using $CaCO_3$ as the neutralizing agent. Fermentations were initiated at pH values ranging from 3.0 to 8.0, with 4-HBA as the carbon source. Strong inhibition was observed at acidic conditions: only 0.37 g/L and 1.52 g/L were produced at pH 3 and 4, respectively (Fig. 4B). In contrast, production increased significantly under mildly acidic to neutral conditions, reaching 3.07 g/L at pH 5.0, 3.47 g/L at pH 6.0, and 3.66 g/L at pH 7.0. Beyond neutrality, titers declined sharply to 0.36 g/L at pH 8.0. These results are consistent with reported pH optima for other malic acid producing fungi. For instance, Kövilein et al. reported an optimum of pH 5.5 for *A. oryzae*, while Zambanini et al. showed that efficient malic acid synthesis by *Ustilago trichophora* TZ1 requires pH values above 5.4 (Kövilein et al., 2022; Zambanini et al., 2016). Mechanistically, low pH imposes proton stress that inhibits central metabolism and often promotes oxalic acid secretion in *A. niger* (Poulsen et al., 2012). Conversely, alkaline conditions destabilize intracellular redox homeostasis, thereby reducing TCA cycle flux (Borin and Oliveira, 2022). These observations reinforce that maintaining pH within the range of 6 – 7 is essential for maximizing malic acid production.

Finally, the effect of $CaCO_3$ concentration was examined at an initial pH of 6.5. In the

absence of $CaCO_3$, negligible malic acid production was observed, confirming that buffering is indispensable. Supplementation with 5, 30, 60, and 90 g/L $CaCO_3$ resulted in titers of 3.54, 3.71, 3.57, and 3.49 g/L, respectively (Fig. 4C). Thus, near maximal production was already achieved at the lowest supplementation level tested (5 g/L), and higher concentrations provided no significant advantage. This indicates that the engineered strain requires only modest buffering capacity to sustain carbon flux toward malate. Similar behavior has been described in other organic acid fermentations, where titers plateaued once sufficient buffering was achieved. For example, increasing $CaCO_3$ concentrations above threshold levels did not enhance yields in malic acid fermentation by *A. niger* or in fumaric acid production by *Rhizopus* species (Gopaliya et al., 2023; Martin-Dominguez et al., 2022).

Under these optimized conditions, the four strains (WT, JJ47, JJ147, and JJ1) were cultivated to compare their malic acid production performance (Fig. 4D). Malic acid titers were markedly higher in the transporter overexpressing strains compared with the wild-type and the *ΔoahA* mutant. Both JJ147 and JJ1 produced no detectable oxalic acid, indicating that the optimized environment effectively suppressed oxalate formation. JJ1 showed the highest malic acid titer, confirming that transporter overexpression alone, together with a buffered medium, is sufficient to enable efficient malate secretion without requiring *oahA* deletion. These results highlight that the combined effects of metabolic engineering and environmental optimization are essential for directing carbon flux toward malate and eliminating competing by-products in *A. niger*.

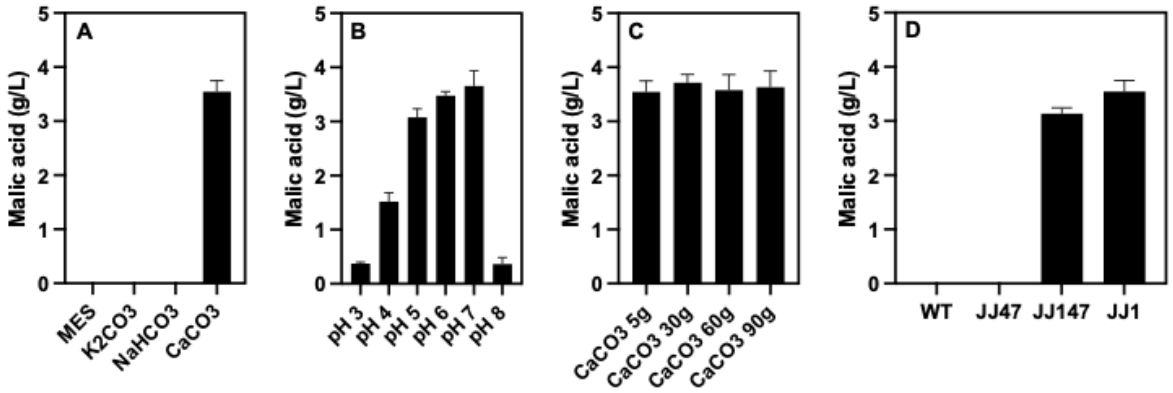


Fig. 4. Effect of neutralizing agents, initial pH, and $CaCO_3$ concentration on malic acid production. (A) Comparison of different neutralizing agents (MES, $CaCO_3$, K_2CO_3, and $NaHCO_3$) on malic acid production. (B) Influence of initial pH (3.0 – 8.0) on malic acid production. (C) Effect of $CaCO_3$ supplementation levels (5 – 90 g/L) on malic acid production. (D) Comparison of different strains on malic acid production. Cultivations were performed in a fermentation medium containing 10 g/L 4-HBA in shake flasks for 72 h at 30 °C.

3.4 Fermentation with model substrates

As lignin is a highly heterogeneous biopolymer, its depolymerization releases a mixture of structurally diverse aromatic monomers. This compositional complexity presents a major challenge for microbial lignin valorization, as different aromatic compounds often vary in assimilation rate, toxicity, and metabolic routing (Xie et al., 2016). Converting such heterogeneous aromatic mixtures into a single bioproduct through metabolic funneling offers a promising route to improve lignin utilization and process efficiency. To explore this concept in a fungal system, representative lignin-derived aromatics 4-HBA and p-CA, were selected as model substrates, along with their 1:1 mixture mimicking

the composition of depolymerized lignin. The engineered strain JJ1 was employed to assess its ability to funnel these aromatic substrates toward malic acid production.

Fermentation profiles of JJ1 grown on individual aromatic substrates revealed distinct kinetic behaviors (Figure 5A–B, D–E). When supplied with 4-HBA, rapid substrate depletion occurred within 48 h, with residual levels falling below detection thereafter. Malic acid accumulation followed substrate consumption, increasing steadily to approximately 3.54 g/L by 72 h. In contrast, cultures utilizing p-CA displayed slower substrate utilization, with concentrations decreasing gradually and complete consumption achieved only at 72 h. Despite the delayed depletion, p-CA supported continued malic acid formation throughout the cultivation period, reaching a higher final titer of 3.90 g/L. The time-course data demonstrate that while 4-HBA was assimilated more rapidly, p-CA sustained extended production activity and resulted in greater malic acid accumulation. These observations highlight substrate-dependent differences in carbon conversion efficiency and overall fermentation kinetics under identical culture conditions.

To further evaluate the strain’s performance under conditions mimicking lignin-derived aromatic mixtures, co-fermentation experiments were conducted using a combination of 4-HBA and p-CA. Fermentation on the mixed 4-HBA and p-CA substrate revealed sequential substrate utilization and balanced product formation (Figure 5C, F). Both aromatic acids were consumed completely within 72 h, but p-CA depletion occurred earlier, with most of the compound consumed by 48 h while 4-HBA persisted longer. Malic acid production increased steadily throughout cultivation, reaching approximately 3.70 g/L at 72 h, comparable to the yield obtained with p-CA alone. The time course

data indicate that JJ1 preferentially assimilated p-CA when both substrates were present, followed by subsequent utilization of 4-HBA. This sequential consumption pattern corresponded with continuous malic acid accumulation, suggesting efficient carbon conversion from the mixed aromatic feedstock. Overall, the results demonstrate that JJ1 can effectively metabolize heterogeneous aromatic mixtures derived from lignin depolymerization while maintaining high levels of malic acid synthesis (23.7% of the theoretical maximum yield, Table S4).

In parallel with substrate consumption and product formation, temporal changes in medium pH were monitored to characterize the overall fermentation dynamics. The pH profiles under all fermentation conditions showed a consistent trend over time (Figure 5A–C). The initial pH remained stable during the first 24 h, followed by a gradual decrease between 24 and 48 h that coincided with the main production phase. Minimum pH values were reached at approximately 48 h, after which pH began to increase again toward the end of fermentation. By 72 h, pH values had risen above their initial levels across all tested conditions. The magnitude of pH change differed slightly depending on the substrate, with cultures grown on p-CA maintaining slightly higher pH values compared to those on 4-HBA. Mixed-substrate fermentations exhibited intermediate profiles, reflecting the combined effects of the two aromatics. These data show a clear progression of pH decrease during active production followed by recovery after substrate depletion, indicating stable and reproducible trends across all culture conditions. As aromatic acids were consumed and malic acid accumulated, pH declined due to net acid generation. A slight rise in pH during the lag phase likely resulted from $CaCO_3$ dissolution. After substrate depletion, the pH increased again as dissolved

$CaCO_3$ neutralized residual acidity. The behavior previously described in *S. cerevisiae* fermentations, where $CaCO_3$ dissolution after glucose depletion led to a pH rebound due to acid consumption and buffering equilibrium (Zelle et al., 2008). This buffering behavior stabilized the culture environment and maintained favorable conditions for organic acid secretion.

Together, these results highlight notable variation in how *A. niger* processes the different aromatic acids. The differences in fermentation behavior between 4-HBA and p-CA are likely due to variations in their chemical structure and metabolic conversion. We used the genome-scale metabolic model to investigate how lignin-derived aromatic compounds can be converted to malic acid in *A. niger*. 4-HBA, a simpler benzoate derivative, is directly hydroxylated to protocatechuate via *p*-hydroxybenzoate hydroxylase, enabling rapid uptake and catabolism through the β-ketoadipate pathway in *Aspergillus* species (Lubbers, 2025). In contrast, p-CA, a hydroxycinnamate, requires CoA activation and β-oxidative side-chain shortening prior to ring cleavage, a process that is energetically more demanding and transcriptionally regulated, resulting in slower overall consumption (Lubbers, 2025). Despite its slower catabolism, p-CA supports higher malic acid production (Table S4) because oxidation of its two-carbon side chain generates additional acetyl-CoA and reducing equivalents, thereby enriching the cellular NADH pool and enhancing flux through the glyoxylate cycle. Meanwhile, 4-HBA catabolism begins with an NAD(P)H-consuming hydroxylation step, which diminishes net reducing power available for malic acid synthesis. Several fungi and bacteria, including *Paecilomyces variotii*, *Pycnoporus cinnabarinus*, and *Bacillus megaterium*, convert p-CA to 4-HBA as an intermediate during aromatic degradation, supporting a

sequential or hierarchical funneling mechanism in which hydroxycinnamates are first transformed into hydroxybenzoates before complete assimilation (Estrada Alvarado et al., 2001; Sachan et al., 2006; Torres Y Torres and Rosazza, 2001). The predicted metabolic flux distribution in *A. niger* producing malic acid from a 1:1 mixture of 4-HBA and p-CA is shown in Figure S2.

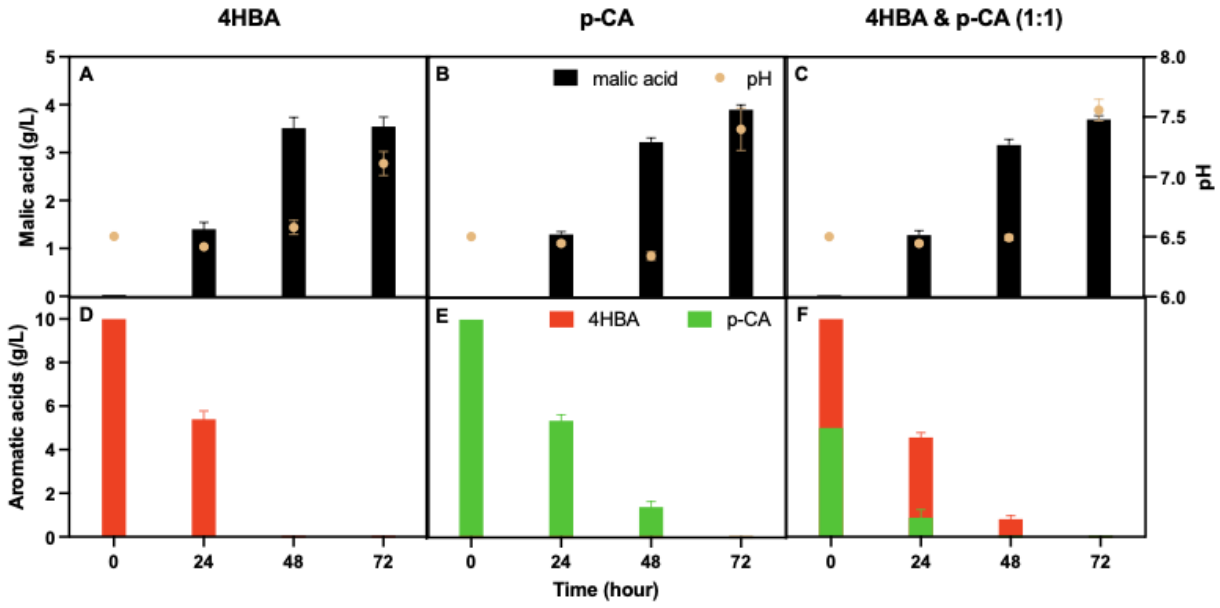


Fig. 5. Conversion of model lignin-derived aromatic acids into malic acid by *A. niger* JJ1. (A–C) Malic acid titers and pH profiles during cultivation with 4-hydroxybenzoic acid (4-HBA), p-coumaric acid (p-CA), or a 1:1 mixture of both substrates. (D–F) Corresponding substrate consumption profiles showing residual concentrations of 4-HBA and p-CA. Cultivations were performed in a fermentation medium containing 10 g/L total aromatic substrate in shake flasks for 72 h at 30 °C.

3.5 Malic acid production by *A. niger* in a real lignin stream

Poplar and sorghum were chosen as representative biomass to examine lignin

depolymerization and microbial conversion because they both are promising energy crops for biofuel production and have been shown previously to produce biocompatible hydrolysates for microbial conversion through ionic liquids pretreatment(Verdía Barbará et al., 2025). The aromatic compositions of BCD poplar (BCDP) and BCD sorghum (BCDS) liquor were quantified to determine the carbon sources available for fermentation (Fig. S3). In BCDP, 4-HBA was the dominant compound at 0.23 g/L, accompanied by smaller amounts of protocatechuic acid (PCA, 0.03 g/L) and ferulic acid (FA, 0.01 g/L). In contrast, BCDS contained a distinct profile enriched in p-coumaric acid (p-CA, 0.33 g/L) and FA (0.09 g/L), with only trace levels of 4-HBA (0.004 g/L) and no detectable PCA. These data highlight the inherent compositional varieties in biomass between hardwood and grass, with poplar lignin primarily generating hydroxybenzoate derivatives and sorghum lignin yielding hydroxycinnamate derivatives upon base-catalyzed depolymerization. The quantified aromatic concentrations were subsequently used to evaluate malic acid production by engineered strain JJ1.

Initial trials using the JJ1 cultivated in the fermentation medium showed limited aromatic acid consumption in BCD poplar (Fig. S4A). When the same test was repeated in MM supplemented with $CaCO_3$ (MM + $CaCO_3$), efficient depletion of aromatic acids was observed (Fig. S4B). This comparison indicated that excessive nutrient supplementation in the fermentation medium might inhibit aromatic uptake, whereas buffered MM provided a more suitable environment for lignin-derived substrate conversion. A likely explanation for the improved aromatic acid utilization in MM relative to the fermentation medium are the differences in nitrogen sources and concentrations. The fermentation medium contains Bacto Peptone, an organic nitrogen source composed of peptides and

amino acids derived from enzymatically hydrolyzed proteins, whereas the MM supplies inorganic nitrogen in the form of nitrate salts from the 20× $NaNO_3$ stock. Organic nitrogen sources might be able to trigger nitrogen metabolite repression in *Aspergillus* species through the *AreA/NmrA* regulatory system (Han et al., 2016; Kudla et al., 1990), thereby down regulating genes required for the assimilation of non preferred carbon substrates such as lignin-derived aromatics. In addition, Huang et al. reported that lignin degradation was more pronounced in minimal M9 medium than in tryptone/yeast extract rich medium for poplar degrading strains, which suggests that nutrient rich conditions may suppress or mask ligninolytic activity (Huang et al., 2013). From a cost perspective, nitrate salts are commodity chemicals available at a fraction of the price of complex organic supplements like Bacto Peptone, which further supports the use of nitrate-based MM for lignin bioconversion processes. Therefore, all subsequent experiments in this section were conducted using MM + $CaCO_3$ as the cultivation medium.

Using this optimized configuration, JJ1 effectively metabolized the aromatic acids present in both BCDP and BCDS liquors (Fig. 6). In BCDP cultures, the total aromatic concentration was approximately 0.25 g/L, consisting primarily of 4-HBA (0.21 g/L), PCA (0.028 g/L) and FA, (0.01 g/L). All components were almost completely depleted within 24 h, resulting in a malic acid titer of 0.65 g/L at 48 h. In BCDS fermentations, the total aromatic content reached 0.38 g/L, dominated by p-CA (0.30 g/L) and FA (0.08 g/L), with only trace 4-HBA (0.003 g/L). p-CA was fully consumed by 24 h and FA by 48 h, yielding 0.82 g/L malic acid. These results demonstrate that JJ1 effectively metabolizes both hardwood and grass derived aromatic acids, maintaining comparable conversion efficiency across distinct lignin feedstocks despite compositional variation.

Building on these individual substrate tests, fermentations were next conducted using an equal mixture of BCDP and BCDS hydrolysates to assess the strain's ability to handle compositional heterogeneity (Figure 6C-F). The initial total aromatic concentration was 0.31 g/L, consisting of PCA (0.014 g/L), 4-HBA (0.12 g/L), p-CA (0.14 g/L), and FA (0.039 g/L). During the first 24 h, p-CA, 4-HBA, and PCA were rapidly and completely depleted, while FA persisted longer and was fully metabolized by 48 h. Malic acid production increased steadily throughout cultivation, reaching 0.70 g/L at 48 h. The sequential depletion pattern indicates preferential utilization of hydroxycinnamate and hydroxybenzoate substrates prior to FA conversion. These findings confirm that JJ1 efficiently metabolizes heterogeneous aromatic mixtures representative of real lignin hydrolysates, maintaining balanced carbon utilization and consistent product formation under mixed-substrate conditions.

Across all fermentations, pH values exhibited minimal variation throughout the 48 h cultivation period, reflecting stable fermentation performance. A slight decrease occurred during the first 24 h, followed by a modest rise by 48 h as aromatic acids were depleted and malic acid accumulation plateaued. The overall pH remained within 6.4 – 6.8, markedly more stable than the wider fluctuations (6.0 – 7.5) observed in fermentations with pure aromatic substrates, which indicates that the buffering capacity of $CaCO_3$ effectively stabilized the fermentation environment, supporting sustained metabolic activity without acidification stress during lignin conversion.

Notably, FA was consistently consumed later than 4-HBA, PCA, and p-CA, revealing differences in substrate complexity and metabolic processing. Unlike 4-HBA and PCA, which directly enter the protocatechuate branch of the β-ketoadipate pathway, and p-

CA, which undergoes CoA activation and β-oxidative side-chain shortening, FA degradation requires additional O-demethylation and side-chain oxidation steps before ring cleavage. These transformations involve inducible enzymes such as feruloyl-CoA synthetase, feruloyl-CoA hydratase/lyase, and vanillate O-demethylase, whose expression in *Aspergillus* species occurs later in cultivation (Campillo et al. 2014; Chou et al. 2024; Dilokpimol et al. 2016; Arentshorst et al. 2022). The delayed induction of these enzymes likely explains the slower depletion of FA observed across all lignin-based fermentations.

Furthermore, the final malic acid titers exceeded the total carbon estimated from quantified monomeric aromatics, suggesting that additional carbon sources contributed to product formation. BCD typically produces complex mixtures containing soluble dimers, oligomers, and aliphatic fragments that are often below analytical detection limits (Park et al., 2020; Rodriguez et al., 2017). Microbial studies have shown that such low-molecular-weight lignin fragments can be internalized or further oxidized during cultivation, while fungi secrete oxidative enzymes such as laccases and peroxidases that depolymerize these intermediates into assimilable monomers (Barnhart-Dailey et al., 2019; Khan et al., 2025; Weng et al., 2021). Moreover, trace organics such as amino acids and short-chain acids present in lignin hydrolysates may have provided auxiliary carbon for metabolism (Park et al., 2020). Together, these results suggest that JJ1 can access a broader range of carbon species within BCD lignin liquor, contributing to higher than expected malic acid yields and reinforcing its potential for direct lignin bioconversion.

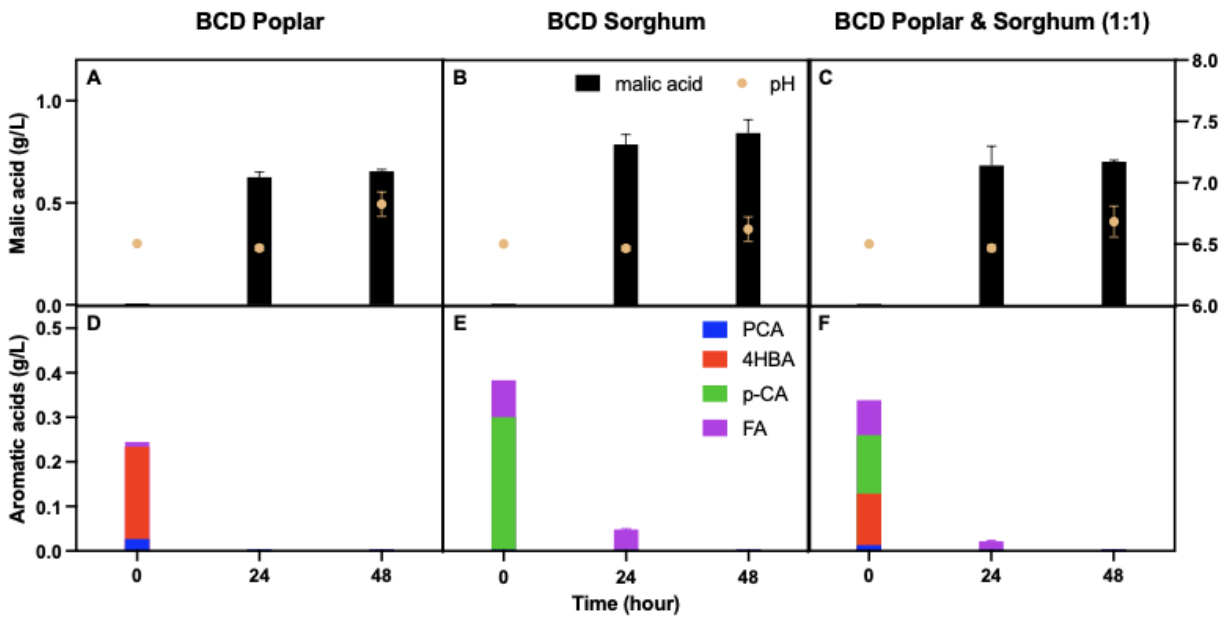


Fig. 6. Conversion of base-catalyzed depolymerized (BCD) lignin streams into malic acid by *A. niger* JJ1. (A–C) Malic acid titers and pH profiles during cultivation with BCD poplar liquor, BCD sorghum liquor, or a 1:1 mixture of both. (D–F) Corresponding substrate consumption profiles showing residual concentrations of major aromatic acids, including protocatechuic acid (PCA), 4-hydroxybenzoic acid (4-HBA), p-coumaric acid (p-CA), and ferulic acid (FA). Cultivations were performed in minimal medium supplemented with $CaCO_3$ in shake flasks for 48 h at 30 °C.

4. Conclusion

In this study, *A. niger* was engineered to convert lignin derived aromatic acids and real BCD lignin streams into malic acid. Overexpression of the C4 dicarboxylate transporter *C4T318* enabled efficient malate secretion, and medium optimization further enhanced production performance. The engineered strain effectively utilized both individual and mixed aromatic substrates as well as poplar and sorghum derived lignin liquors,

maintaining stable fermentation and balanced carbon utilization. This study represents the first demonstration of direct fungal conversion of real lignin streams into malic acid and establishes *A. niger* as a robust microbial platform for sustainable lignin valorization and biorefinery integration.

Acknowledgements

The work conducted at the Joint BioEnergy Institute was supported by the U.S. Department of Energy, Office of Science, Biological and Environmental Research Program, through contract DE-AC02-05CH11231 between Lawrence Berkeley National Laboratory and the U.S. Department of Energy. Sandia National Laboratories is a multi-mission laboratory managed and operated by National Technology and Engineering Solutions of Sandia, LLC., a wholly owned subsidiary of Honeywell International, Inc., via the U.S. Department of Energy's National Nuclear Security Administration under contract DE-NA0003525. The United States Government retains and the publisher, by accepting the article for publication, acknowledges that the United States Government retains a non-exclusive, paid-up, irrevocable, worldwide license to publish or reproduce the published form of this manuscript, or allow others to do so, for United States Government purposes. Any subjective views or opinions that might be expressed in this paper do not necessarily represent the views of the U.S. Department of Energy or the United States Government.

CRediT authorship contribution statement

Jiayuan Jia: Writing – original draft, Writing – review and editing, Conceptualization, Data curation, Investigation, Methodology, Validation, Visualization. **Ziyu Dai:** Writing – review and editing, Conceptualization, Investigation, Methodology, Validation. **Yinglei Han:** Writing – original draft, Writing – review and editing, Investigation, Methodology, Validation. **Hemant Choudhary:** Writing – review and editing, Investigation, Methodology, supervision. **Guoliang Yuan:** Writing – review and editing, Methodology,

Resources. **John M. Gladden:** Formal analysis, Supervision. **Blake A. Simmons:** Formal analysis, Supervision, Funding acquisition. **Scott E. Baker:** Conceptualization, Formal analysis, Supervision. **Jon K. Magnuson:** Supervision, Funding acquisition, Project administration. **Joonhoon Kim:** Writing – review and editing, Conceptualization, Supervision, Funding acquisition, Project administration.

Declaration of competing interest

BAS has a financial interest in Illium Technologies, Caribou Biofuels, and Erg Bio. All other authors declare the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

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