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Advancing Malic Acid Production of *Aspergillus oryzae*: Combined Process Optimization and Application of Various Substrates

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ABSTRACT

In light of climate change and growing resource scarcity, microbial production of organic acids offers a sustainable alternative to fossil-based chemical synthesis. In this study, malic acid production by *Aspergillus oryzae* was optimized through cultivation temperature adjustment and biotin supplementation, while organic acid formation from various carbon sources was systematically characterized. The process conditions applied during substrate screening, including pH control using Na_2CO_3 and NaOH, Zn^{2+} supplementation and hypoxia, were based on previously established strategies to stimulate malic acid production. Cultivation at 35°C increased respiratory activity compared to 32°C, resulting in an average productivity of 0.17 g L^{-1} h^{-1}. Biotin supplementation enhanced productivity by 20% and increased the carbon yield, defined as the proportion of consumed carbon recovered in malic acid, by 5%. Under optimized cultivation conditions, the highest malic acid productivity was achieved in cultivation with glucose as substrate and Na_2CO_3 as pH-neutralizing agent, reaching 57.57 g L^{-1} malic acid with a yield of 0.66 g g^{-1} and an overall productivity of 0.24 g L^{-1} h^{-1}, while fructose and glycerol resulted in substantially lower productivities. Furthermore, we demonstrate the ability to perform carbon balancing even in the presence of carbonate-based neutralizing agents. This is achieved by quantifying and subtracting the CO_2 generated during neutralization reactions from the total emissions, enabling precise determination of microbial CO_2 production and calculation of carbon yields. By systematically combining optimization strategies reported in previous studies, this work achieves productivity and carbon efficiency exceeding those of the individual approaches reported so far.

1 | Introduction

Malic acid is a C4 dicarboxylic acid applied in food, pharmaceutical, and cosmetic industries (Aldrich et al. 1979; Bellon 2003; Gore et al. 2010). Industrial malic acid production is predominantly based on chemical processes using fossil-derived precursors, typically yielding racemic mixtures (Kövilein et al. 2019; Winstrom et al. 1968). In contrast to fossil-based chemical synthesis, microorganisms such as *Aspergillus oryzae* enable the production of enantiopure malic acid from renewable feedstocks (Kövilein et al. 2019; Wei et al. 2021). In *A. oryzae*, malic acid accumulation primarily relies on the reductive cytosolic branch (rTCA) (Peleg et al. 1989). The importance of this metabolic pathway is demonstrated by increased malic acid productivity following overexpression of pyruvate carboxylase and malate dehydrogenase, which are key

enzymes in malic acid production, as shown in Figure 1 (Liu et al. 2017).

Pyruvate carboxylase activity depends on the availability of the cofactor biotin (Jitrapakdee et al. 2008). Increasing cofactor availability through medium supplementation may therefore represent an alternative strategy to stimulate this enzyme and thereby enhance malic acid production. Consistent with this idea, enhanced malic acid production through biotin supplementation has been demonstrated in an engineered *Saccharomyces cerevisiae* strain (Kang et al. 2022). However, this strategy, to the best of our knowledge, has not been demonstrated for *A. oryzae*.

Temperature is a key cultivation parameter accelerating metabolic reactions, with enzyme activities often roughly doubling with every 10°C increase (Elias et al. 2014). Industrial processes tolerating higher cultivation temperatures allow for reduced cooling demands, resulting in energy savings. Previous studies investigated temperature effects on malic acid production by *A. oryzae* in shake flasks (SF) buffered with $CaCO_3$. Both Kövilein et al. and Ochsenreither et al. found that malic acid productivity increased at 35°C, although Kövilein et al. additionally reported that the highest product-to-by-product ratio occurred at 32°C (Kövilein et al. 2022b; Ochsenreither et al. 2014). However, conventional SF experiments lack continuous process monitoring and feedback control, considerably impeding interpretation of temperature effects on microbial physiology and product formation.

Accurate carbon tracing is essential for evaluating substrate-specific conversion efficiencies in organic acid production. However, the use of carbonate-based neutralizers such as Na_2CO_3 introduces inorganic carbon, interfering with the attribution of substrate carbon to product molecules. This is

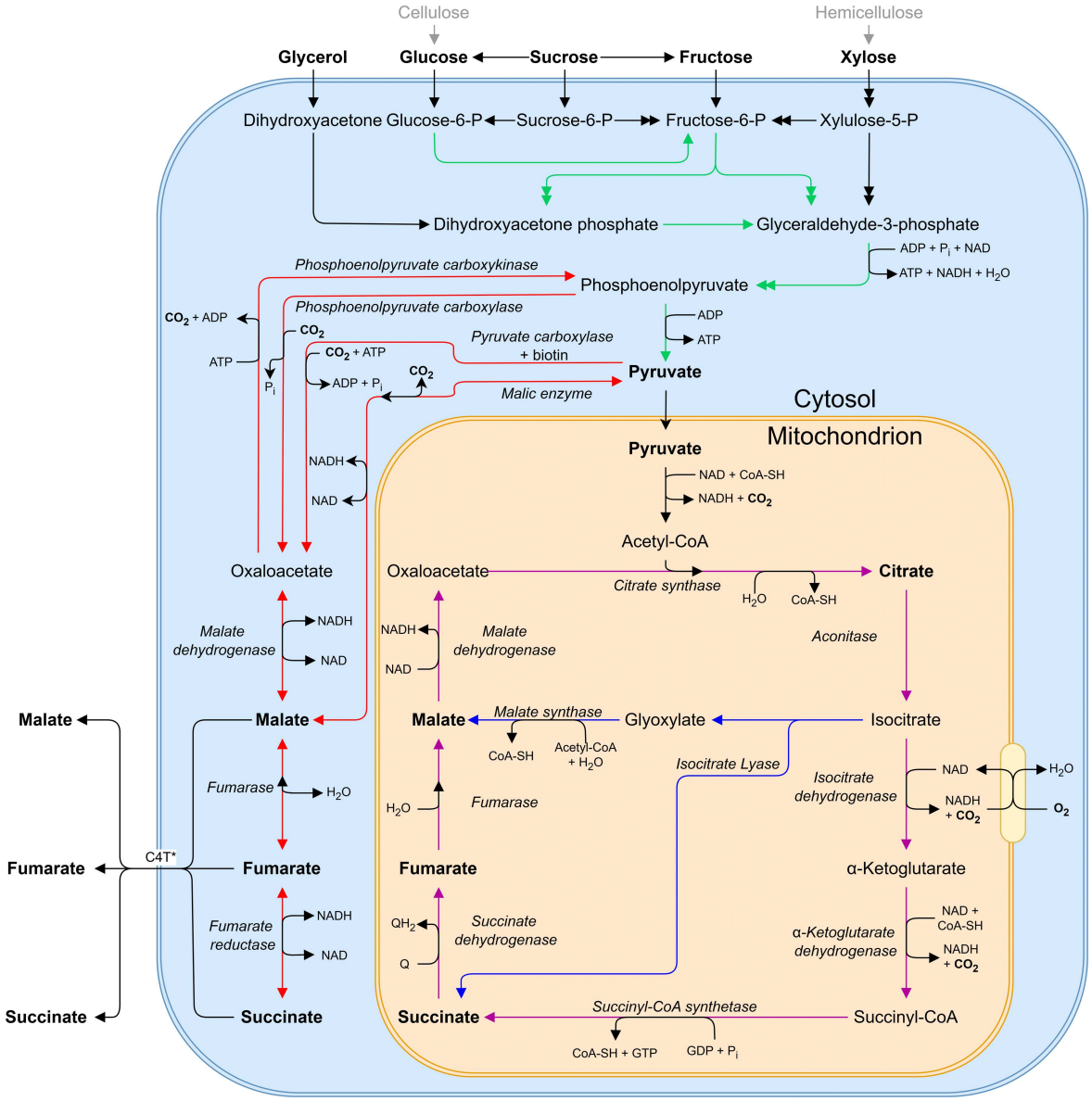

FIGURE 1 | Substrate and organic acid metabolism of *Aspergillus oryzae*. Double arrows summarize multiple metabolic steps. Pathway information was adapted from Kyoto Encyclopedia of Genes and Genomes to complement standard metabolic routes (Kanehisa et al. 2016). Only key pathways relevant to this study are shown; some reversible and alternative steps are omitted. Redox cofactors are shown in a simplified manner and proton balances are omitted. *C4-dicarboxylic acid transporter (Yang et al. 2017). Metabolites quantified in this study are written in bold font.

particularly relevant when analyzing profiles of CO_2 emissions, as the release of CO_2 from neutralization masks microbial CO_2 production. Although Na_2CO_3 has been used as a neutralizing agent in malic acid production by Hartmann et al., its analytical implications remain insufficiently addressed (Hartmann et al. 2026a).

To address these gaps, this study evaluates the impact of biotin supplementation and reassesses temperature effects on malic acid production in *A. oryzae* under well-controlled conditions. Established optimization strategies, including demand-based pH regulation using Na^+-based neutralizers, operation at an elevated pH of 7.00, Zn^{2+} supplementation and hypoxic conditions, are integrated with biotin supplementation and temperature adjustment to form a consolidated optimization approach (Hartmann et al. 2026a; Hartmann et al. 2026b). This consolidated strategy is evaluated across representative carbon sources, including sucrose, fructose, glycerol, and xylose. In addition, a method to correct CO_2 emissions for carbon tracing during carbonate-based neutralization is established. These findings provide a basis for improved process understanding and further optimization of microbial malic acid production.

2 | Methods

2.1 | Microorganism and Media

A. oryzae DSM 1863 was obtained from the German Collection of Microorganisms and Cell Cultures (DSMZ, Braunschweig, Germany). Conidia production and storage followed the procedure described by Kövilein et al., as modified by Hartmann et al. (Hartmann et al. 2026a; Kövilein et al. 2021).

The composition of media for both preculture and main culture followed protocols previously established by Kövilein et al. and Hartmann et al. (Hartmann et al. 2026a; Kövilein et al. 2022a). The preculture medium contained: 40 g L^{-1} glucose monohydrate, 4 g L^{-1} $(NH_4)_2SO_4$, 0.75 g L^{-1} KH_2PO_4, 0.98 g L^{-1} K_2HPO_4, 0.1 g L^{-1} $MgSO_4{\cdot}7H_2O$, 0.1 g L^{-1} $CaCl_2{\cdot}2H_2O$, 5 mg L^{-1} NaCl and 5 mg L^{-1} $FeSO_4{\cdot}7H_2O$. All major components were sterilized together by autoclaving for 20 min at 121°C while trace elements were added aseptically prior to inoculation as 2 mL L^{-1} Hutner's Trace Elements (HTE) (Hartmann et al. 2026a; Hill and Kafer 2001). The HTE solution was sterilized by filtration with a pore diameter of 0.2 μm.

For temperature-varied main cultures, a basal medium was prepared containing: 120 g L^{-1} glucose monohydrate, 1.2 g L^{-1} $(NH_4)_2SO_4$, 0.1 g L^{-1} KH_2PO_4, 0.17 g L^{-1} K_2HPO_4, 0.1 g L^{-1} $MgSO_4{\cdot}7H_2O$, 0.1 g L^{-1} $CaCl_2{\cdot}2H_2O$, 5 mg L^{-1} NaCl and 60 mg L^{-1} $FeSO_4{\cdot}7H_2O$. Crude $FeSO_4{\cdot}7H_2O$ was used for precultures, while $FeSO_4{\cdot}7H_2O$ dissolved in 0.1 M H_2SO_4 was used for main cultures. $(NH_4)_2SO_4$, KH_2PO_4, K_2HPO_4 and NaCl were autoclaved together with 100 μL of antifoam agent Contraspum A 4050 HAc (Zschimmer & Schwarz GmbH & Co. KG, Lahnstein, Germany). Glucose monohydrate was autoclaved separately, while stock solutions of $FeSO_4{\cdot}7H_2O$, $CaCl_2{\cdot}2H_2O$ and $MgSO_4{\cdot}7H_2O$ were sterilized by filtration, and all components were added aseptically after autoclaving.

To investigate the potential of cofactor supplementation, biotin supplementation experiments were conducted in comparison to a non-supplemented reference at 32°C. The supplementation level applied in the stirred tank reactor (STR) experiments was selected based on a preliminary shake flask screening study (data not shown), in which biotin concentrations were varied within a range of 1–100 μg L^{-1}. Cultivation performance was assessed to identify the supplementation level that maximized malic acid formation and glucose-to-malic acid yield while minimizing vitamin addition. Accordingly, in this study, sterile-filtered biotin was added to the basal medium after autoclaving to a concentration of 20 μg L^{-1}.

For cultivations employing alternative carbon sources or neutralized with Na_2CO_3 under optimized conditions, the basal medium was modified accordingly. Autoclaved glucose monohydrate and glycerol, as well as sterile-filtered fructose, sucrose and xylose were used, each normalized to a carbon content equivalent to 109 g L^{-1} glucose. Additionally, 44 mg L^{-1} $ZnSO_4{\cdot}7H_2O$ and 20 μg L^{-1} biotin, both sterile-filtered, were aseptically added after autoclaving (Hartmann et al. 2026b).

2.2 | Preculture Conditions

Precultures and inoculation of main cultures were performed as described by Hartmann et al. based on the cultivation protocol originally reported by Kövilein et al. (Hartmann et al. 2026a; Kövilein et al. 2022b). Main cultures were inoculated with 7.5 g L^{-1} washed biomass.

2.3 | Main Culture Conditions

Cultivations were conducted in 2.5 L STR (Minifors, Infors AG, Bottmingen, Switzerland) with a working volume of 1.4 L. The reactors were equipped with two Rushton turbines with diameters of 4.5 cm and operated at 400 rpm as standard cultivation protocol. As shown in Table 1, temperature-dependent experiments were carried out at 32°C, 35°C, and 38°C while one duplicate cultivation with biotin supplementation was performed at 32°C. Cultivations under optimized conditions with various carbon sources were conducted at 35°C. Aeration was maintained at 0.7 L_n min^{-1} air in most experiments, where L_n denotes liters under normal conditions. In specific cultivations at 35°C and 38°C under otherwise identical reference conditions, aeration and agitation were automatically controlled to maintain 36% dissolved oxygen tension (DOT). Detailed time profiles of aeration and agitation for those experiments are provided in Supporting 1. Reference, temperature-variable without DOT control and biotin-supplemented cultivations were neutralized to pH 6.50 ± 0.05 using 4 M NaOH and 4 M H_3PO_4. Cultivations with DOT control experienced higher evaporation due to increased aeration, so NaOH was applied at 3 M to compensate for the resulting volume loss and maintain pH 6.50 ± 0.05. Cultivations under optimized conditions were maintained at pH 7.00 ± 0.05 using 4 M NaOH and 4 M H_3PO_4 or, in one duplicate, 2 M Na_2CO_3. Neutralizers were stored on balances throughout the cultivation, allowing the time-resolved consumption to be calculated based on gravimetrically recorded mass, corresponding densities and pump rates. The reported neutralizer consumption corresponds exclusively to base addition, as the amount of H_3PO_4 used for pH control was below the gravimetric detection limit. For comparative purposes, the consumed neutralizer volume was divided by the initial

TABLE 1 | Overview of STR cultivations regarding reference and optimized conditions. Reference conditions: pH 6.50, 32°C, without biotin and Zn^{2+} supplementation. Optimized conditions: pH 7.00, 35°C, with biotin and Zn^{2+} supplementation.

Condition	pH [-]	Alkali conc. [M]	Alkali identity	Temperature [°C]	DOT control	Biotin	Zn^{2+}
32°C (Reference)	6.50	4	NaOH	32	✗	✗	✗
35°C	6.50	4	NaOH	35	✗	✗	✗
38°C	6.50	4	NaOH	38	✗	✗	✗
35°C, DOT controlled	6.50	3	NaOH	35	✓	✗	✗
38°C, DOT controlled	6.50	3	NaOH	38	✓	✗	✗
32°C + biotin	6.50	4	NaOH	32	✗	✓	✗
Reference, NaOH, Glucose	6.50	4	NaOH	32	✗	✗	✗
Optimized, NaOH, Glucose	7.00	4	NaOH	35	✗	✓	✓
Optimized, Na_2CO_3, Glucose	7.00	2	Na_2CO_3	35	✗	✓	✓
Optimized, NaOH, Fructose	7.00	4	NaOH	35	✗	✓	✓
Optimized, NaOH, Sucrose	7.00	4	NaOH	35	✗	✓	✓
Optimized, NaOH, Xylose	7.00	4	NaOH	35	✗	✓	✓
Optimized, NaOH, Glycerol	7.00	4	NaOH	35	✗	✓	✓

working culture volume. The pH, DOT and off-gas composition were monitored using sensors and a gas analyzer (EasyFerm Plus and VisiFerm, Hamilton Bonaduz AG, Bonaduz, Switzerland; BlueVary, BlueSens gas sensor GmbH, Herten, Germany), all calibrated according to the manufacturer's instructions. To avoid mycelial adherence to reactor surfaces, internal baffles were removed. Additionally, convex DOT sensor caps (ODO cap H2, Hamilton Bonaduz AG) were employed to reduce fouling. The lower impeller was positioned 1 cm above the shaft base with a spacing of 6.5 cm to the upper impeller. A curved aeration tube with a single outlet of 4 mm in diameter was used to minimize the risk of sparger blockage. Sampling was performed by withdrawing 10 mL of culture broth containing biomass. All STR cultivations were carried out in biological duplicates.

2.4 | Analytics

Broth samples obtained from STR cultivations were filtered using a 0.5 mm stainless steel mesh. The retained solids were placed in a Petri dish, scanned at 2400 dpi using a flatbed scanner (Perfection V600 Photo, Epson Deutschland GmbH, Düsseldorf, Germany). 2D scans are provided in Supporting 3. The biomass was then returned to the steel mesh, rinsed with demineralized water and dried at 80°C for at least 2 days prior to gravimetric determination of cell dry weight (CDW) (Ferreira et al. 2014; Uwineza et al. 2021). For samples containing small biomass filaments that passed through the mesh, rinsed filtrates were additionally centrifuged at 4,800 × g for 15 min and the recovered solids were added to the biomass for drying. Broth filtrates from the STR cultivations were stored at −20°C until further analysis. The elemental biomass composition used for carbon balance calculations was published elsewhere (Hartmann et al. 2026a).

Metabolite quantification was performed according to Hartmann et al. and Kövilein et al. with a few adaptations (Hartmann et al. 2026a; Kövilein et al. 2021). For HPLC analysis, broth filtrates were diluted 10-fold with 0.66 M H_2SO_4, incubated at 80°C and 1000 rpm in a 3 mm orbital diameter for 20 min, then centrifuged at 17,000 × g for 5 min. For carbohydrate quantification in sucrose cultivations, this incubation step was omitted and samples were diluted 10-fold with demineralized water instead to prevent acid-catalyzed hydrolysis of sucrose. The supernatant was analyzed using a standard HPLC system (Agilent 1100 Series, Agilent Technologies, Santa Clara, California, United States) equipped with a Rezex ROA Organic Acid H+ (8%) column (300 × 7.8 mm) including a corresponding guard column (Phenomenex, Aschaffenburg, Germany). For most metabolites, chromatographic separation was carried out isocratically with 5 mM H_2SO_4 as eluent at a flow rate of 0.5 mL min^{-1}, a column temperature of 30°C and a 10 µL injection volume. Malic acid and carbohydrates from cultivations using fructose, xylose or sucrose were quantified using 15 mM H_2SO_4 as eluent at a flow rate of 0.4 mL min^{-1}, a column temperature of 10°C and an injection volume of 10 µL. Substrates were detected by refractive index while organic acids were quantified using UV absorbance at 220 nm. Pyruvic acid was only quantified when glucose and glycerol were used as substrates.

2.5 | Calculations

Metabolite mass balances, dynamic changes in cultivation volume and gas transfer rates were calculated using Microsoft Excel (Version 2108, Microsoft Corporation, Redmond, Washington, United States) and OriginPro (Version 2023, OriginLab Corporation, Northampton, Massachusetts, United States) according to methods provided in a previous study (Hartmann et al. 2026a). The oxygen transfer rate (OTR), carbon dioxide transfer rate (CTR) and respiratory quotient (RQ) were calculated from the compositions of the in- and off-gas streams using the methodology established by Knoll et al. and applied by Hartmann et al. (Hartmann et al. 2026a; Knoll et al. 2007; Knoll et al. 2005). Throughout this study, CO_2 emissions refer to CTR values derived from off-gas analysis. Culture volume was tracked to maintain a mass balance, accounting for sampling, neutralizer additions and evaporative losses measured via

humidity. For comparison across cultivation conditions, gas transfer rates, neutralizer consumption and metabolite amounts were expressed relative to the initial reactor volume. The selectivity of product formation was evaluated by assigning carbon from substrates to individual metabolites (Hartmann et al. 2026a). To compare metabolite production, molar productivities of all quantified organic acids were summed.

When Na_2CO_3 is used as a neutralizing agent, CO_2 released by chemical neutralization, defined as $CTR_{neutralization}$, must be subtracted from the total CO_2 transfer rate CTR_{total} to obtain the microbial CO_2 transfer rate $CTR_{microbial}$ in Equation 1.

$$CTR_{microbial}(t) = CTR_{total}(t) - CTR_{neutralization}(t) \quad (1)$$

CTR_{total} was determined from off-gas analysis by balancing the molar fractions of O_2 and CO_2. The molar concentration of Na_2CO_3 in the reactor resulting from base addition, $c_{Na_2CO_3,R}$ was calculated according to Equation 2 based on the molar concentration $c_{Na_2CO_3,B}$ and the cumulative volume of base added $V_{Na_2CO_3,B}$. divided by the initial reactor volume $V_{0,R}$.

$$c_{Na_2CO_3,R}(t) = c_{Na_2CO_3,B}\frac{V_{Na_2CO_3,B}(t)}{V_{0,R}} \quad (2)$$

$CTR_{neutralization}$ was obtained as the time derivative of $c_{Na_2CO_3,R}$, assuming complete conversion of Na_2CO_3-derived carbonate to CO_2 upon neutralization, as shown in Equation 3.

$$CTR_{neutralization}(t) = \frac{d}{dt}c_{Na_2CO_3,R}(t) \quad (3)$$

For Na_2CO_3-neutralized cultivations, the CTR shown in Figure 5 corresponds to $CTR_{microbial}$ and was used for the carbon tracing analysis presented in Figure 7. Figure 8 compares the $CTR_{microbial}$, $CTR_{neutralization}$ and CTR_{total} obtained under Na_2CO_3 neutralization with the CTR measured for NaOH-neutralized cultivations under optimized conditions.

3 | Results

3.1 | Organic Acid Production at Different Temperatures and With Biotin Supplementation

Temperatures were selected based on conditions reported by Kövilein et al. (Kövilein et al. 2022b). The cultivation at 32°C corresponds to the reference cultivation applied by Hartmann et al. (Hartmann et al. 2026a). Across all cultivations shown in Figure 2, OTR and CTR increased rapidly during the initial growth phase. The formation of a transient plateau in the CTR, particularly evident until 48 h, was previously attributed to depletion of Zn^{2+}, likely due to carry-over from the preculture (Hartmann et al. 2026b). The subsequent decline in CTR indicated nitrogen limitation resulting from the high C/N ratio of the medium, which limited further biomass formation, as reflected by the stagnation of CDW in Figure 3, and promoted organic acid production (Hartmann et al. 2026a; Knuf et al. 2013). This shift toward organic acid production was also reflected in a decreasing RQ, indicating the synthesis of oxidized products from glucose. Continuous base consumption further accompanied this metabolic transition. Due to increased respiratory activity at 35°C and 38°C and constant aeration rates, cultures grown at 35°C and 38°C exhibited lower DOT values compared to those at 32°C. At 35°C, malic and succinic acid productivities increased by 48% and 59%, respectively, compared to 32°C. At 38°C, fumaric acid productivity increased markedly by 260% compared to 32°C.

Since low DOT, as observed in cultivations at 35°C and 38°C, has previously been shown to enhance production of malic acid and related rTCA branch-associated organic acids, additional cultivations were performed with DOT control to decouple temperature effects from differences in oxygen availability (Diano et al. 2009; Hartmann et al. 2026b; Meijer et al. 2007; Terabayashi et al. 2012). Under these conditions, the productivities of rTCA-associated acids declined compared to uncontrolled DOT cultivations, whereas citric acid productivity increased at 35°C.

At controlled DOT levels, malic acid productivity at 35°C was 29% higher than in the reference cultivation at 32°C and 14% higher than at 38°C with controlled DOT as shown in Table 2. As depicted in Figure 4, the carbon fraction from glucose converted to malic acid was highest at 35°C under uncontrolled DOT. However, this yield decreased under DOT-controlled conditions. Although citric acid formation was highest at 35°C under DOT-controlled conditions, this drawback could be mitigated, as shown by Hartmann et al. by increasing the pH and inducing the production phase under hypoxic conditions (Hartmann et al. 2026a; Hartmann et al. 2026b). Due to its superior malic acid productivity and a yield comparable to that at 32°C, 35°C was selected for cultivation under optimized conditions.

Off-gas analysis of the biotin-supplemented cultivation at 32°C revealed similar respiratory activity profiles compared to the reference process without biotin. The only notable difference observed in online analytics was an increase in base consumption, indicating a higher overall organic acid production despite otherwise unchanged respiration patterns. The productivity stimulation by biotin was confirmed via HPLC analysis, showing increased productivities of malic, succinic and fumaric acid. Additionally, the fraction of glucose carbon converted into malic acid was elevated compared to the reference, indicating a more carbon-efficient malic acid production in the presence of biotin. In summary, biotin supplementation enhanced malic acid productivity and redirected carbon flux toward rTCA-associated acids. Due to these beneficial effects, biotin was included in all subsequent cultivations aimed at producing malic acid under optimized conditions.

3.2 | Optimized Organic Acid Production With Different Substrates

The selection of optimized cultivation parameters for malic acid production was based on a combination of previously validated and newly optimized conditions. Specifically, the choices of pH, Zn^{2+} supplementation and hypoxia were concluded by earlier studies, while the choice of temperature and biotin supplementation was refined in the context of this work (Hartmann et al. 2026a; Hartmann et al. 2026b). In addition, Na_2CO_3 was employed as a neutralizing agent in one duplicate, following evidence suggesting a potential increase in malic acid productivity compared to NaOH (Hartmann et al. 2026a). Its use in this

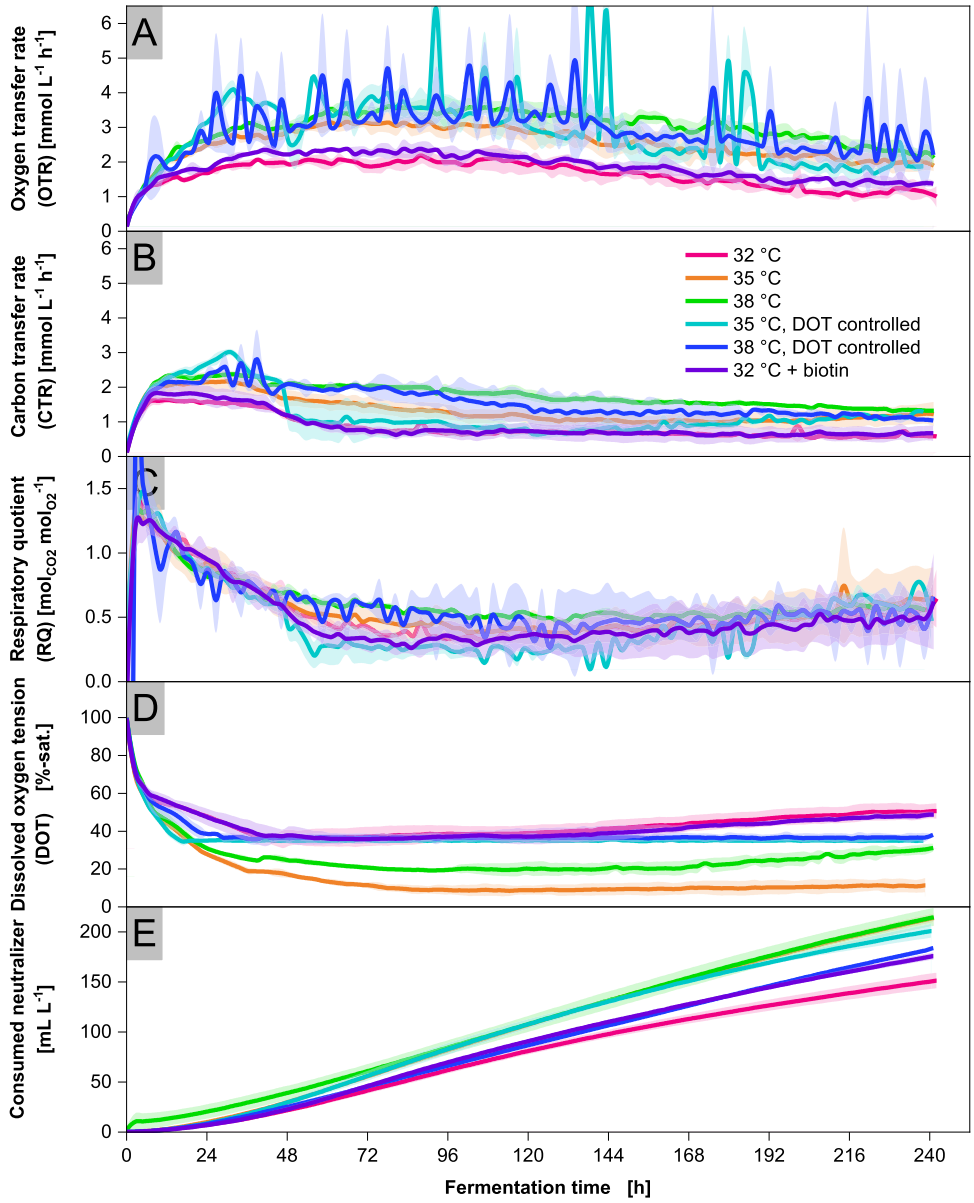

FIGURE 2 | Online monitoring of cultivations with *Aspergillus oryzae* DSM 1863 in 2.5 L STR at varying temperatures and with biotin supplementation. Cultivations were performed with X_0 of 7.5 g L^{-1} biomass, 109 g L^{-1} glucose, V_0 of 1.4 L, 400 rpm and 0.7 L_n min^{-1} air for DOT uncontrolled cultivations. Agitation frequency and aeration rate of DOT controlled cultivations are provided in Supporting 1. pH was maintained with 3 M NaOH in cultivations with controlled DOT or 4 M NaOH and 4 M H_3PO_4 at 6.50 ± 0.05 for cultivations with uncontrolled DOT. The reference cultivation at 32°C originates from the previously published study (Hartmann et al. 2026a). The online signals were averaged to obtain 100 data points. Means are represented by solid lines, while deviations are shown as shaded areas of the same color. Data represent means ± standard deviations of biological duplicates.

study served a dual purpose: not only to explore its impact under optimized conditions, but also to assess the viability of correcting CTR measurements for CO_2 released during carbonate-neutralization.

Figure 5 shows a rapid increase in CTR, reflecting sufficient Zn^{2+} availability and exceeding the plateau level observed in the reference cultivation, reaching threefold higher values. In all cultivations, flattening CTR slopes were observed toward the end of the initial rapid growth phases, indicating continued fungal growth under emerging oxygen limitation. This effect was particularly pronounced in xylose and glycerol cultivations, where the CTR slope began to decline after 17 h. Notably, the RQ in the glycerol-based cultivation was markedly lower from the beginning. This observation could be explained by the higher degree of reduction of glycerol relative to glucose. In cultivations with fructose, xylose and glycerol, fungal growth, indicated by CTR, proceeded more slowly compared to glucose and sucrose. Among these substrates, fructose supported the fastest growth, likely due to its identity as a hexose and stereoisomer of glucose, which enabled partial overlap in metabolic pathways and may have reduced the extent of metabolic adaptation required. In contrast, xylose and glycerol differ more substantially in structure and metabolism, which likely caused the slower growth.

The optimized cultivation neutralized with Na_2CO_3, as shown in Figure 6, achieved an average malic acid productivity of

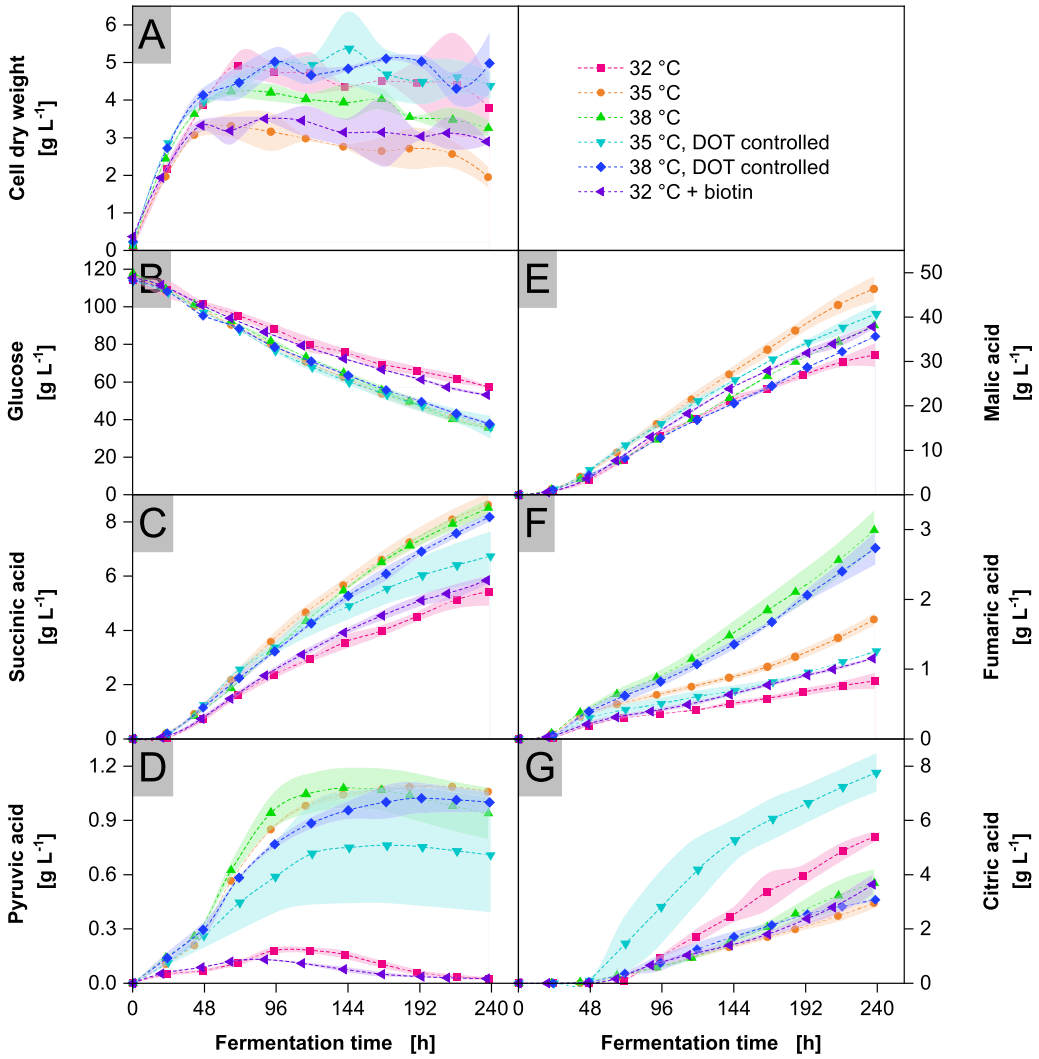

FIGURE 3 | Metabolites in cultivations with *Aspergillus oryzae* DSM 1863 in 2.5 L STR at varying temperatures and with biotin supplementation. Cultivations were performed with X_0 of 7.5 g L^{-1} biomass, 109 g L^{-1} glucose, V_0 of 1.4 L, 400 rpm and 0.7 L_n min^{-1} air for DOT uncontrolled cultivations. Agitation frequency and aeration rate of DOT controlled cultivations are provided in Supporting 1. pH was maintained with 3 M NaOH in cultivations with controlled DOT or 4 M NaOH and 4 M H_3PO_4 at 6.50 ± 0.05 for cultivations with uncontrolled DOT. The reference cultivation at 32°C originates from the previously published study (Hartmann et al. 2026a). Data represent means ± standard deviations of biological duplicates. Dashed lines and Akima-spline-connected standard deviations are provided for visual guidance.

TABLE 2 | Key performance parameters of malic acid production with *Aspergillus oryzae* DSM 1863 at varying temperatures and with biotin supplementation. Averaged productivities and yields were calculated based on endpoint measurements. MA = malic acid.

Condition	$c_{MA,\ max}$[g L^{-1}]	$Y_{MA/S}$[mg g^{-1}]	P_{MA}[mg L^{-1} h^{-1}]	P_{MA}[mM h^{-1}]
32°C	31.44 ± 2.67	549 ± 48	132 ± 11	0.98 ± 0.08
35°C	46.31 ± 2.82	573 ± 20	195 ± 12	1.45 ± 0.09
38°C	38.12 ± 0.34	464 ± 8	160 ± 1	1.19 ± 0.01
35°C, DOT controlled	40.75 ± 2.11	524 ± 11	170 ± 9	1.27 ± 0.07
38°C, DOT controlled	35.62 ± 0.16	468 ± 10	149 ± 1	1.11 ± 0.01
32°C + biotin	37.79 ± 0.47	607 ± 10	159 ± 2	1.19 ± 0.01

241 mg L^{-1} h^{-1}, representing an 83% increase over the reference cultivation reported by Hartmann et al., highlighting the substantial impact of the combined process improvements (Hartmann et al. 2026a). Among the tested conditions, this glucose-based cultivation showed the highest malic acid productivity, followed closely by the corresponding NaOH-neutralized setup and by the xylose-based cultivation. In line with the neutralization profile, malic acid formation in the

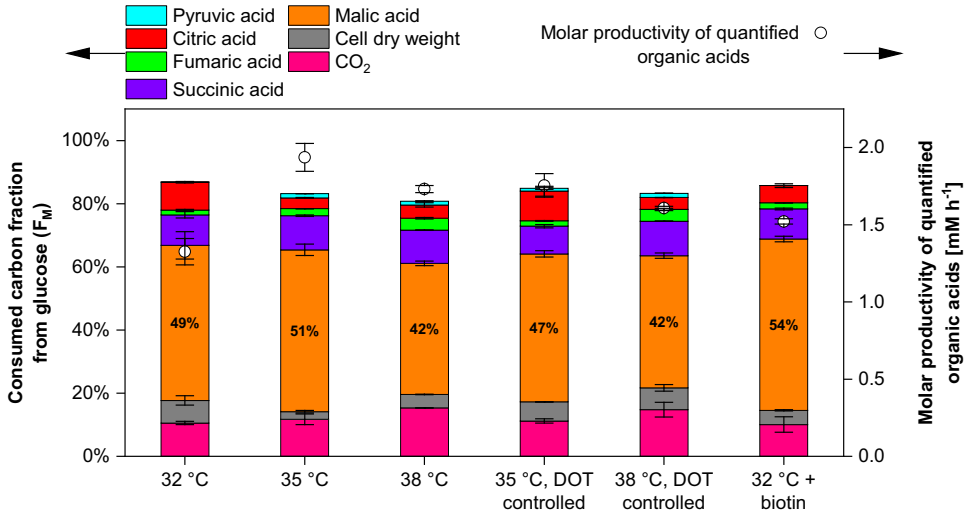

FIGURE 4 | Consumed carbon fraction from glucose and molar productivity of quantified organic acids in cultivations with *Aspergillus oryzae* DSM 1863 in 2.5 L STR at varying temperatures and with biotin supplementation. Fractions and productivities were calculated for endpoint measurements. Cultivations were performed with X_0 of 7.5 g L^{-1} biomass, 109 g L^{-1} glucose, V_0 of 1.4 L, 400 rpm and 0.7 L_n min^{-1} air for DOT uncontrolled cultivations. Agitation frequency and aeration rate of DOT controlled cultivations are provided in Supporting 1. pH was maintained with 3 M NaOH in cultivations with controlled DOT or 4 M NaOH and 4 M H_3PO_4 at 6.50 ± 0.05 for cultivations with uncontrolled DOT. The reference cultivation at 32°C originates from the previously published study (Hartmann et al. 2026a). Data represent means ± standard deviations of biological duplicates.

xylose cultivation proceeded more slowly during the initial phase but increased steadily over time, ultimately reaching productivity levels comparable to those of the glucose-based process, as illustrated in Supporting 2. All optimized cultivations showed considerably increased production of fumaric acid compared to the reference and, except for the glycerol condition, also of succinic acid. In contrast, citric acid formation was almost completely suppressed, likely due to the synergistic effect of all optimized process parameters.

As shown in Table 3 and Figure 7, the highest yields for malic acid were achieved in the glucose-based cultivation neutralized with Na_2CO_3 and in the xylose-based process. In contrast, the lowest malic acid yields were observed with fructose and glycerol as carbon sources. The decline in malic and succinic acid productivity observed in the sucrose-based cultivation after 6 days coincided with glucose depletion and a switch to fructose metabolism. The lower performance during this fructose consumption phase was consistent with the reduced productivity observed in cultivations grown solely on fructose compared to glucose.

Figure 8 shows that the $CTR_{microbial}$ profiles obtained for Na_2CO_3- and NaOH-neutralized cultivations are highly similar in both magnitude and temporal profile. Under the assumption of complete conversion of carbonate to CO_2 during neutralization, this similarity indicates that the choice between hydroxide- and carbonate-based neutralizing agents does not substantially affect microbial CO_2 emission.

4 | Discussion

4.1 | Organic Acid Production at Different Temperatures and With Biotin Supplementation

The effect of cultivation temperature on malic acid production shows both consistent and divergent trends across studies. Ochsenreither et al. reported a substantial increase in malic acid productivity when raising the temperature from 30°C to 35°C, with a 71% improvement and a yield increase from 0.55 g g^{-1} to 0.58 g g^{-1} (Ochsenreither et al. 2014). Kövilein et al. reported a 9% productivity gain when increasing the temperature from 32°C to 35°C, accompanied by a yield decrease from 0.60 g g^{-1} to 0.49 g g^{-1} (Kövilein et al. 2022b). Our results followed a similar pattern, with productivity rising by 29% between 32°C and 35°C with DOT control, while yield declined from 0.55 g g^{-1} to 0.52 g g^{-1}. The larger productivity increase observed in our study compared to Kövilein et al. may be attributed to improved process control in the bioreactor. Additionally, the use of $CaCO_3$ as buffering agent may have influenced pH dynamics and metabolic responses. This trade-off between productivity and yield at higher temperatures, evident in our study and that of Kövilein et al., was not observed by Ochsenreither et al. likely because their comparison involved 30°C rather than 32°C as the lower temperature.

Elevated temperatures enhance malic acid productivity, possibly reflecting enhanced central carbon flux, as suggested by transcriptomic analyses of *Aspergillus kawachii* reporting increased expression of glycolysis-associated genes at higher cultivation temperatures (Futagami et al. 2015). At the same time, reduced expression of pyruvate carboxylase reported for *A. kawachii* under elevated temperatures likely contributes to the pyruvic acid accumulation and reduced malic acid yield we observed. This shift in carbon flux likely contributed to the increased respiratory activity observed. In addition, activation of stress-associated pathways may redirect carbon and energy toward protective functions, consistent with our previous observation of increased CO_2 yield under stress conditions (Hagiwara et al. 2016; Hartmann et al. 2026a). However, whether the increased respiratory activity primarily reflects a stress response or simply elevated metabolic activity cannot be conclusively determined. Considering the trade-off between productivity and

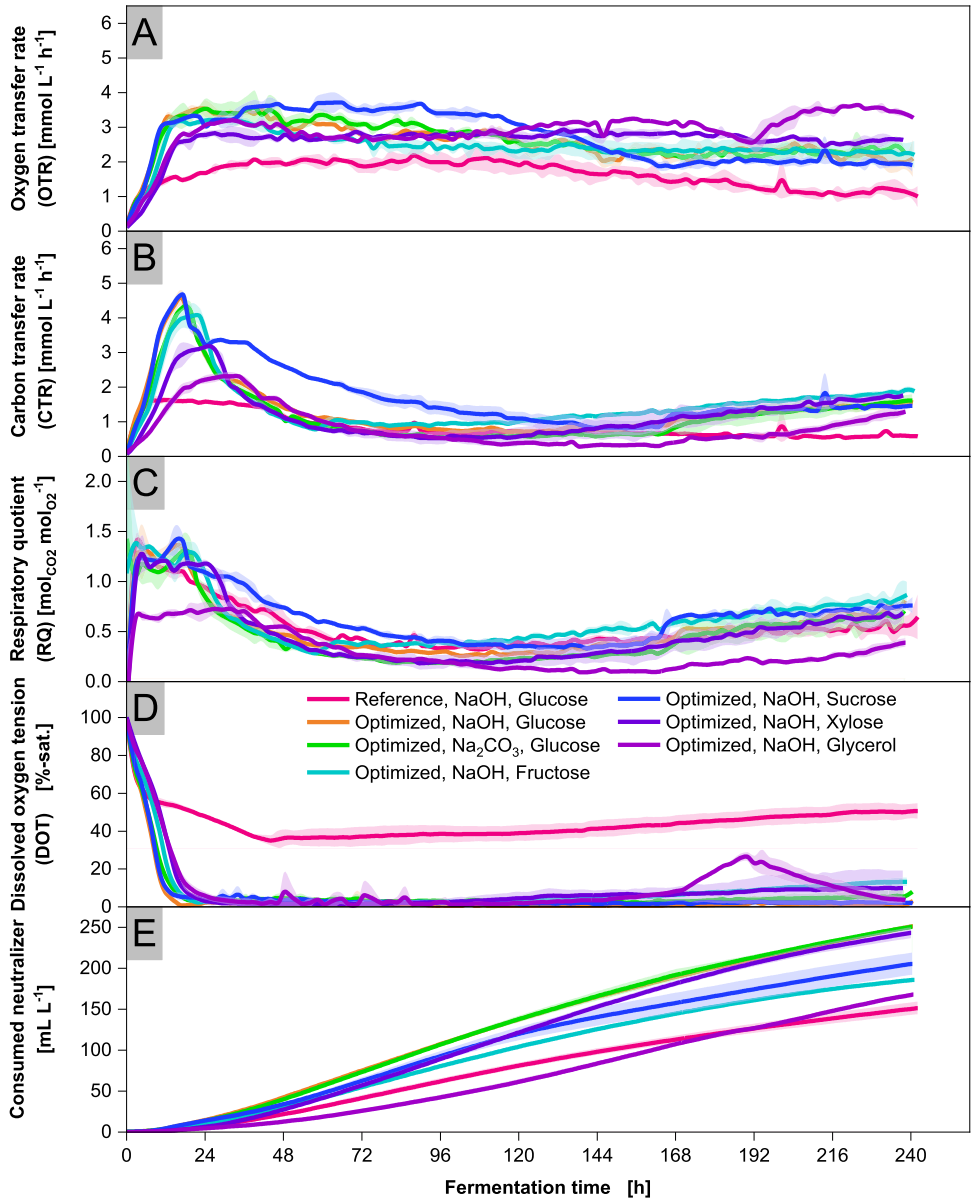

FIGURE 5 | Online monitoring of cultivations with *Aspergillus oryzae* DSM 1863 in 2.5 L STR with varying substrates and neutralizers. The $CTR_{microbial}$ profile shown was derived from the CTR_{total} of the Na_2CO_3-neutralized cultivation by subtracting the neutralization-related CO_2 contribution $CTR_{neutralization}$. Cultivations were performed with X_0 of 7.5 g L^{-1} biomass, 109 g L^{-1} glucose, 109 g L^{-1} fructose, 104 g L^{-1} sucrose, 109 g L^{-1} xylose, 110 g L^{-1} glycerol, V_0 of 1.4 L, 32°C in reference and 35°C in optimized cultivations, 400 rpm and 0.7 L_n min^{-1} air with 44 mg L^{-1} $ZnSO_4 \cdot 7H_2O$ and 20 µg L^{-1} biotin in optimized cultivations. pH was maintained with 4 M NaOH and 4 M H_3PO_4 at 6.50 ± 0.05 in reference and 7.00 ± 0.05 in optimized cultivations. The reference cultivation at 32°C with glucose originates from the previously published study (Hartmann et al. 2026a). The online signals were averaged to obtain 100 data points. Means are represented by solid lines, while deviations are shown as shaded areas of the same color. Data represent means ± standard deviations of biological duplicates.

yield, 35°C emerged as the most favorable cultivation temperature, balancing high productivity with only minor yield reduction.

Beyond temperature effects, biotin supplementation positively influenced malic acid formation, indicating a role of cofactor availability in metabolic performance. As a cofactor of pyruvate carboxylase, a key enzyme converting pyruvate to oxaloacetate in the rTCA branch, biotin likely enhanced carbon flux toward malic acid (Chi et al. 2016; Jitrapakdee et al. 2008). A similar effect was reported by Kang et al. in an engineered *S. cerevisiae* strain, in which biotin improved productivity and yield at a supplementation concentration of 0.6 mg L^{-1} (Kang et al. 2022). Consistently, Khan et al. reported enhanced Ca-malate formation in *Penicillium viticola* when corn steep liquor (CSL), which contains biotin, was used as a nutrient source (Khan et al. 2014; Xiao et al. 2012). In a separate study, Liu et al. demonstrated increased pyruvate carboxylase activity under CSL supplementation (Liu et al. 2015). These findings align with the positive effect of biotin supplementation observed in the present study, thereby highlighting microbial biotin metabolism as a potential target for improving production independently of external vitamin supply.

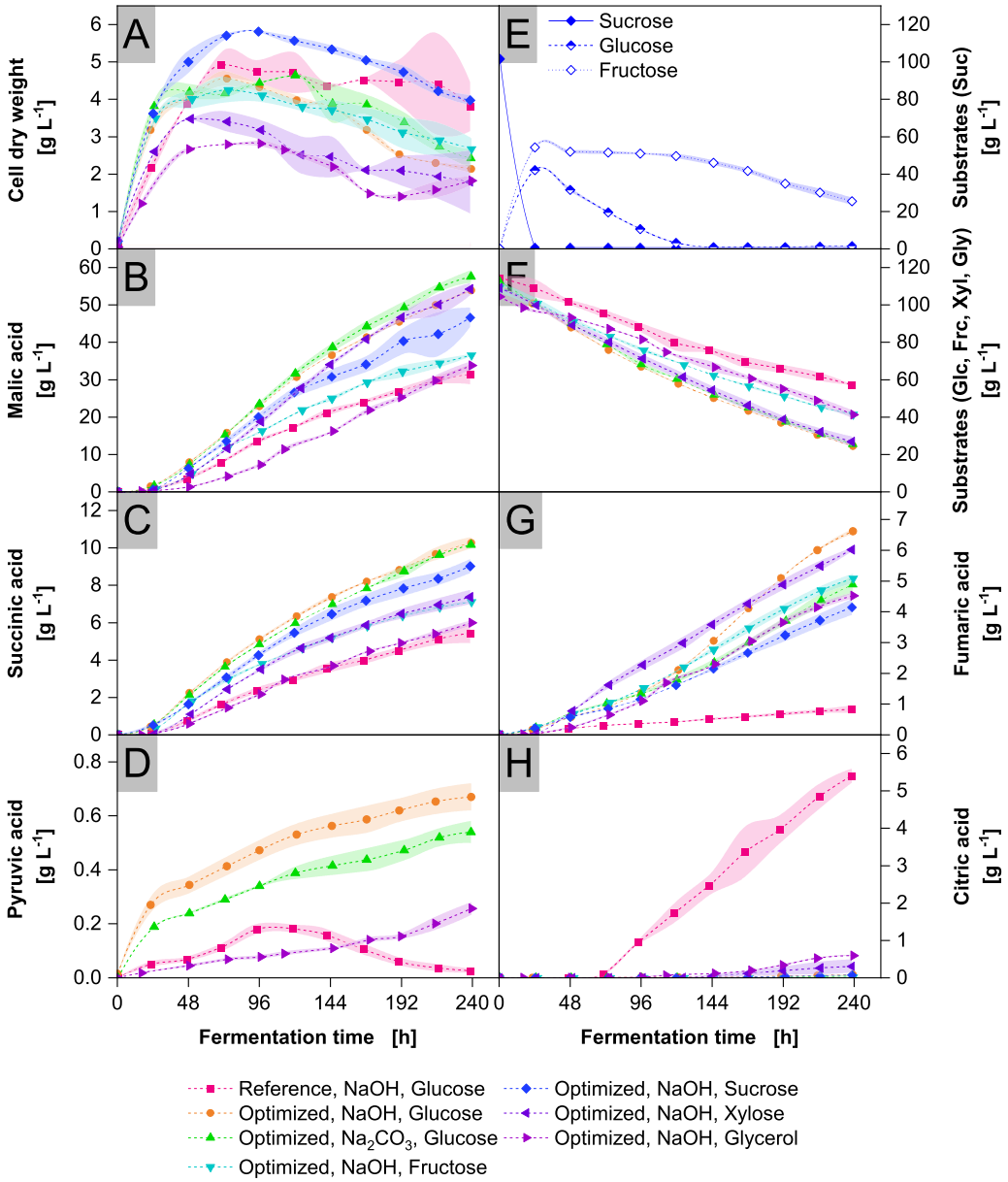

FIGURE 6 | Metabolites in cultivations with *Aspergillus oryzae* DSM 1863 in 2.5 L STR with varying substrates and neutralizers. Cultivations were performed with X_0 of 7.5 g L^{-1} biomass, 109 g L^{-1} glucose, 109 g L^{-1} fructose, 104 g L^{-1} sucrose, 109 g L^{-1} xylose, 110 g L^{-1} glycerol, V_0 of 1.4 L, 32°C in reference and 35°C in optimized cultivations, 400 rpm and 0.7 L_n min^{-1} air with 44 mg L^{-1} $ZnSO_4 \cdot 7H_2O$ and 20 µg L^{-1} biotin in optimized cultivations. pH was maintained with 4 M NaOH and 4 M H_3PO_4 at 6.50 ± 0.05 in reference and 7.00 ± 0.05 in optimized cultivations. The reference cultivation at 32°C with glucose originates from the previously published study (Hartmann et al. 2026a). Data represent means ± standard deviations of biological duplicates. Dashed lines and Akima-spline-connected standard deviations are provided for visual guidance.

4.2 | Optimized Organic Acid Production With Different Substrates

The carboxylation of pyruvic acid to oxaloacetic acid, catalyzed by pyruvate carboxylase, is considered the rate-limiting step in the biosynthesis of malic acid (Knuf et al. 2013). Both carbonate and biotin supplementation support pyruvate carboxylase activity (Jitrapakdee et al. 2008). Accordingly, the use of Na_2CO_3 as a neutralizing agent increased malic acid productivity by 7% in the present work compared to NaOH. Biotin supplementation enhanced productivity by 20% under reference conditions. Elevating the pH to 7.00 reduces energy demand for pH homeostasis and reduces organic acid influx, which contributed to a 25% increase as reported by Hartmann et al. (Dechant et al. 2010; Fernández-Niño et al. 2015; Hartmann et al. 2026a; Orij et al. 2012; Piper et al. 2001; Stratford et al. 2013; Ullah et al. 2012; Young et al. 2010). The temperature increase to 35°C resulted in a 29% improvement in productivity. Zn^{2+} supplementation prevents growth limitation, and under hypoxic conditions, electron carriers are likely redirected toward the reductive TCA branch, while the glyoxylate pathway is likely up-regulated, together causing a 33% increase (Diano et al. 2009; Hartmann et al. 2026b; Meijer et al. 2007; Terabayashi et al. 2012). Notably, the integration of all optimization strategies culminated in an 83% increase in malic acid productivity compared to the reference process, underscoring

TABLE 3 | Key performance parameters of malic acid production with *Aspergillus oryzae* DSM 1863 with varying substrates and neutralizers. Averaged productivities and yields were calculated based on endpoint measurements.

Condition	$c_{MA,\ max}$[g L^{-1}]	$Y_{MA/S}$[mg g^{-1}]	P_{MA}[mg L^{-1} h^{-1}]	P_{MA}[mM h^{-1}]
Reference, NaOH, Glucose	31.44 ± 2.67	549 ± 48	132 ± 11	0.98 ± 0.08
Optimized, NaOH, Glucose	53.86 ± 0.47	627 ± 7	225 ± 2	1.68 ± 0.01
Optimized, Na_2CO_3, Glucose	57.57 ± 1.70	662 ± 12	241 ± 7	1.80 ± 0.05
Optimized, NaOH, Fructose	36.56 ± 0.31	539 ± 27	153 ± 1	1.14 ± 0.01
Optimized, NaOH, Sucrose	46.63 ± 2.58	614 ± 33	196 ± 11	1.46 ± 0.08
Optimized, NaOH, Xylose	54.19 ± 1.65	659 ± 1	227 ± 7	1.69 ± 0.05
Optimized, NaOH, Glycerol	33.80 ± 1.07	536 ± 6	141 ± 4	1.05 ± 0.03

Abbreviation: MA, malic acid.

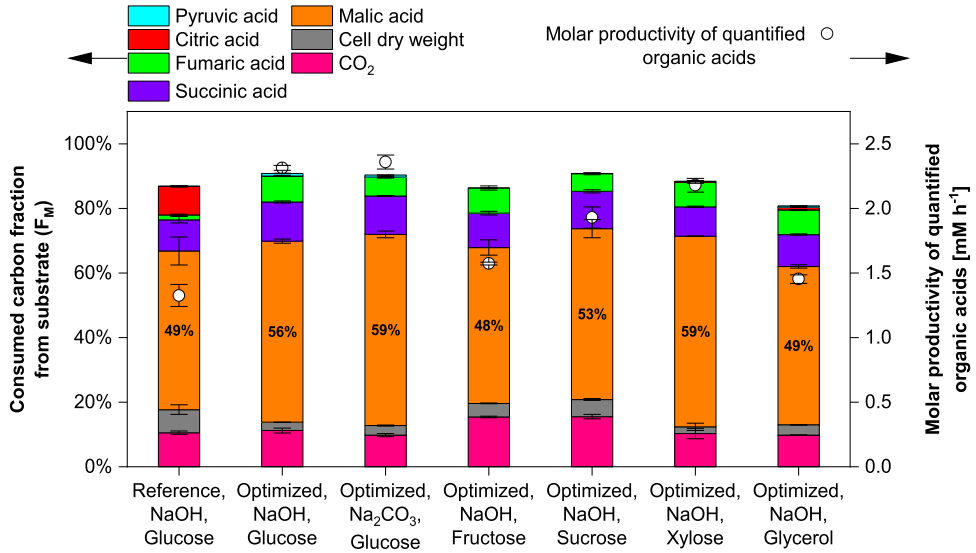

FIGURE 7 | Consumed carbon fraction from substrates and molar productivity of quantified organic acids in cultivations with *Aspergillus oryzae* DSM 1863 in 2.5 L STR with varying substrates and neutralizers. Fractions and productivities were calculated for endpoint measurements. For Na_2CO_3-neutralized cultivations, the consumed fraction from glucose-derived carbon released as CO_2 was calculated using $CTR_{microbial}$. Cultivations were performed with X_0 of 7.5 g L^{-1} biomass, 109 g L^{-1} glucose, 109 g L^{-1} fructose, 104 g L^{-1} sucrose, 109 g L^{-1} xylose, 110 g L^{-1} glycerol, V_0 of 1.4 L, 32°C in reference and 35°C in optimized cultivations, 400 rpm and 0.7 L_n min^{-1} air with 44 mg L^{-1} $ZnSO_4 \cdot 7H_2O$ and 20 µg L^{-1} biotin in optimized cultivations. pH was maintained with 4 M NaOH and 4 M H_3PO_4 at 6.50 ± 0.05 in reference and 7.00 ± 0.05 in optimized cultivations. The reference cultivation at 32°C with glucose originates from the previously published study (Hartmann et al. 2026a). Data represent means ± standard deviations of biological duplicates.

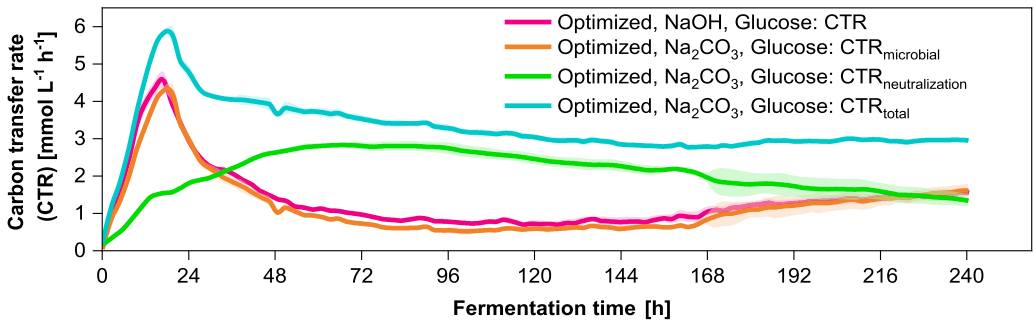

FIGURE 8 | CTR, $CTR_{microbial}$, $CTR_{neutralization}$ and CTR_{total} of cultivations with *Aspergillus oryzae* DSM 1863 in 2.5 L STR neutralized with NaOH and Na_2CO_3. Cultivations were performed with X_0 of 7.5 g L^{-1} biomass, 109 g L^{-1} glucose, V_0 of 1.4 L, 35°C, 400 rpm and 0.7 L_n min^{-1} air with 44 mg L^{-1} $ZnSO_4 \cdot 7H_2O$ and 20 µg L^{-1} biotin. pH was maintained with 4 M NaOH or 2 M Na_2CO_3 with 4 M H_3PO_4 at 7.00 ± 0.05. The online signals were averaged to obtain 100 data points. Means are represented by solid lines, while deviations are shown as shaded areas of the same color. Data represent means ± standard deviations of biological duplicates.

the synergistic potential of the combined approach. Additionally, citric acid formation was reduced, maximizing product selectivity.

Ochsenreither et al. achieved a productivity of 259 mg L^{-1} h^{-1} malic acid from glucose after 168 h when cultivating the same strain in SF with $CaCO_3$ buffer at 35°C (Ochsenreither et al. 2014). Under the same cultivation time and temperature, we reached a comparable productivity of 262 mg L^{-1} h^{-1} in a STR using Na_2CO_3 for demand-based pH control and supplementing Zn^{2+} and biotin, demonstrating the feasibility of achieving similar productivities with water-soluble neutralizing agents compared to established processes utilizing the environmentally debated $CaCO_3$ buffer (Yang et al. 2011).

The lower productivity for malic acid with fructose compared to glucose observed in this study was also reported by Dörsam et al. (Dörsam et al. 2017). Similar trends were reported for *A. niger* in citric acid production, as well as for *Lactobacillus rhamnosus* in lactic acid production (Abedi and Hashemi 2020; Hossain et al. 1984). These observations suggest that the reduced productivity with fructose may result from limitations upstream of fructose-6-phosphate, where both sugars converge in glycolysis. We hypothesize that the differences are explained by the substrate specificities of glucokinase and hexokinase in *Aspergillus* species, as demonstrated by Fleck and Brock (Fleck and Brock 2010). While glucose phosphorylation is reported to be catalyzed by both enzymes, fructose phosphorylation depends primarily on the hexokinase. Consequently, limitations in hexokinase expression or activity may lead to slower fructose utilization and thus reduced productivity.

In contrast to the findings by Ochsenreither et al. and Dörsam et al., who both reported markedly lower malic acid productivity with xylose compared to glucose in *A. oryzae* SF cultivations using $CaCO_3$ as buffering agent, we observed comparable productivities for both carbon sources (Dörsam et al. 2017; Ochsenreither et al. 2014). Although the same strain was used in all studies, differences in cultivation mode and media composition likely contributed to the observed variation.

In our study, malic acid production from glycerol was considerably lower than from glucose. Similar observations were made by Ochsenreither et al. for *A. oryzae* and by Zhang et al. for *A. niger* (Ochsenreither et al. 2014; Zhang et al. 2025). Zhang et al. identified slow intracellular glycerol catabolism as a major bottleneck, driven by limited glycerol transport and low flux through phosphorylation-oxidation pathways. By co-expressing the high-affinity glycerol transporter STL1 and enhancing key enzymatic steps of glycerol metabolism, they accelerated glycerol uptake and conversion and increased malic acid productivity. The reduced productivity observed in our study with *A. oryzae* could therefore be attributed to similar metabolic constraints, such as low transporter activity or limited expression of glycerol-metabolizing enzymes.

Following sucrose hydrolysis, *A. oryzae* consumes glucose prior to fructose, indicative of classic glucose-induced catabolite repression. However, such sequential sugar consumption is not universal across *Aspergillus* species. For example, *A. niger* was shown to consume glucose and fructose simultaneously under certain conditions (Bizukojc and Ledakowicz 2004). Moreover, recent studies indicate that sugar uptake in *A. niger* can occur independently of CreA-mediated repression, suggesting that transport and metabolism in *Aspergilli* are regulated separately rather than through canonical catabolite repression mechanisms (Mäkelä et al. 2018).

To the best of our knowledge, Salek et al. were the first to address pH control in microbial cultivation using $CaCO_3$ through a comprehensive model-based approach (Salek et al. 2015). Their work demonstrated the complexity of quantifying CO_2 emissions from $CaCO_3$ consumption. In contrast, our study extends this concept to a different use case by focusing on soluble carbonate sources such as Na_2CO_3, where CO_2 release can be calculated directly from the added volume of neutralizer. This simplification enables straightforward correction of CO_2 emission rates to determine $CTR_{microbial}$, thereby supporting accurate carbon flow analysis in cultivations employing demand-based neutralization using soluble carbonates.

5 | Conclusion

This study provides a comprehensive evaluation of key process parameters influencing malic acid production with *A. oryzae*. We identified 35°C as the optimal cultivation temperature, resulting in higher productivities than at 32°C and 38°C under both controlled and uncontrolled DOT conditions. Furthermore, our findings showed enhancement of malic acid production by biotin supplementation. When combined with previously established optimization strategies, these measures led to a considerable improvement in process performance. Xylose supported malic acid production at levels comparable to glucose, while sucrose, fructose and glycerol resulted in substantially lower productivities. We also demonstrated that CO_2 emissions from Na_2CO_3-based neutralization could be accounted for by assuming complete conversion of carbonate to CO_2, enabling carbon flow analysis even with carbonate-based neutralizers. These findings demonstrate the power of consolidating dispersed process optimizations into a unified strategy for advancing biotechnological production.

Author Contributions

Lukas Hartmann: conceptualization, investigation, methodology, formal analysis, visualization, writing – original draft, writing – review and editing. **Anke Neumann:** conceptualization, supervision, project administration, writing – review and editing. **Dirk Holtmann:** conceptualization, supervision, resources, writing – review and editing. **Björn Vater:** investigation, writing – review and editing. **Thomas Hahn:** funding acquisition, project administration, writing – review and editing. **Susanne Zibek:** conceptualization. **Katrin Ochsenreither:** conceptualization, supervision, funding acquisition, writing – review and editing.

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Ethics Statement

The authors have nothing to report.

Conflicts of Interest

The authors declare no conflicts of interest.

AI Disclosure Statement

During the preparation of this work, the authors used ChatGPT (OpenAI, San Francisco, California, United States) to improve language quality and clarity. The authors reviewed and edited the output as needed and take full responsibility for the published article.

Data Availability Statement

Data available on request from the authors.

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Supporting Information

Additional supporting information can be found online in the Supporting Information section.

Supporting File: bit70201-supp-0001-TempVitSubstrates_Aspergillus_oryzae_Supplements.docx.