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Influence of Zn^{2+} and Oxygen Supply on Malic Acid Production and Growth of *Aspergillus oryzae*

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ABSTRACT

Malic acid is a valuable platform chemical traditionally derived from fossil-based resources. Microbial cultivation with *Aspergillus oryzae* offers a sustainable alternative based on renewable feedstocks. In this study, a well-established minimal medium for malic acid production, commonly used in previous research to ensure reproducibility, was employed. Despite its widespread use, respiration monitoring combined with stepwise nutrient pulsing unexpectedly revealed a nutrient depletion after 8 h of cultivation. Zn^{2+} limitation was identified through a combination of respiration monitoring and systematic stepwise nutrient pulsing. Supplementation of Zn^{2+} increased oxygen consumption, leading to hypoxic conditions. This induced hypoxia enhanced malic acid production and influenced the overall organic acid profile. Different dynamic oxygen concentration strategies were tested to evaluate their effect on malic acid productivity, showing that allowing growth into hypoxia and maintaining hypoxia throughout the production phase resulted in the best performance. By combining Zn^{2+} supplementation, maintaining a culture pH of 7.00 and Zn^{2+}-induced hypoxia, final malic acid concentrations were elevated from 31.44 g L^{-1} to 45.28 g L^{-1}, with a yield of 0.61 g malic acid per g of glucose and an average productivity of 0.19 g L^{-1} h^{-1}.

1 | Introduction

Malic acid and its precursor maleic anhydride are currently produced as racemate from fossil raw materials like benzene and butane (Kerr 1993; Kövilein et al. 2019; Udovich and Meyers 1981; Winstrom et al. 1968). With an annual production of 105 kt in 2023, malic acid is used in the food and beverage industry as an acidifier and flavoring agent, buffer in cosmetic products, chelator in cleaning agents and as an additive in drug formulations (Aldrich et al. 1979; Bellon 2003; ChemAnalyst 2024; Gore et al. 2010; Hornstein 1969; Sakurai and Sugimoto 2001). However, the foreseeable depletion of fossil resources and the environmental drawbacks of petrochemical synthesis underscore the need for bio-based production of platform chemicals (Bozell and Petersen 2010). Microbial cultivation not only relies on renewable feedstocks but also yields enantiomerically pure malic acid, eliminating costly fumarase-based enantiomer purification (Ortiz et al. 2017). A frequently used fungus in research for microbial production of malic acid is the obligate aerobe *Aspergillus oryzae*, convincing with a broad substrate spectrum suitable for the utilization of agro-industrial side streams (Dörsam et al. 2017; Lee et al. 2016). The long-standing use of this *Ascomycete* in the industrial production of hydrolytic enzymes, soy sauce, sake and miso illustrates both its technological scalability and its recognition as a generally recognized as safe (GRAS) organism specifically for food-related applications (Akaike et al. 2020; EFSA CEP Panel et al. 2022; Frisvad et al. 2018; Kusumoto et al. 2021; Liang et al. 2009; Sun et al. 2024).

Optimization of aerobic cultivations generally benefits from monitoring respiratory activity through continuous measurement of dissolved oxygen tension (DOT) and off-gas composition to determine oxygen (OTR) as well as carbon dioxide transfer rate (CTR), providing real-time insights into cultivation progress and

performance (Anderlei et al. 2004; Finger et al. 2023; Heyman et al. 2020). However, the potential for monitoring respiratory activity in *A. oryzae* cultivations for malic acid production remains underexplored, partly due to the widespread use of offline shake flask (SF) experiments and the common practice of using $CaCO_3$ as neutralizing agent (Schmitt et al. 2021). The CO_2 released during the acid-carbonate reaction is challenging to distinguish from respiratory CO_2, complicating accurate metabolic characterization. Therefore, we established malic acid production neutralized with NaOH, allowing monitoring of respiratory CO_2 release (Hartmann et al. 2026).

To complement online analytics and to ensure consistent process conditions, the cultivations in this study rely on a defined minimal medium first published by Abe et al. in 1962 (Abe et al. 1962). Using this minimal medium and based on respiration monitoring, the cultivation process can be divided into three distinct phases (Hartmann et al. 2026): In the initial growth phase, the fungus proliferates during nutrient-excess conditions, as indicated by a steep decline in DOT and a simultaneous increase in OTR and CTR. In the second phase, the DOT continues to decline nearly linearly, while OTR and CTR remain at plateau levels, indicating a shift from unlimited to constrained growth. The third phase marks the transition into an ammonium-depleted stationary state indicated by a gradual decrease in OTR and CTR. During this stage, most organic acids are produced. Based on observations from our previous study, where growth restriction during the second phase had been assumed to result from nutrient depletion, the present study aims to investigate this hypothesis in more detail.

Identifying such limitations is critical for optimizing bioprocess efficiency, yet conventional analytical methods often require time-consuming *post-hoc* assays to detect nutrient deficiencies. In this study, we utilize the DOT as a rapid response indicator to identify the cause of the observed growth limitation by pulsing medium components during the restricted growth phase. The constant oxygen supply during pulsing experiments ensures that changes in the DOT directly reflect the oxygen consumption of the culture. This approach allows immediate distinction between nutrient sufficiency and deficiency.

Once the depleting nutrient is identified, it is supplemented to alleviate the growth restriction. Due to the constant oxygen supply and increased oxygen demand caused by supplementation, malic acid is produced during hypoxic conditions. To differentiate the effects of the supplemented nutrient and varying oxygen availability, the volumetric mass transfer coefficient (k_La) is characterized for the used reactor set-up and manipulated to intentionally create normoxic and hypoxic environments in the presence and absence of the supplemented nutrient (Özbek and Gayik 2001). In essence, this study demonstrates both a rapid and straightforward method for identifying nutrient depletions and highlights the oxygen dependency of malic acid production.

2 | Methods

2.1 | Microorganism and Media

A. oryzae DSM 1863 was obtained from DSMZ strain collection (Deutsche Sammlung von Mikroorganismen und Zellkulturen GmbH, Braunschweig, Germany). All media were prepared using demineralized water. Conidia production was carried out in alignment with the procedure described by Kövilein et al. (Kövilein et al. 2021). Sporulation of *A. oryzae* was performed on minimal medium, containing 15 g L^{-1} glucose monohydrate, 6 g L^{-1} $NaNO_3$, 22.37 g L^{-1} KCl, 0.52 g L^{-1} $MgSO_4{\cdot}7H_2O$, 1.52 g L^{-1} KH_2PO_4, 15 g L^{-1} agar and 2 mL L^{-1} of Hutner's Trace Elements (HTE) (Barratt et al. 1965; Song et al. 2001). The medium's pH was adjusted to 6.5 using NaOH prior to sterilization by autoclaving at 121°C for 20 min. The concentrated trace element solution comprised 5 g L^{-1} $FeSO_4{\cdot}7H_2O$, 50 g L^{-1} Na_2EDTA, 22 g L^{-1} $ZnSO_4{\cdot}7H_2O$, 11 g L^{-1} H_3BO_3, 5 g L^{-1} $MnCl_2{\cdot}4H_2O$, 1.6 g L^{-1} $CoCl_2{\cdot}6H_2O$, 1.6 g L^{-1} $CuSO_4{\cdot}5H_2O$ and 1.1 g L^{-1} $(NH_4)_6Mo_7O_{24}{\cdot}4H_2O$ at pH 6.5 and sterilized by filtration using a 0.2 µm membrane filter (Hill and Kafer 2001). Sporulation occurred over 6 days at 30°C. The resulting conidia were collected in 50% (v/v) glycerol, passed through Miracloth (Merck KGaA, Darmstadt, Germany), quantified using a hemocytometer and stored at -20°C in aliquots.

The media compositions used for preculture and main culture were based on previously described procedures (Kövilein et al. 2022). 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 by autoclaving, while trace elements were added afterward in the form of 2 mL L^{-1} HTE solution. For one specific experiment, the addition of HTE in the preculture medium was replaced with demineralized water. This HTE-lacking biomass was used for inoculation of one reactor duplicate.

For the main culture, the medium was composed of 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} $FeSO_4{\cdot}7H_2O$. For sterilization, $(NH_4)_2SO_4$, KH_2PO_4, K_2HPO_4 and NaCl were autoclaved together, with 100 µL of the antifoam agent Contraspum A 4050 HAc (Zschimmer & Schwarz GmbH & Co KG, Lahnstein, Germany), which was exclusively added for STR experiments. Glucose monohydrate was autoclaved separately, while $FeSO_4{\cdot}7H_2O$, $CaCl_2{\cdot}2H_2O$ and $MgSO_4{\cdot}7H_2O$ were sterile-filtered.

2.2 | Preculture Conditions

Precultures were cultivated in 500 mL baffled SF containing 100 mL medium, inoculated with 2×10^5 conidia mL^{-1}. The SF were incubated for 24 h at 30°C at 100 rpm in an orbit diameter of 25 mm. Subsequently, the resulting biomass was harvested using Miracloth filtration and rinsed with demineralized water. STR main cultures were inoculated with 7.5 g L^{-1} of the washed fungal biomass (Kövilein et al. 2022).

One specific STR duplicate was inoculated with biomass for which the volume of HTE in the preculture was replaced with demineralized water. In addition, the impact of trace element supplementation on biomass growth in the preculture was investigated. In one setup, the preculture was supplemented as usual with 2 mL L^{-1} HTE. In a second setup, the same volume of a 22 g L^{-1} $ZnSO_4{\cdot}7H_2O$ solution was added instead of HTE. In a third setup, the volume corresponding to HTE was replaced with demineralized water.

2.3 | Main Culture Conditions

STR cultivations were conducted in 2.5 L bioreactors (Minifors, Infors AG, Bottmingen, Switzerland) with a working volume of

1.4 L. All cultivations were performed at 32°C using two Rushton turbines with diameters of 4.5 cm. Operational settings of all STR cultivations are shown in Supplement 1. In specific cultivations, the k_La was tuned via specific agitation and aeration settings to meet microbial oxygen consumption, resulting in normoxic or hypoxic conditions. Aeration rate was increased first, with agitation frequency adjusted only as necessary to minimize potential effects of shear stress. Normoxic and hypoxic conditions were operationally defined based on DOT trends, with normoxic conditions characterized by sustained elevated DOT levels and hypoxic conditions being indicated by a rapid and persistent decrease in DOT. Some cultures underwent a controlled shift in DOT resulting in hypoxic and normoxic conditions during growth and production. All cultivations were aerated with sterile air. The pH was controlled at 6.50 ± 0.05 using 4 M NaOH and 4 M H_3PO_4. One specific cultivation was maintained at pH 7.00 ± 0.05. Neutralizers were kept on balances, enabling calculation of the consumed volume from recorded mass, density and pump flow over time. For comparability across experiments, the neutralizer volume was divided by the initial cultivation volume of 1.4 L. The volume of H_3PO_4 added for pH control was minimal and fell below the detection limit for gravimetric quantification. The pH sensor (EasyFerm Plus, Hamilton Bonaduz AG, Bonaduz, Switzerland) was calibrated with standard buffer solutions at pH 4.00 and 7.00. The DOT sensor (VisiFerm, Hamilton Bonaduz AG) was two-point calibrated using 100% N_2 and 100% air. Off-gas analysis was conducted using a gas sensor (BlueVary, BlueSens gas sensor GmbH, Herten, Germany), calibrated with air and used to monitor O_2 and CO_2 partial pressures as well as absolute humidity. To minimize fungal adhesion on reactor surfaces, baffles were omitted. Additionally, convex DOT sensor caps (ODO cap H2, Hamilton Bonaduz AG, Bonaduz, Switzerland) were applied to reduce fouling by biomass. To avoid sparger clogging, aeration was provided through a curved metal tube with a single opening of 4 mm in diameter. The lower impeller was installed 1 cm above the end of the stirring shaft, with a vertical distance of 6.5 cm to the upper impeller. Sampling was performed by withdrawing 10 mL of culture broth containing fungal biomass. In specific experiments, the main culture medium composition was additionally supplemented with 2 mL L^{-1} HTE or 2 mL L^{-1} of a 22 g L^{-1} $ZnSO_4 \cdot 7H_2O$ solution. An overview of the cultivation conditions is provided in Table 1. Most STR cultivations were performed in biological duplicates, while pulse experiments were conducted over shorter periods in singlicates.

2.4 | Nutrient Pulse Experiments

To investigate the effect of nutrient pulsing during constraint growth, individual reactor cultures were grown to the phase of nutrient depletion with operating conditions equivalent to the reference cultivation. The amount of pulsed nutrients corresponded to 50% of the initially supplied quantity, following the protocol for the main culture medium, or matched the concentration present in 1 mL L^{-1} HTE. It should be noted that the pulsed trace element solutions were freshly prepared prior to each addition, omitting EDTA as used in HTE.

2.5 | k_La Determination

To determine k_La, the gassing-out method was employed (Blažej et al. 2004). Initially, N_2 was introduced into the system to establish anoxic conditions. Subsequently, various combinations of aeration rate and agitation frequency were applied to capture the operational window of the conditions prevailing in this study. The k_La was calculated from the change in DOT using Equation 1, with an oxygen saturation concentration $c^*_{O_2}$ of 7.10 mg L^{-1} during the present operating conditions, and c_{O_2} as the measured oxygen concentration.

$$\ln\left(c^*_{O_2} - c_{O_2}\right) = -k_La\, t + \ln(c^*_{O_2}) \quad (1)$$

It should be noted that the k_La determination was conducted during abiotic conditions, fungal aggregates might alter the k_La during cultivation (Galaction et al. 2003).

2.6 | Analytics

Samples from STR experiments were filtered through a 0.5 mm mesh, the retained solids were rinsed with demineralized water, scanned at 2400 dpi (Perfection V600 Photo, Epson Deutschland GmbH, Düsseldorf, Germany), dried at 80°C for a minimum of

TABLE 1 | Overview of STR cultivations performed in duplicates.

STR cultivation	Trace elements in preculture	Trace elements in main culture	Growth	Production	pH [-]
Reference	HTE	—	Normoxia	Normoxia	6.50
Hypoxia	HTE	—	Hypoxia	Hypoxia	6.50
+ HTE	HTE	HTE	Hypoxia	Hypoxia	6.50
Preculture w/o HTE	—	Zn^{2+} (pulsed)	Normoxia	mostly Normoxia	6.50
+ Zn^{2+}	HTE	Zn^{2+}	Hypoxia	Hypoxia	6.50
+ Zn^{2+}, complete normoxia	HTE	Zn^{2+}	Normoxia	Normoxia	6.50
+ Zn^{2+}, partly hypoxia	HTE	Zn^{2+}	Normoxia	Hypoxia	6.50
+ Zn^{2+}, partly normoxia	HTE	Zn^{2+}	Hypoxia	Normoxia	6.50
+ Zn^{2+}, pH 7.00	HTE	Zn^{2+}	Hypoxia	Hypoxia	7.00

Note: HTE refers to Hutner's Trace Elements.

2 days and weighed to determine the cell dry weight (CDW) (Ferreira et al. 2014; Uwineza et al. 2021). This method was also applied to determine the CDW in the preculture experiment investigating the effects of different trace element supplements. The filtrates of the STR samples were frozen at -20°C for metabolite analysis. The biomass composition of *A. oryzae* used for carbon retracing was published previously (Hartmann et al. 2026).

Ammonium concentrations were determined photometrically using the Spectroquant assay kit (114752, Merck KGaA, Darmstadt, Germany), following the manufacturer's protocol. The assay was miniaturized to a total volume of 200 µL and conducted in microtiter plates using filtrate from each reactor, measured in singlicates (Kövilein et al. 2022).

For metabolite quantification, protocols were adapted from Kövilein et al. and previously published by Hartmann et al. (Hartmann et al. 2026; Kövilein et al. 2021). Filtrates of STR experiments were diluted 10-fold with 0.66 M H_2SO_4, incubated at 80°C and 1000 rpm in an orbit diameter of 3 mm for 20 min and centrifuged for 5 min at 17,000 × *g*. The resulting 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%) analytical column (300 × 7.8 mm) and guard column (Phenomenex, Aschaffenburg, Germany). Isocratic separation was performed with 5 mM H_2SO_4 as the mobile phase at a flow rate of 0.5 mL min^{-1}, an injection volume of 10 µL and a column temperature of 30°C. Glucose was detected via refractive index detection, while organic acids were quantified using a UV detector at 220 nm.

2.7 | Calculations

Used software and calculations of metabolite mass balances, time-dependent cultivation volume and gas transfer rates were previously published (Hartmann et al. 2026). Volume changes due to sampling, neutralization and evaporation were quantitatively considered to avoid dilution effects. Evaporation was determined based on the absolute humidity in the off-gas, while OTR, CTR and respiratory quotient (RQ) were calculated from the off-gas composition established by Knoll et al. in separate studies and described by Hartman et al. (Hartmann et al. 2026; Knoll et al. 2007; Knoll et al. 2005). Gas transfer rates and metabolite masses were divided by the initial culture volume, enabling direct comparison across different cultivation conditions. Product selectivity was assessed based on the fraction of consumed carbon allocated to different products from glucose conversion. Total molar productivities of organic acids were calculated to quantify and compare their overall formation.

Statistical significance of CDW values yielded by different preculture supplementation conditions was evaluated using one-way ANOVA followed by Tukey's post-hoc test. Differences were considered weakly significant at $p < 0.05$, significant at $p < 0.01$ and strongly significant at $p < 0.001$.

3 | Results

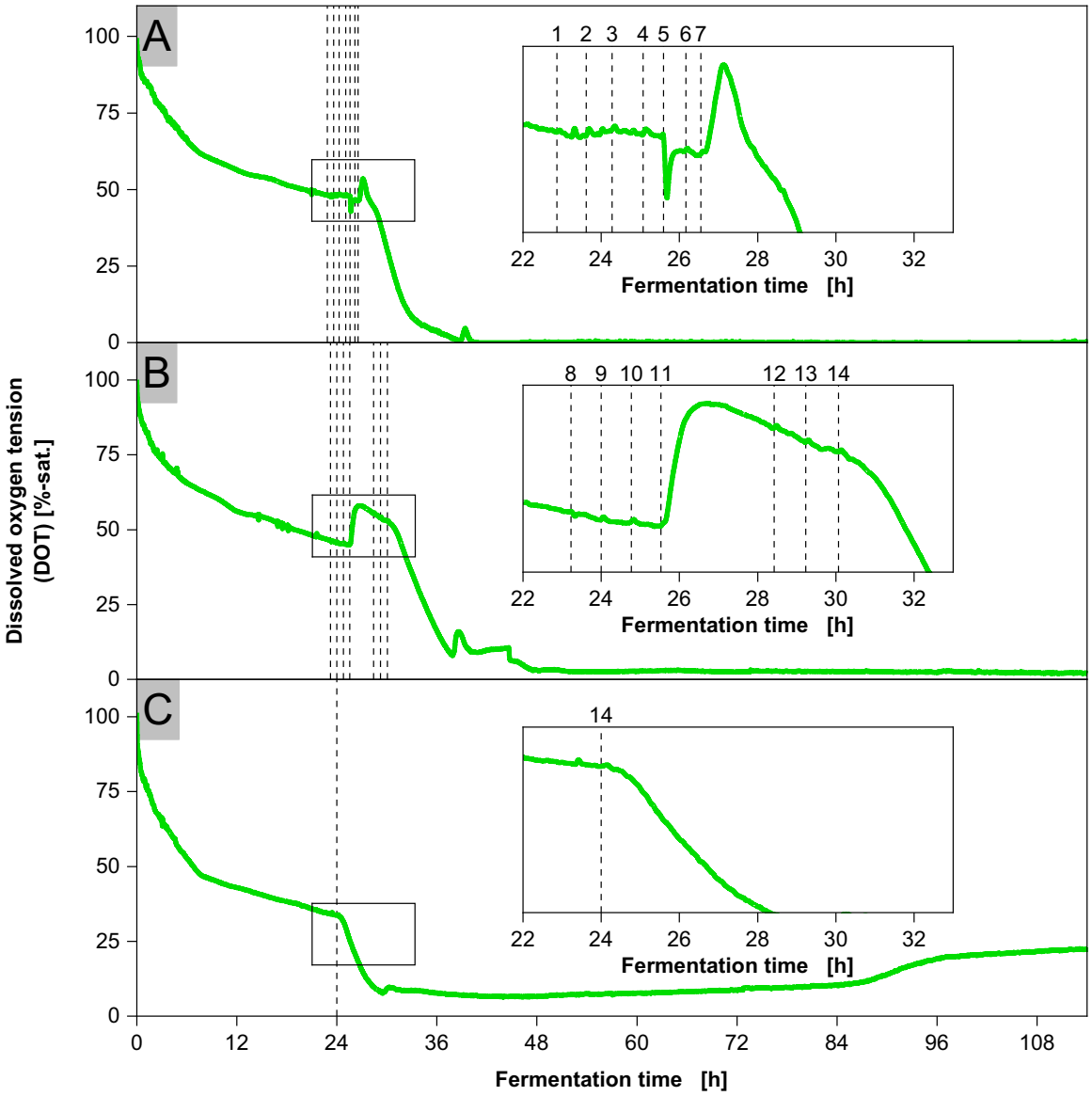
3.1 | Identification of Growth Limiting Nutrient

In order to identify a potential depleted nutrient, the reference cultivation previously published was reproduced until the onset of the limited growth phase. Then, individual medium components were sequentially pulsed. According to the hypothesis, addition of the depleted nutrient should alleviate growth restrictions and restore respiratory activity. As shown in Figure 1A, the respiratory activity remained unchanged upon the addition of all nutrients present in the main culture medium. The transient drop in DOT following the addition of $FeSO_4 \cdot 7H_2O$ stored in H_2SO_4 (5) was most likely attributed to a short increase in oxygen consumption due to increased energy demand for pH homeostasis. This theory also explains the lack of DOT decrease after addition of freshly prepared $FeSO_4 \cdot 7H_2O$ without H_2SO_4 (8). Consequently, the limiting nutrient originates from a source other than the main culture medium. As trace elements in HTE were the only other components contacting the biomass, even after washing the mycelium following preculture harvesting, HTE was considered the most likely source of the depleting nutrient. Pulsing HTE initially caused a decline in respiratory activity within the first half-hour after addition, followed by a sharp increase. Consequently, one or more components of HTE likely cause restricted growth upon depletion in the initial phase of the main cultivation.

In order to identify the limiting nutrient in Figure 1B, trace elements present in HTE were individually added during restricted growth. EDTA, as present in HTE, was tested separately in advance and resulted in unchanged restricted growth (data not shown). After the addition of $CuSO_4 \cdot 5H_2O$, the DOT increased similarly to the addition of HTE, but then decreased considerably slower compared to HTE. About half an hour after the addition of $ZnSO_4 \cdot 7H_2O$, the DOT dropped sharply, similar to the peak observed after the addition of HTE. The direct DOT increase after the addition of Cu^{2+} indicated an inhibitory effect of the applied concentration. To test for potential interactions between the responses of multiple trace elements and the addition of Zn^{2+}, the metabolic response to the sole addition of Zn^{2+} was examined in Figure 1C. The DOT decrease started half an hour after the addition and the increase in respiratory activity corresponds to the response observed after the addition of Zn^{2+} in Figure 1B and of HTE in Figure 1A. As evidenced by the observed changes in respiratory activity following its addition, Zn^{2+} was identified as the limiting nutrient in the main culture organic acid production medium.

3.2 | Supplementing Trace Elements for Organic Acid Production

The initial phase of unrestricted growth observed in the reference cultivation supported the hypothesis of sufficient Zn^{2+} availability in the medium until the CTR plateau develops. Since Zn^{2+} was present only in the preculture medium, which was removed by thoroughly washing the fungal mycelium before inoculation, Zn^{2+} was suspected to enter the main culture via the biomass inoculum. To verify this hypothesis, biomass originating from the preculture prepared without trace element supplementation was used as inoculum for one STR duplicate. With HTE, four SFs per reactor typically provide 10.5 g of wet biomass for inoculation. However, as shown in Figure 2, the absence of trace elements in the preculture significantly reduced the fungal biomass harvested after 24h, the effect was strongly significant when trace elements were omitted entirely and still weakly significant when only Zn^{2+} was



#	Additive	Amount [mg]	#	Additive	Amount [mg]
1	$(NH_4)_2SO_4$	840.0	8	$FeSO_4 \cdot 7H_2O$ (freshly soluted)	7.0
2	KH_2PO_4 & K_2HPO_4	70.0 & 119.0	9	H_3BO_3	15.4
3	$MgSO_4 \cdot 7H_2O$	70.0	10	$CoCl_2 \cdot 6H_2O$	2.2
4	$CaCl_2 \cdot 2H_2O$	70.0	11	$CuSO_4 \cdot 5H_2O$	2.2
5	$FeSO_4 \cdot 7H_2O$ (in 0.1 M H_2SO_4)	42.0	12	$(NH_4)_6Mo_7O_{24} \cdot 4H_2O$	1.5
6	NaCl	3.5	13	$MnCl_2 \cdot 4H_2O$	7.0
7	Hutner´s Trace Elements (HTE)	1.4 [mL]	14	$ZnSO_4 \cdot 7H_2O$	30.8

FIGURE 1 | Dissolved oxygen tension (DOT) in pulse cultivations with *Aspergillus oryzae* DSM 1863 in 2.5 L STR. Amounts of pulsed medium components correspond to 50% of the initial amounts or 1 mL L^{-1} HTE respectively. Numbers and dotted lines mark pulsed additives. Cultivations were performed with X_0 of 7.5 g L^{-1} biomass, 109 g L^{-1} glucose, V_0 of 1.4 L, 32°C, 400 rpm and 0.7 L_n min^{-1} air. pH was maintained at 6.50 ± 0.05 with 4 M NaOH and 4 M H_3PO_4. The online signals were smoothed over 100 data points.

supplemented. Without HTE, two additional SFs per reactor were required to produce the demanded biomass for inoculation.

The absence of trace elements in the preculture resulted in strongly inhibited growth of *A. oryzae* in main culture, as reflected in respiratory activity, shown in Figure 3, as well as in CDW and ammonium concentrations shown in Figure 4.

Compared to the biomass of the reference cultivation, fungal pellets, as illustrated in Figure 5, exhibited an aberrant phenotype, characterized by smaller pellet size and increased

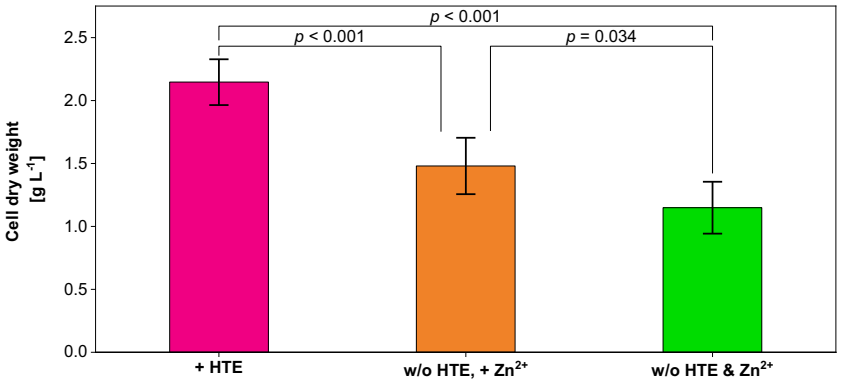


FIGURE 2 | Cell dry weight (CDW) of precultures with *Aspergillus oryzae* DSM 1863 in 500 mL baffled SF with varying supply of trace elements. Cultivations were performed with X_0 of 2×10^5 conidia mL^{-1}, 36 g L^{-1} glucose, V_0 of 100 mL, 30°C, 100 rpm in an orbit diameter of 25 mm. Data represent means ± standard deviations of biological hexaplicates.

aggregation. Zn^{2+} supplementation restored respiratory activity, enabled unrestricted growth and initialized acid production. These findings indicate Zn^{2+} introduction via the inoculum at the start of the reference cultivation.

The proposed transfer of Zn^{2+} via the inoculum prompted an investigation into whether other metal ions might influence the main culture. To this end, the medium composition was supplemented with either HTE or $ZnSO_4 \cdot 7H_2O$. Initially in Figure 3, both supplementations resulted in cultivations developing respiratory activity similar to the reference, supporting the hypothesis of Zn^{2+} availability in the reference cultivation. During the limited growth phase of the reference, CTR and OTR in cultivations supplemented with trace elements increased to two to three times the initial levels observed in the reference. This elevated metabolic activity was reflected in faster increase of CDW and consumption of ammonium. Meanwhile, the DOT dropped below 10% and remained at this level until the end of the cultivation. Until the CTR peaks at 20 h, both cultures exhibited a similar growth trajectory, suggesting that the addition of Zn^{2+} was sufficient for unlimited biomass growth. At the time of low DOT, a flattening slope in the CTR became evident, while the OTR began to stagnate several hours earlier, indicating the onset of growth limitation due to hypoxia. Subsequently, the respiratory activity of the duplicate with HTE remained higher than in the cultures supplemented with Zn^{2+} while also being the only cultivation that displayed biomass growth continuing beyond ammonium depletion. Compared to the reference and HTE-supplemented duplicate in Figure 4, the addition of Zn^{2+} increased the production of malic, succinic and fumaric acids, while the production of citric acid decreased. As indicated in Figure 5, the addition of Zn^{2+} resulted in less dense pellets with longer hyphal extensions, HTE addition led to denser pellets, with part of the biomass aggregated into small pellets.

In order to analyze the impact of hypoxia without the supplementation of additional trace elements, hypoxic conditions were to be established during cultivation. For this purpose, k_La was characterized by applying a spectrum of agitation frequencies and aeration rates, as shown in Figure 6. Generally, k_La increases with aeration intensity; however, the effect levels off at higher aeration rates. Furthermore, elevated stirring frequencies systematically enhance mass transfer across the entire aeration spectrum.

Based on the obtained data, an initial aeration rate of 0.25 vvm and an agitation rate of 300 rpm were selected to ensure hypoxic conditions during the initial growth phase. However, as the DOT level after 17 h exceeded initial predictions, a cascade control regulating the DOT at 5% via the aeration rate was implemented, as shown in Figure 3 and Supplement 1. The reduced OTR and CTR compared to the reference confirm the onset of oxygen limitation, resulting in a slower increase of CDW and decelerated ammonium consumption. With the base addition starting to increase considerably only after 3 days, the production of succinic and malic acid, as shown in Figure 4, began more slowly in comparison to the reference. Although initial growth was slower during hypoxic conditions, the overall malic acid productivity was 14% higher compared to the reference, as shown in Table 2.

As illustrated in Supplement 2, reference and hypoxia cultures reached a comparable maximum productivity; however, hypoxia enabled a prolonged maintenance of a higher productivity during the later stages of cultivation. After 240 h, productivity during hypoxia was approximately twice as high as in the reference culture. The overall increase in productivity upon Zn^{2+} addition shown in Figure 4 can thus be attributed both to the faster biomass growth and to a hypoxia-stimulated malic acid production phase. A comparable trend was observed for succinic acid production. The observed reduction in citric acid production during hypoxia in the presence of Zn^{2+} was also evident during hypoxic conditions lacking Zn^{2+}, indicating that the decrease was primarily caused by limited oxygen availability. With respect to biomass morphology shown in Figure 5, cultures exposed to hypoxic conditions exhibited a visibly looser and less compact structure than those grown during normoxia.

The impact of oxygen availability with supplementation of Zn^{2+} was subsequently analyzed by using automated control of the aeration rate and a manual increase in agitation frequency to keep the culture in complete normoxic conditions. As a result, the maximum respiratory activity, as shown in Figure 3, increased 6-fold compared to the reference culture. After the depletion of ammonium, growth ceased and the respiratory activity returned to the level observed in the culture without DOT leveling. Increased oxygen availability reduces the productivity of malic, succinic and fumaric acids, while citric acid production increases, reaching concentrations comparable with the reference and HTE-supplemented culture.

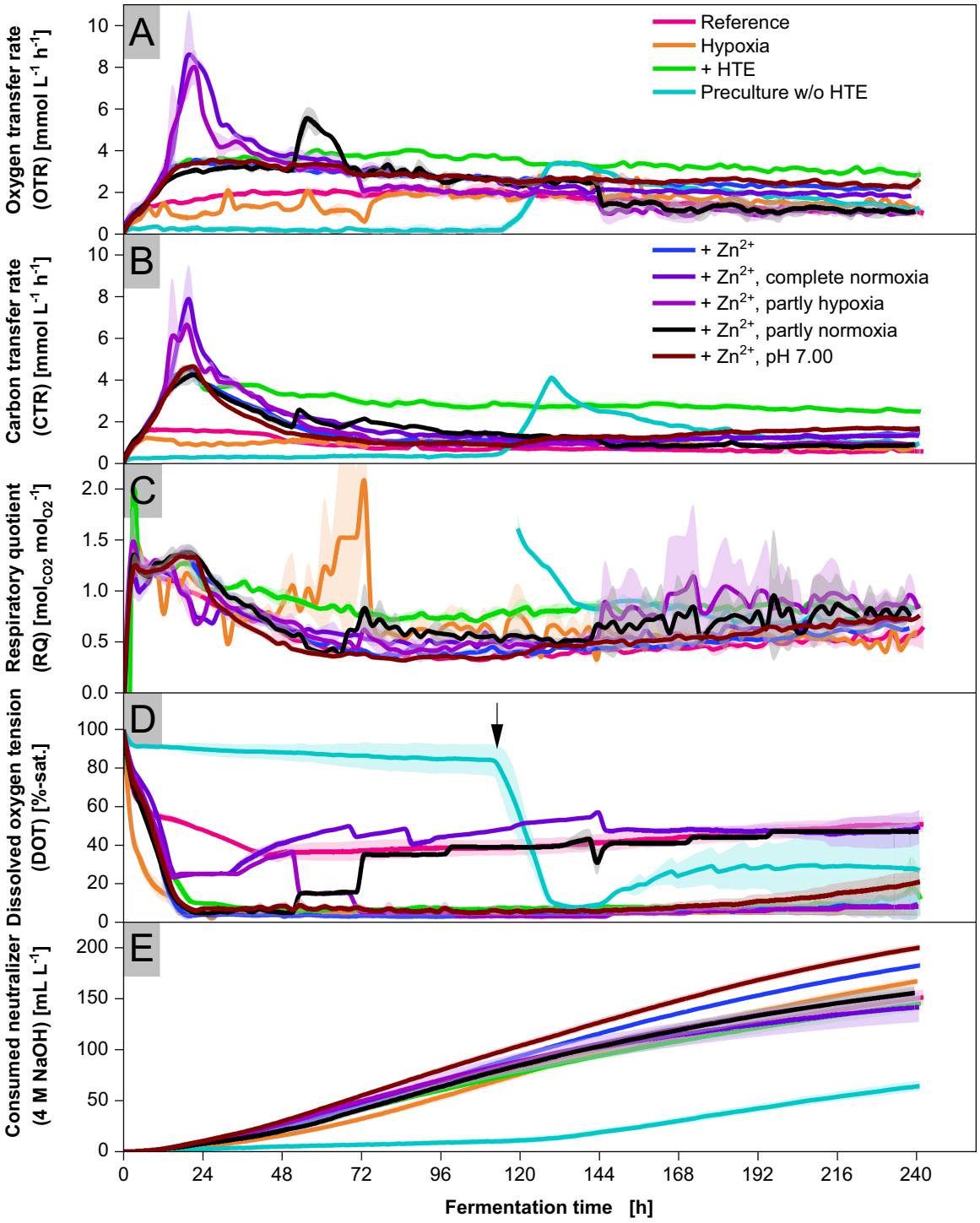


FIGURE 3 | Online monitoring of cultivations with *Aspergillus oryzae* DSM 1863 in 2.5 L STR at varying oxygen tensions, trace element supply and pH. Cultivations were performed with X_0 of 7.5 g L^{-1} biomass, 109 g L^{-1} glucose, V_0 of 1.4 L, 32°C, agitation frequency and aeration rate contained in Supplement 1. If not stated otherwise, pH was maintained with 4 M NaOH and 4 M H_3PO_4 at 6.50 ± 0.05. The black arrow indicates addition of Zn^{2+} equivalent of 1 mL L^{-1} HTE. The reference cultivation originates from the previously published study (Hartmann et al. 2026). The online signals were averaged over 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.

In order to elucidate the effect of oxygen availability on malic acid productivity during Zn^{2+} sufficiency, one duplicate of cultivations involved an initial hypoxic phase followed by a switch to normoxia after 3 days, while another duplicate of cultivations employed the inverse oxygen profile, as shown in Figure 3. Both duplicates, with partial hypoxia as well as partial normoxia in production phase, produced 24% and 19% higher malic acid concentrations, respectively, after 10 days compared to the duplicate in complete normoxia. However, when compared to the cultivations during continuous hypoxia with Zn^{2+} supplementation, the malic acid production was 14% and 17% lower, respectively. For optimal malic acid production, the

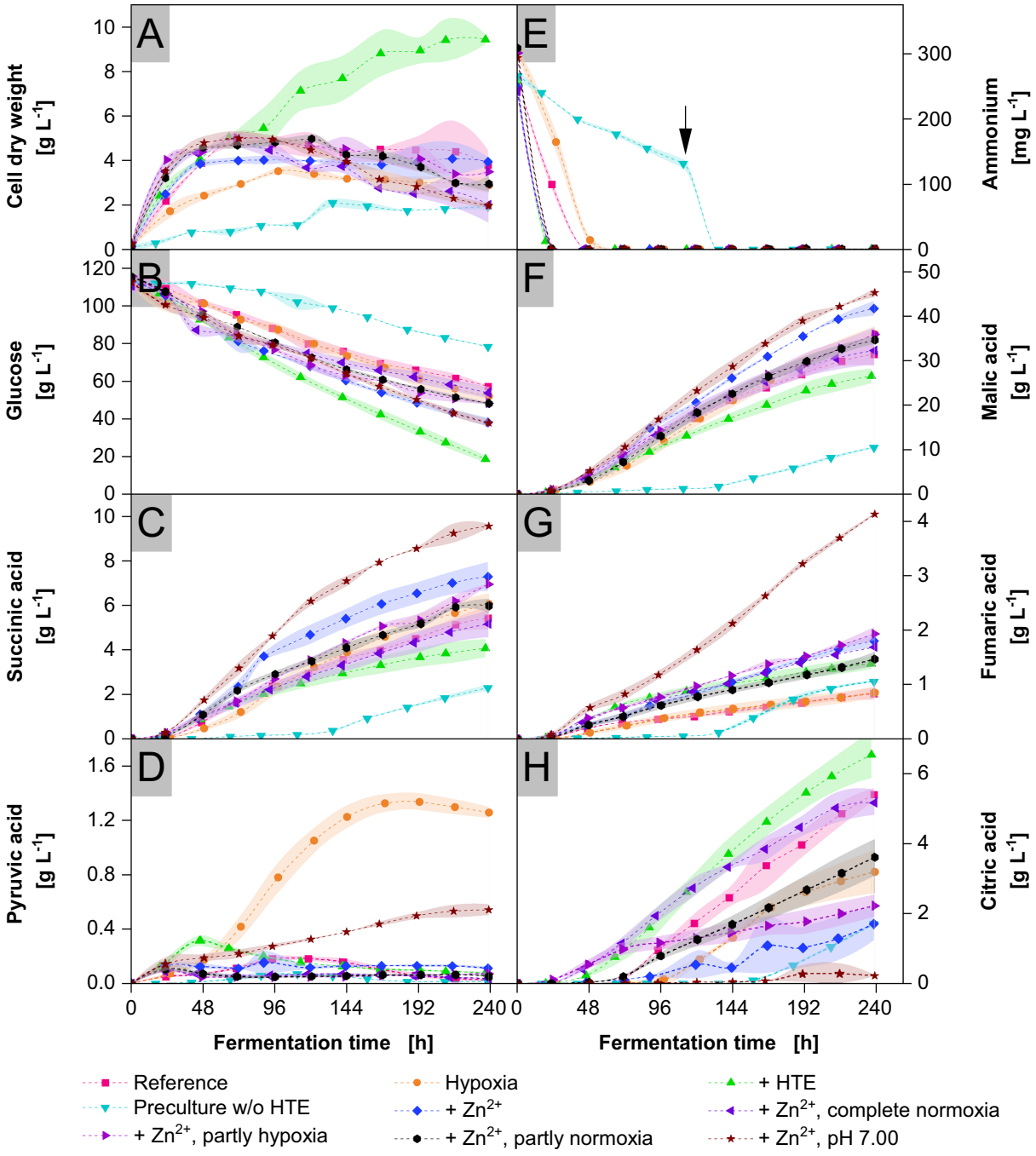


FIGURE 4 | Metabolites in cultivations with *Aspergillus oryzae* DSM 1863 in 2.5 L STR at varying oxygen tensions, trace element supply and pH. Cultivations were performed with X_0 of 7.5 g L^{-1} biomass, 109 g L^{-1} glucose, V_0 of 1.4 L, 32°C, agitation frequency and aeration rate contained in Supplement 1. If not stated otherwise, pH was maintained with 4 M NaOH and 4 M H_3PO_4 at 6.50 ± 0.05. The black arrow indicates addition of Zn^{2+} equivalent of 1 mL L^{-1} HTE. Concentrations for ammonium consider volume adjustments by evaporation and neutralizer but do not account for loss by sampling. The reference cultivation originates from the previously published study (Hartmann et al. 2026). Data represent means ± standard deviations of biological duplicates. Dashed lines and Akima-spline-connected standard deviations are provided for visual guidance.

ideal oxygen supply appears to be an early onset of hypoxia, followed by its sustained maintenance. In contrast, citric acid production appears to be more oxygen-sensitive. In both Zn^{2+}-supplemented experiments with initial normoxia, the citric acid production remained very similar until oxygen availability was reduced in one set-up. Upon the establishment of hypoxia, citric acid productivity considerably decreased, akin to the cultivations maintained during continuous hypoxia. On the other hand, cultivations with initial hypoxia produced very little citric acid and their productivity increased to the level of the cultivation during continuous normoxia after oxygen availability was elevated. In summary, to suppress citric acid formation as a by-product, a continuous hypoxic environment is required.

To further enhance malic acid production, the pH was increased from 6.50 to 7.00 in a Zn^{2+}-supplemented culture, as Hartmann et al. reported elevated malic acid production at this pH (Hartmann et al. 2026). While respiratory activity, shown in Figure 3, remained largely unchanged compared to the set-up

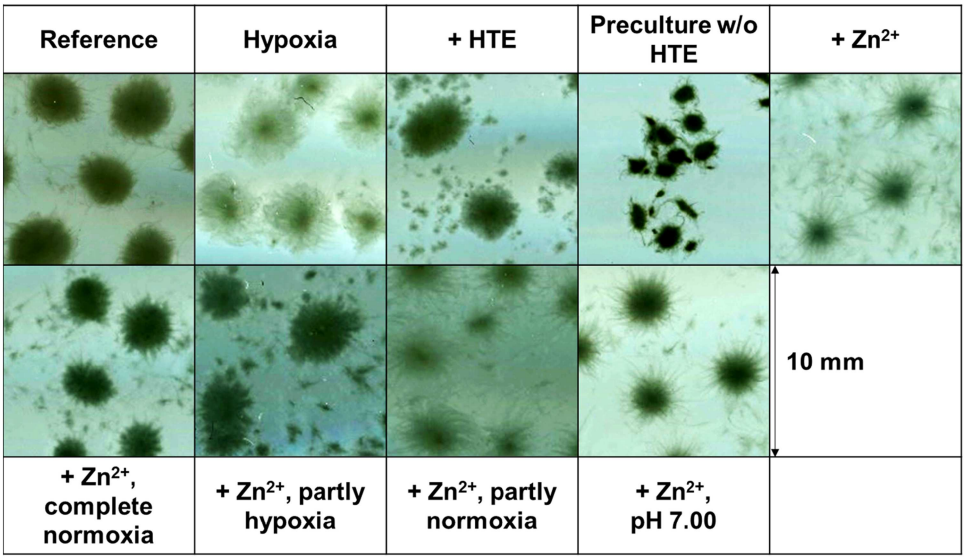


FIGURE 5 | 2D scans of *Aspergillus oryzae* DSM 1863 in STR cultivations at varying oxygen tensions, trace element supply and pH. All images represent biomass harvested after 2 days of cultivation. Contrast and brightness of the images are adjusted to enhance visibility. All images are processed using the same settings.

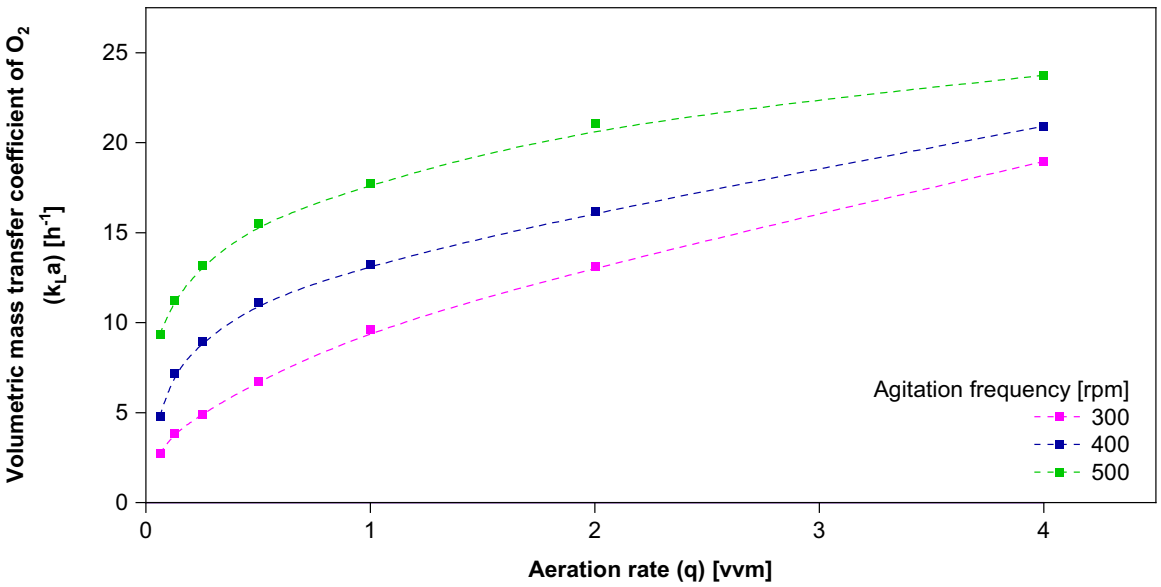


FIGURE 6 | Volumetric oxygen transfer coefficient (k_La) in 2.5 L STR with 1.4 L main culture medium, 32°C and pH 6.50 ± 0.05 with 4 M NaOH and 4 M H_3PO_4. Calculations are based on the DOT increase following aeration with air after deoxygenation with N_2.

at pH 6.50 with Zn^{2+} supplementation, the increased NaOH consumption mirrored the stimulated production of organic acids, as shown in Figure 4. Similar to reports by Hartmann et al. citric acid production was further reduced (Hartmann et al. 2026).

Cultivating entirely in normoxia with Zn^{2+} supplementation directed 23% of carbon into CO_2, while Zn^{2+} supplementation at pH 7.00 increased malic acid yield to 54% of consumed carbon. The latter generated the highest molar productivity of quantified organic acids, as shown in Figure 7. In general, a higher fraction of carbon is channeled into malic acid during hypoxic conditions compared to the respective normoxic counterparts.

4 | Discussion

The use of highly pure chemicals and meticulously cleaned equipment reduces unintended trace element contamination and reveals true medium limitations. Consequently, the avoidance of trace element contamination in modern laboratory environments can markedly influence the performance of long-established media - such as the one used in this study - potentially leading to deviations from previously reported results obtained during historically less defined conditions. Therefore, the supplementation of trace elements eliminates sensitivity of unintended contaminations, ensuring that otherwise trace element-starved cultures are not inadvertently affected by residual impurities of instruments or medium

TABLE 2 | Key performance parameters of malic acid production upon trace element supplementation in cultivation with *Aspergillus oryzae* DSM 1863.

Condition	c_{max} [g L^{-1}]	$Y_{MA/S}$ [mg g^{-1}]	P_{MA} [mg L^{-1} h^{-1}]	P_{MA} [mM h^{-1}]
Reference	31.44 ± 2.67	549 ± 48	132 ± 11	0.98 ± 0.09
Hypoxia	35.93 ± 1.79	616 ± 17	150 ± 7	1.12 ± 0.06
+ HTE	26.52 ± 1.63	276 ± 20	112 ± 7	0.84 ± 0.06
Preculture w/o HTE	10.47 ± 0.21	306 ± 7	44 ± 1	0.33 ± 0.01
+ Zn^{2+}	41.73 ± 1.66	559 ± 41	175 ± 7	1.31 ± 0.06
+ Zn^{2+}, complete normoxia	29.01 ± 2.93	469 ± 65	121 ± 12	0.91 ± 0.1
+ Zn^{2+}, partly hypoxia	35.91 ± 1.53	533 ± 32	150 ± 6	1.12 ± 0.05
+ Zn^{2+}, partly normoxia	34.62 ± 0.86	515 ± 1	145 ± 4	1.08 ± 0.03
+ Zn^{2+}, pH 7.00	45.28 ± 0.98	605 ± 4	189 ± 4	1.41 ± 0.04

Note: Averaged productivities and yields were calculated based on endpoint measurements.

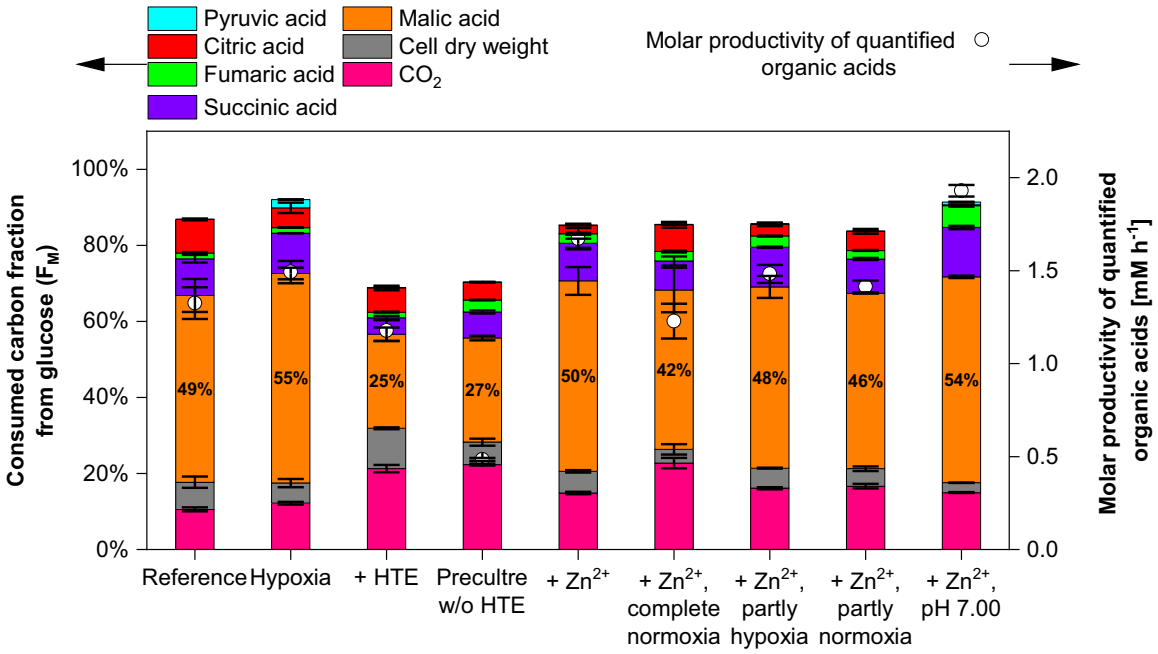


FIGURE 7 | Consumed carbon fraction from glucose and molar productivity of quantified organic acids in cultivations with *Aspergillus oryzae* DSM 1863 in 2.5 L STR at varying oxygen tensions, trace element supply and pH. Fractions and productivities were calculated for endpoint measurements. Cultivations were performed with X_0 of 7.5 g L^{-1} biomass, 109 g L^{-1} glucose, V_0 of 1.4 L, 32°C, agitation frequency and aeration rate contained in Supplement 1. If not stated otherwise, pH was maintained with 4 M NaOH and 4 M H_3PO_4 at 6.50 ± 0.05. Data represent means ± standard deviations of biological duplicates.

compounds (Shimoga Nadig et al. 2025). Shimoga Nadig et al. highlight the importance of trace element supplementation for process reproducibility. Frequently used microbial cultivation media like M9, MOPS and MTM provide trace element supplementation (Emerson and Tang 2007; Geiser et al. 2014; Neidhardt et al. 1974). However, especially for historically established minimal media with a narrow nutrient profile, online monitoring of microbial respiratory activity offers a sensitive and straightforward approach to detect performance deviations arising from nutrient scarcity.

Such monitoring not only allows the identification of nutrient limitations but also reveals inhibitory effects of an excess of trace elements. In *A. oryzae*, Cu^{2+} addition at 6.3 μM reduced respiratory activity, exceeding the 1.6 μM threshold known to strongly inhibit sclerotia formation and indicating toxic effects (Katayama and Maruyama 2023). This toxicity arises from metalloenzyme inactivation, enhanced reactive oxygen species formation, interactions with metallophilic ligands and nucleic acids, disruption of mitochondrial function through lipid and protein oxidation, as well as redox imbalance, displacement of FeS-cluster enzymes, and inactivation of key mitochondrial enzymes such as ferredoxin and aconitase (Antsotegi-Uskola et al. 2017; Cobine et al. 2021; Fridovich 1978, 1983; Lippert 1992; Macomber and Imlay 2009). Respiratory activity is restored via a combination of passive adsorption to cell wall components and active detoxification through metallothioneins, phytochelatins and Cu^{2+}-transporting ATPases (Ballou 1976; Cizewski Culotta et al. 1995; Fu et al. 1995; Hu et al. 2004; Negi

and Das 2023; Priyanka and Dwivedi 2023; Shine et al. 2015; Venkateswerlu and Stotzky 1986).

The considerably restricted growth observed in the STR duplicate lacking HTE supplementation in the preculture highlights the biomass-associated metal ion transfer from preculture to main culture. This transfer can be facilitated by cell wall-associated metal ion adsorption as well as vacuolar Zn^{2+} storages, a process in *S. cerevisiae* mediated by the vacuolar Zn^{2+} importers Zrc1 and Cot1 (DalCorso et al. 2021; Lin et al. 2008; Miyabe et al. 2000, 2001; Zhai et al. 2022). Moreover, the restored metabolic activity upon Zn^{2+} addition shows the inherent lack of this trace element in the main medium composition as well as the essential role of Zn^{2+} in central metabolism. Zn^{2+} serves as a cofactor in hydrolytic enzymes as well as a structural stabilizer (Calera and Haas 2008; McCall et al. 2000; Weston 2005). Moreover, the metal ion locates in Cys_2His_2 Zn^{2+}-finger and Zn_2Cys_6 binuclear cluster domain transcription factors (Tsuji et al. 2000). For example, Zn_2Cys_6 transcription factors regulate kojic acid production and salt tolerance in *A. oryzae* (Marui et al. 2011; Yu et al. 2025). In response to Zn^{2+} deficiency, microorganisms adapt by switching to Zn^{2+}-independent protein variants to conserve scarce metal ions for essential cellular functions. For instance, in the presence of Zn^{2+} sufficiency, *Escherichia coli* expresses the Zn^{2+}-finger protein L31 via the *rpmE* gene, whereas during Zn^{2+} limitation, the Zn^{2+}-independent L31p variant is expressed encoded by *ykgM* (Graham et al. 2009; Rasmussen et al. 2022). During Zn^{2+} deficiency, *Saccharomyces cerevisiae* downregulates the expression of Zn^{2+}-dependent alcohol dehydrogenases Adh1 and Adh3 while upregulating Adh4, potentially conserving Zn^{2+} by either utilizing Fe^{2+} as a cofactor instead of Zn^{2+} or by the lower dependency of Zn^{2+} in Adh4 than in Adh1 and Adh3 (Bird et al. 2006; Drewke and Ciriacy 1988; Eide 2009; Lyons et al. 2000; Wu et al. 2008). In response to Zn^{2+} depletion, *Aspergillus fumigatus* upregulates membrane transporters to enhance Zn^{2+} uptake, a process regulated by the Cys_2His_2 Zn^{2+}-finger protein *ZafA*, while also inducing *GliZ*, a Zn_2Cys_6 transcription factor that regulates expression of genes involved in gliotoxin biosynthesis. (Moreno et al. 2007; Seo et al. 2019; Vicentefranqueira et al. 2005). Such adaptation may serve to prepare the cells for Zn^{2+} acquisition once availability is restored, as was evidenced by the sharp rise in respiratory activity upon Zn^{2+} supplementation.

Yet, despite its physiological importance, the effect of Zn^{2+} on malic acid production remains largely unexplored. Still, the ion is present in production media, typically as part of trace element mixtures, albeit in highly variable concentrations. Studies on malic acid production, such as those by Geyer et al. Ochsenreither et al. and Hartmann et al. with *A. oryzae* and Battat et al. with *Aspergillus flavus* used the medium reported by Abe et al. omitting the addition of Zn^{2+} (Abe et al. 1962; Battat et al. 1991; Geyer et al. 2018; Hartmann et al. 2026; Ochsenreither et al. 2014). In contrast, Zambanini et al. employed the MTM medium described by Geiser et al. for malic acid production with *Ustilago trichophora*, containing 0.1 mg L^{-1} Zn^{2+} (Geiser et al. 2014; Zambanini et al. 2016). Similarly, Jiang et al. applied 0.1 mg L^{-1} Zn^{2+} for malic acid production using *E. coli* (Jiang et al. 2020). The medium of Chen et al. contains 0.3 mg L^{-1} Zn^{2+} in addition to tryptone to produce malic acid with *Trichoderma reesei* (Chen et al. 2023). Zelle et al. used a trace element composition based on Verduyn et al. for malic acid production with *S. cerevisiae*, including 1.0 mg L^{-1} Zn^{2+} (Verduyn et al. 1992; Zelle et al. 2008). Our study contains 10.0 mg L^{-1} Zn^{2+} in the medium composition. Xia et al. and Zou et al. produced malic acid with *Aureobasidium pullulans* using 40.5 mg L^{-1} Zn^{2+} (Xia et al. 2017; Zou et al. 2013). However, McHargue and Calfee reported Zn^{2+} promoted growth of *A. flavus*, with 5 mg L^{-1} - half the concentration we initially added to cultivations - being the most beneficial (McHargue and Calfee 1931). In their study, higher concentrations reduced the growth stimulating effect, suggesting metal-ion-associated toxicity. Therefore, lower Zn^{2+} concentrations in our cultivations could have enhanced growth even more.

The preculture strategy was originally established by Ochsenreither et al. in 2014, further applied by Dörsam et al. in 2016 as well as 2017 and Kövilein et al. in 2021, all of whom cultivated without the addition of Zn^{2+} or HTE in both pre- and main cultures (Dörsam et al. 2016, 2017; Kövilein et al. 2021; Ochsenreither et al. 2014). The addition of HTE to the composition of the preculture medium was first introduced by Kövilein et al. in 2022, demonstrating a substantial increase in harvested biomass upon HTE supplementation (Kövilein et al. 2022). Our findings extend this observation by identifying Zn^{2+} as one of the contributing trace elements to enhanced fungal growth in preculture. In contrast to our study, the previously mentioned studies reported considerable malic acid production even in the absence of trace element supplementation in preculture. One possible explanation could be unintended metal ion contamination, which may have compensated for the Zn^{2+} depletion identified in our study. For instance, ions originating from the conidia preparation may have contributed to sufficient trace element supply, as the agar medium used for sporulation contains HTE. Similarly, in our study, trace metal contamination may account for the stronger growth of the preculture without trace elements, compared with the main culture inoculated from it, as trace elements carried over by the conidia solution were sufficient to support growth in the preculture, but after washing, their transfer to the main culture was insufficient to achieve similar growth.

The supplementation of HTE leads to increased CO_2 production and enhanced biomass formation. As ammonium, the primary nitrogen source, is depleted during biomass growth, most likely nitrogen-free biomass accumulates. This is also reflected in the higher level of RQ, which, compared to cultivation with supplemented Zn^{2+}, indicates the synthesis of more reduced products like intracellular storage compounds. A candidate could be lipids, since *A. oryzae* is also being investigated as an oleaginous species for lipid production (Anantayanon et al. 2021; Lv et al. 2021; Wannawilai et al. 2024). While nitrogen depletion, as applied in our study, promotes malic acid production by increasing transcription of genes associated with the reductive TCA (rTCA) branch and reducing transcription in the oxidative TCA (oTCA) cycle, it also stimulates lipid accumulation (Knuf et al. 2013; Lv et al. 2021). In addition, trace element availability is known to influence lipid synthesis across diverse microorganisms. For instance, the precisely balanced addition of Fe^{3+}, Zn^{2+}, Mn^{2+}, Mo^{6+} and EDTA has been shown to enhance lipid production in *Nannochloropsis oculata*, whereas Cu^{2+} and Co^{2+} were found to have inhibitory effects (Dou et al. 2013). In *Yarrowia lipolytica*, sufficient supplementation of Fe^{2+}, K^{+}, Mg^{2+}, Mn^{2+} and Zn^{2+}

promotes lipid production, a similar trend was observed in *Scenedesmus* spp. (Kumar et al. 2020; Rocha et al. 2019). As shown in our study for organic acid production with *A. oryzae*, targeted Zn^{2+} supplementation is preferable to the broad addition of various trace element species, as it selectively modulates metabolic pathways to minimize by-product formation.

2D scans after 2 days of cultivation indicate a trend toward the formation of loose pellet structures during low DOT conditions, characterized by long hyphal extensions at the pellet edges. Since the mycelium density in the pellet architecture determines the extent of mass transfer limitation inside core regions, we hypothesize that the fungus may reduce mycelium density in response to hypoxia. Similar phenomena of fluffier phenotypes have been reported for *Aspergillus awamori*, in which a lower DOT is associated with a reduced biomass-to-pellet-volume ratio (Cui et al. 1998). A previous study demonstrated that malic acid production in *A. oryzae* can be enhanced through morphology engineering by optimizing pellet volume via adjustments in process parameters such as aeration and agitation as well as modulation of cell cycle regulators such as CDC14 (Chen et al. 2019). In contrast, the comparable malic acid productivities of the STR duplicates under inversely controlled normoxic and hypoxic conditions of our study suggest that productivity of malic acid is not directly linked to morphology. Also, filamentous microorganisms exhibit non-Newtonian, shear-thinning behavior during cultivation (Bliatsiou et al. 2020). Hence, filamentous growth during hypoxia very likely increases viscosity of culture broth, reducing gas-liquid mass transfer (Gabelle et al. 2012). This biotic effect may lead to an overestimation of k_La values determined during abiotic conditions.

During Zn^{2+}-supplemented hypoxia, malic acid productivity increases while citric acid formation is markedly reduced. This effect appears to result from the limited oxygen availability rather than Zn^{2+} itself, as the trend reverses during normoxic conditions. This enhancement of rTCA branch-associated organic acid formation during hypoxic conditions, as previously reported by Hartmann et al. is likely attributed to the limited availability of oxygen as a terminal electron acceptor, channeling NADH oxidation through the production of reduced organic acids (Diano et al. 2009; Hartmann et al. 2026; Meijer et al. 2007). Furthermore, hypoxia stimulates the glyoxylate cycle, redirecting isocitric acid toward malic acid synthesis, limiting NADH production and potentially leading to the observed decrease in citric acid production (Terabayashi et al. 2012). A comparable mechanism has been proposed for *Ustilago maydis* during hypoxia, where the diversion of cis-aconitic towards itaconic acid formation serves to avoid NADH production (Volkmar et al. 2025). Therefore, and similar to our duplicate with partial normoxia, increasing DOT elevates citric acid productivity, as also shown for *Candida lipolytica* and *A. niger* (Cui et al. 1998; Rane and Sims 1994). A notable exception to this trend is our cultivation with HTE supplementation, which produced considerable amounts of citric acid despite hypoxia. This may result from a shift in electron flow due to increased availability of metal cofactors, influencing key enzymes of the oTCA cycle and redirecting metabolism toward citric acid.

The duplicate with added Zn^{2+} at pH 7.00 confirms findings from Hartmann et al. where this pH increases malic acid productivity and reduces citric acid formation compared to pH 6.50 (Hartmann et al. 2026). Specifically, Hartmann et al. reported an increase in malic acid total productivity from 131 mg L^{-1} h^{-1} to 164 mg L^{-1} h^{-1}, corresponding to a rise by 25%, whereas in the present study, productivity only increased from 174 mg L^{-1} h^{-1} to 188 mg L^{-1} h^{-1}, corresponding to an increase by 8%. This attenuated effect may result from product inhibition at high malic acid concentrations, which could diminish the impact of beneficial conditions (Brink et al. 2022; Schmitt et al. 2021). Key metabolic mechanisms that stimulate malic acid production under these optimized conditions are as follows: Zn^{2+} most likely increases the global carbon flux by activating Zn^{2+}-dependent transcription factors, and supplementation with Zn^{2+} as a targeted trace element prevents excessive biomass-contained carbon accumulation during nitrogen depletion (Tsuji et al. 2000). Nitrogen depletion reduces the carbon flux toward protein biosynthesis and redirects it into the rTCA branch while suppressing the oTCA cycle (Knuf et al. 2013). Hypoxia reinforces this metabolic shift by limiting NADH reoxidation via respiration, promoting NAD^+ regeneration through oxaloacetate reduction to malic acid, and stimulating glycolytic flux, redirecting carbon from isocitric acid within the oTCA cycle toward malic acid synthesis (Diano et al. 2009; Meyer et al. 2021; Terabayashi et al. 2012). Maintaining near-neutral pH reduces the toxicity of accumulated organic acids and lowers energy demand for pH homeostasis (Kane 2016; Piper et al. 2001).

5 | Conclusion

In summary, continuous monitoring of fungal respiratory activity revealed a phase of growth limitation and subsequent pulsed supplementation of medium components identified Zn^{2+} as the depleting nutrient. In conjunction, evidence for biomass-associated transfer of metal ions from the biomass-growing preculture to the acid-producing main culture was demonstrated. As a consequence of Zn^{2+}-induced hypoxia and elevated pH levels, malic acid productivity increased. Beyond optimizing organic acid production, these insights highlight the broader potential of respiratory activity monitoring for process improvement.

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. **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.

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.

Supplement 1: Operational settings in cultivation with *Aspergillus oryzae* DSM 1863. All cultivations not listed were conducted under standard conditions of 400 rpm and 0.7 L_n min^{-1}. Data were averaged over 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. **Supplement 2:** Productivities of malic acid in cultivations with *Aspergillus oryzae* DSM 1863 in 2.5 L STR at varying oxygen tensions, trace element supply and pH. Cultivations were performed with X_0 of 7.5 g L^{-1} biomass, 109 g L^{-1} glucose at V_0 of 1.4 L, 32°C, agitation frequency and aeration rate contained in Supplement 1. If not stated otherwise, pH was maintained with 4 M NaOH and 4 M H_3PO_4 at 6.50 ± 0.05. The reference cultivation originates from the previously published study (Hartmann et al. 2026). Data represent means ± standard deviations of biological duplicates. Dashed lines and Akima-spline-connected standard deviations are provided for visual guidance.