From acetate to value-added products: L-malic acid production with *Aspergillus oryzae*

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Preamble

Parts of this dissertation are based on peer-reviewed, open-access research articles. The content of the chapters is therefore partly identical to these publications. All articles were drafted in the context of this thesis and describe the major findings of the microbial production of L-malic acid from acetate using the filamentous fungus *Aspergillus oryzae*. The layout and formatting of the publications and included figures have been adapted to the style of this dissertation.

Chapter 1 describes the theoretical background, and the subchapter “Malic acid” is partly based on the publication:

Kövilein, A.*; Kubisch, C.*; Cai, L.; Ochsenreither, K. (2020). Malic acid production from renewables: a review. *Journal of Chemical Technology and Biotechnology* 95 (3), 513-526. DOI: 10.1002/jctb.6269.

*Co-first authorship

Chapters 2 and 3 present the results of shake flask experiments for the evaluation of several aspects of L-malic acid production from acetate such as the carbon source concentration, the type of nitrogen source, the cultivation temperature or different process modes. These chapters are based on the following publications:

Kövilein, A.; Umpfenbach, J.; Ochsenreither, K. (2021). Acetate as substrate for L-malic acid production with *Aspergillus oryzae* DSM 1863. *Biotechnology for Biofuels* 14, 48. DOI: 10.1186/s13068-021-01901-5.

Kövilein, A.; Zadravec, L.; Hohmann, S.; Umpfenbach, J.; Ochsenreither, K. (2022). Effect of process mode, nitrogen source and temperature on L-malic acid production with *Aspergillus oryzae* DSM 1863 using acetate as carbon source. *Frontiers in Bioengineering and Biotechnology* 10, 1033777, DOI: 10.3389/fbioe.2022.1033777.

In Chapter 4 the possibility to control the morphology of *A. oryzae* during malic acid production from acetate in shake flasks and 2.5-L bioreactors by entrapment in the natural polymers alginate, κ-carrageenan and agar is evaluated. This chapter is based on the publication:

Kövilein, A.; Aschmann, V.; Hohmann, S.; Ochsenreither, K. (2022). Immobilization of *Aspergillus oryzae* DSM 1863 for L-Malic Acid Production. *Fermentation* 8 (1), 26. DOI: 10.3390/fermentation8010026.

In Chapter 5 the results of 2.5-L bioreactor fermentations are presented during which acetic acid served for pH control and substrate supply. This chapter is based on the publication:

Kövilein, A.; Aschmann, V.; Zadravec, L.; Ochsenreither, K. (2022). Optimization of L-malic acid production from acetate with *Aspergillus oryzae* DSM 1863 using a pH-coupled feeding strategy. *Microbial Cell Factories* 21, 242. DOI: 10.1186/s12934-022-01961-8.

List of publications

Peer-reviewed publications

Kövilein, A.*; Kubisch, C.*; Cai, L.; Ochsenreither, K. (2020). Malic acid production from renewables: a review. *Journal of Chemical Technology and Biotechnology* 95 (3), 513-526. DOI: 10.1002/jctb.6269.

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Kövilein, A.; Umpfenbach, J.; Ochsenreither, K. (2021). Acetate as substrate for L-malic acid production with *Aspergillus oryzae* DSM 1863. *Biotechnology for Biofuels* 14, 48. DOI: 10.1186/s13068-021-01901-5.

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Kövilein, A.; Aschmann, V.; Zadravec, L.; Ochsenreither, K. (2022). Optimization of L-malic acid production from acetate with *Aspergillus oryzae* DSM 1863 using a pH-coupled feeding strategy. *Microbial Cell Factories* 21, 242. DOI: 10.1186/s12934-022-01961-8.

Kubisch, C.; **Kövilein, A.**; Aliyu, H.; Ochsenreither, K. (2022). RNA-Seq Based Transcriptome Analysis of *Aspergillus oryzae* DSM 1863 Grown on Glucose, Acetate and an Aqueous Condensate from the Fast Pyrolysis of Wheat Straw. *Journal of Fungi* 8 (8), 765. DOI: 10.3390/jof8080765.

Poster presentation

Kövilein, A.; Ochsenreither, K. L-Malic acid production with *Aspergillus oryzae* using acetate as carbon source. Himmelfahrtstagung on Bioprocess Engineering 2021 - New Bioprocesses, New Bioproducts, Online Event, May 2021.

Abstract

Given the increasing scarcity of non-renewable resources and the challenges associated with greenhouse gas emissions, a shift from our mainly fossil-based economy to a bioeconomy becomes increasingly important. Therefore, manufacturing methods using fossil raw materials need to be replaced by bio-based processes. Research on product synthesis from renewable feedstock has been mainly focused on saccharides such as glucose as carbon source, thus competing with food and feed production. The utilization of acetate could represent a promising alternative since many substrates derived from biomass contain acetic acid due to the cleavage of acetyl groups from hemicellulose during pretreatment. These include lignocellulosic hydrolysates and products of fast pyrolysis in which acetate can be a main constituent. Furthermore, acetic acid can be obtained through the fermentation of syngas by acetogens. Thus, acetate-containing substrates derived from biomass represent an important feedstock in a bioeconomy. Malic acid belongs to the chemicals which are currently synthesized from petroleum or natural gas. The dicarboxylic acid is mainly used as acidulant and taste enhancer in the food industry but can furthermore serve as a component in personal care and cleaning products, pharmaceuticals, or polymers. As an intermediate of the tricarboxylic acid cycle, malic acid has great potential to be produced microbially. However, despite the availability of various production organisms, fermentation processes for malic acid production are not yet economically competitive compared to chemical manufacturing. This work therefore evaluated microbial L-malic acid production from acetate as an option for bio-based synthesis of the organic acid. Being a potent natural malate producer and robust microorganism towards inhibitors contained in biomass-derived substrates, *Aspergillus oryzae* DSM 1863 was selected as production strain.

First experiments were performed in shake flasks to evaluate several aspects of malic acid production from acetate such as the optimal substrate concentration and temperature, or the effect of the type of nitrogen source. The initial acetate concentration was found to influence the morphology and the organic acid spectrum produced by *A. oryzae*. In media with substrate concentrations up to 30 g/L, biomass pellets were formed, whereas a filamentous morphology was obtained at 40 g/L acetic acid and above. Regarding malic acid production, 45 g/L of the carbon source was identified as most suitable, resulting in a maximum product titer of 8.44 ± 0.42 g/L with a production rate of 0.044 ± 0.002 g/L/h and a yield of 0.19 ± 0.01 g/g. At a higher substrate concentration of 55 g/L the maximum production rate was reduced, suggesting substrate inhibition. Analysis of the side products showed a correlation between the concentration of the carbon source and the acid spectrum. Low initial acetate concentrations were not suitable for malate synthesis due to an increased production of oxalic acid. Compared to cultivations with glucose, the utilization of 45 g/L acetic acid increased the concentration of the side product succinate, resulting in about 35-40% of the total acid concentration while malic acid amounted to about 55%. The cultivation temperature during malate production was also found to affect the side product spectrum. Although product formation accelerated with

increasing temperature, the share of malic acid decreased in favor of succinic acid. Therefore, of the four cultivation temperatures tested in the range of 29-38 °C, a temperature of 32 °C was found to be optimal. Evaluation of different nitrogen sources showed an accelerated onset of germination and enhanced biomass titer with the complex compounds which was increased up to about 3.9-fold compared to cultures with ammonium sulfate. During the acid production phase, especially the utilization of yeast extract accelerated malate synthesis. Product synthesis was also accelerated by the supplementation of acetate medium with low concentrations of glucose in the range of 5-15 g/L. It was found that both carbon sources were metabolized concurrently which is an interesting observation for the future utilization of biomass-derived substrate for malic acid production.

Whereas acetate concentrations of around 45 g/L resulted in the highest malic acid titer and productivity, a filamentous-lumpy morphology was observed most likely due to a high ion concentration. As a pelleted morphology ensures a lower viscosity of the fermentation broth, leading to a better heat, oxygen, and nutrient transfer, it is often preferred. Therefore, the possibility of whole-cell immobilization by entrapment in natural polymers was evaluated as a tool for morphological control during L-malic acid production with *A. oryzae*. Since this has not been reported in the literature before, experiments were performed both in media with acetate and glucose for comparison. *A. oryzae* was entrapped in different concentrations of alginate, agar and κ-carrageenan and malic acid was detected with all conditions. However, differences were observed between the two carbon sources in terms of production rate, maximum product titer and matrix stability. In glucose medium, all immobilization matrices were stable and similar product concentrations were obtained with immobilized particles compared to cultivations with naturally formed biomass pellets. On the other hand, when medium containing acetate was used, production with entrapped *A. oryzae* was lower than with free mycelium in shake flask cultures. Furthermore, incubation in acetate medium had a negative effect on the stability of the entrapped particles as disintegration of alginate beads and biomass detachment from the agar and κ-carrageenan matrices was observed in later stages of the fermentation. Despite these challenges, entrapment of *A. oryzae* proved advantageous in 2.5-L bioreactor cultivations with acetate by preventing the biomass from attaching to the bioreactor installations, resulting in increased malic acid titers.

Due to substrate inhibition observed in a batch process, the maximum malate concentration from acetate is limited to less than 10 g/L. Therefore, a fed-batch or repeated-batch process is more suitable. These possibilities were first evaluated in shake flask cultivations with ammonium sulfate or yeast extract as nitrogen source. In the repeated-batch process, the production rate was successfully maintained at a high level for a cultivation time of 240 h during which three medium exchanges were performed. Furthermore, the highest absolute malate amount was quantified. However, process optimization is required to maintain the productivity for an increased number of cycles. Compared to the batch process, the period of malic acid production was extended, and a higher maximum titer was obtained in the fed-batch process (11.49 ± 1.84 g/L with $(NH_4)_2SO_4$ and 12.08 ± 1.25 g/L with yeast extract). The production rate in this process declined in later stages of

cultivation, most likely due to a repeated drop of the pH to values between 5.5-6.0 as a consequence of the manual addition of acetic acid. Therefore, the possibility of using a pH-coupled feed of acetic acid in 2.5-L bioreactor fermentations was assessed. An advantage of processes based on acetate is that the increase of pH caused by the consumption of the substrate can be counteracted by using acetic acid for pH control, simultaneously supplying additional carbon. With a pH-coupled feed of 10 M acetic acid, a maximum malic acid titer of 29.53 ± 1.82 g/L was obtained after 264 h, which is a 5.3-fold increase compared to the batch process without pH control. Furthermore, the yield was increased to 0.29 ± 0.01 g/g and the highest reported overall production rate of 0.112 ± 0.007 g/L/h was obtained. Since malate production ceased in later process stages despite carbon availability, a possible product inhibition was evaluated. Experiments with up to 50 g/L malic and succinic acid added to the medium showed that *A. oryzae* is capable of organic acid production despite the presence of high product concentrations.

In conclusion, this work provides a comprehensive overview of various aspects of L-malic acid production from acetate with wild-type *A. oryzae* DSM 1863. While using acetate as a carbon source, several challenges were identified such as a pH-dependent substrate inhibition, an effect of acetate-containing media on the morphology, and a higher share of side products compared to glucose-based cultivations. Therefore, strategies were investigated and developed to enable efficient malate production despite these challenges, and options for further optimization were highlighted. Especially the development of suitable process management strategies such as the use of acetic acid for pH control and substrate supply is of particular importance to enhance malate production from acetate.

Zusammenfassung

Angesichts der zunehmenden Verknappung fossiler Ressourcen und der Herausforderungen im Zusammenhang mit Treibhausgasemissionen gewinnt die Umstellung unserer hauptsächlich auf Erdöl, Kohle und Erdgas basierenden Wirtschaft auf eine Bioökonomie zunehmend an Bedeutung. Im Zuge dessen müssen herkömmliche Syntheseverfahren durch biobasierte Prozesse ersetzt werden. Die Forschung zur Herstellung verschiedenster Produkte aus erneuerbaren Rohstoffen hat sich bisher hauptsächlich auf Saccharide wie Glukose als Kohlenstoffquelle konzentriert und steht damit in Konkurrenz zur Lebens- und Futtermittelproduktion. Die Nutzung der Kohlenstoffquelle Acetat stellt dazu eine vielversprechende Alternative dar. Aus Biomasse gewonnene Substrate enthalten häufig Essigsäure, die durch Abspaltung von Acetylgruppen aus der Hemicellulose entsteht. Dazu gehören beispielsweise Lignocellulosehydrolysate und Pyrolyseprodukte, in denen Acetat ein Hauptbestandteil sein kann. Außerdem kann Essigsäure durch Synthesegasfermentation mittels acetogenen Bakterien gewonnen werden. Daher sind acetathaltige, aus Biomasse gewonnene Substrate eine vielversprechende Kohlenstoffquelle im Kontext der Bioökonomie. Äpfelsäure gehört zu den Chemikalien, die derzeit aus fossilen Rohstoffen synthetisiert werden. Die Dicarbonsäure wird hauptsächlich als Säuerungsmittel und Geschmacksverstärker in der Lebensmittelindustrie verwendet, kann aber auch als Bestandteil von Kosmetikartikeln, Reinigungsprodukten, Arzneimitteln oder zur Synthese von Polymeren genutzt werden. Als Zwischenprodukt des Tricarbonsäurezyklus hat Äpfelsäure ein großes Potenzial, mikrobiell hergestellt zu werden. Trotz der Verfügbarkeit verschiedener Produktionsorganismen ist die fermentative Äpfelsäuresynthese im Vergleich zur chemischen Herstellung jedoch noch nicht wirtschaftlich. Im Rahmen dieser Arbeit wurde daher die mikrobielle L-Äpfelsäureproduktion aus Acetat als Möglichkeit einer biobasierten Synthese der organischen Säure untersucht. *Aspergillus oryzae* DSM 1863 wurde dafür als Produktionsstamm gewählt, da dieser filamentöse Pilz ein potenter natürlicher Malatproduzent und ein robuster Mikroorganismus gegenüber Inhibitoren ist, die in aus Biomasse gewonnenen Substraten enthalten sein können.

Zunächst wurden Experimente im Schüttelkolbenmaßstab durchgeführt, um verschiedene grundlegende Aspekte der Äpfelsäureproduktion aus Acetat zu untersuchen. Dazu gehörten die Bestimmung der optimalen Substratkonzentration im Batch-Verfahren, sowie der Einfluss der Kultivierungstemperatur und der Art der Stickstoffquelle. Es wurde festgestellt, dass die initiale Acetatkonzentration die Morphologie und das Spektrum der von *A. oryzae* produzierten organischen Säuren beeinflusst. In Medien mit Substratkonzentrationen bis zu 30 g/L wurden Biomassepellets gebildet, wohingegen eine filamentöse Morphologie ab 40 g/L Essigsäure beobachtet wurde. Für die Malatproduktion im Batch-Prozess erwies sich eine Substratkonzentration von 45 g/L Essigsäure als am besten geeignet, womit eine maximale Äpfelsäurekonzentration von 8,44 ± 0,42 g/L, eine Produktionsrate von 0,044 ± 0,002 g/L/h, sowie eine Ausbeute von 0,19 ± 0,01 g/g erreicht wurden. Bei einer höheren Konzentration von 55 g/L Essigsäure war die maximale Produktionsrate

verringert, was auf eine Substrathemmung hindeutet. Die Analyse der Nebenprodukte zeigte einen Einfluss der initialen Substratkonzentration auf das Säurespektrum. Niedrige Acetatkonzentrationen führten beispielsweise zu einem höheren Anteil an Oxalsäure. Im Vergleich zur Kultivierung mit Glukose erhöhte sich bei der Verwendung von 45 g/L Essigsäure der Anteil des Nebenprodukts Succinat, das etwa 35-40% der Gesamtsäurekonzentration ausmachte, während der Anteil der Äpfelsäure etwa 55% betrug. Es wurde außerdem festgestellt, dass auch die Kultivierungstemperatur während der Malatproduktion das Spektrum der Nebenprodukte beeinflusst. Obwohl sich die Produktbildung mit steigender Temperatur beschleunigte, nahm dabei der Anteil von Malat zugunsten von Succinat ab, weshalb eine Kultivierungstemperatur von 32 °C als optimal bewertet wurde. Die Untersuchung des Einflusses verschiedener Stickstoffquellen auf das Wachstum von *A. oryzae* zeigte einen früheren Beginn der Konidienkeimung und einen um bis zu 3,9-fach höheren Biomassetiter bei Verwendung von komplexen Stickstoffquellen im Vergleich zu Kulturen mit Ammoniumsulfat. Während der Säureproduktionsphase beschleunigte insbesondere die Verwendung von Hefeextrakt die Malatsynthese. Weiterhin wurde die Äpfelsäureproduktion auch durch Supplementierung des Acetatmediums mit niedrigen Glukosekonzentrationen im Bereich von 5-15 g/L beschleunigt. Dabei wurde festgestellt, dass *A. oryzae* beide Kohlenstoffquellen gleichzeitig verstoffwechselte, was eine interessante Beobachtung für die künftige Nutzung von biomassebasierten Substraten ist.

Während mit einer Acetatkonzentration von 45 g/L die höchsten Äpfelsäure- und Biomassetiter erzielt wurden, führte die Verwendung dieser Substratkonzentration zu einer hauptsächlich filamentösen Morphologie. Diese Beobachtung ist wahrscheinlich auf eine hohe Ionenkonzentration in acetatreichen Medien zurückzuführen. Eine pelletartige Morphologie wird jedoch häufig bevorzugt, da diese zu einer geringeren Viskosität des Fermentationsmediums führt, wodurch ein besserer Wärme-, Sauerstoff- und Nährstofftransport gewährleistet werden kann. Daher wurde die Möglichkeit der Immobilisierung von *A. oryzae* durch Einschluss in natürliche Polymere als Werkzeug zur morphologischen Kontrolle während der Äpfelsäureproduktion mit *A. oryzae* untersucht. Da dies in der Literatur zuvor nicht beschrieben war, wurden Versuche zum Vergleich sowohl in Medien mit Acetat als auch mit Glukose durchgeführt. Dazu wurden Konidien in verschiedenen Konzentrationen von Alginat, Agar und κ-Carrageen eingeschlossen. Eine Malatbildung wurde unter allen Bedingungen nachgewiesen, es ließen sich jedoch Unterschiede zwischen den beiden Kohlenstoffquellen hinsichtlich der Produktionsrate, des maximalen Produkttiters und der Matrixstabilität feststellen. In glukosehaltigem Medium waren alle Polymere stabil, und mit immobilisiertem *A. oryzae* wurden ähnliche Produktkonzentrationen erzielt wie mit natürlich gebildeten Biomassepellets. Bei Verwendung eines acetathaltigen Mediums hingegen war die Produktion in Schüttelkolbenkulturen geringer als mit freiem Myzel und die Inkubation der Polymerstrukturen in diesem Medium hatte nach längerer Zeit eine negative Wirkung auf deren Stabilität. In späteren Stadien der Fermentation tendierten die Alginatstrukturen dazu, sich aufzulösen und es wurde eine Ablösung der Biomasse von den Agar- und κ-Carrageen-Strukturen

beobachtet. Trotz dieser Herausforderungen erwies sich der Polymereinschluss von *A. oryzae* in 2,5-L-Bioreaktorkulturen mit acetathaltigem Medium als vorteilhaft, da eine Ablagerung der Biomasse an Reaktoreinbauten verhindert wurde, was zu einer verbesserten Produktion führte.

Aufgrund der festgestellten Substrathemmung mit Acetat als Kohlenstoffquelle im Batch Prozess ist die maximale Malatkonzentration auf Werte von weniger als 10 g/L begrenzt. Um diese Hemmung durch eine initial hohe Substratkonzentration zu umgehen, ist ein Fed-Batch- oder Repeated-Batch-Verfahren geeignet. Diese Möglichkeiten wurden zunächst in Schüttelkolbenkulturen mit Ammoniumsulfat oder Hefeextrakt als Stickstoffquelle untersucht. Im Repeated-Batch-Verfahren konnte die Produktionsrate über eine Kultivierungszeit von 240 Stunden, was drei Medienwechseln entsprach, erfolgreich auf einem hohen Niveau gehalten werden. Dabei wurde die höchste absolute Malatmenge verglichen mit den zwei anderen Prozessstrategien quantifiziert. Eine Prozessoptimierung ist jedoch erforderlich, um die Produktivität auch über eine höhere Anzahl von Zyklen aufrechtzuerhalten. Auch das Fed-Batch Verfahren zeigte Vorteile gegenüber dem Batch-Verfahren, da der Produktionszeitraum verlängert und höhere maximale Produktkonzentrationen (11,49 ± 1,84 g/L mit $(NH_4)_2SO_4$ und 12,08 ± 1,25 g/L mit Hefeextrakt) erzielt wurden. Die Produktionsraten sanken jedoch im Verlauf der Kultivierung, was höchstwahrscheinlich auf ein wiederholtes Absinken des pH-Werts auf Werte zwischen 5,5 und 6,0 infolge der manuellen Zugabe von Essigsäure zurückzuführen ist. Daher wurde die Möglichkeit der Verwendung einer pH-gekoppelten Zufuhr von Essigsäure in 2,5-L-Bioreaktor-Fermentationen untersucht. Ein Vorteil von acetatbasierten Prozessen ist, dass der aufgrund des Substratverbrauchs verursachte Anstieg des pH-Werts durch die Verwendung von Essigsäure zur pH-Kontrolle bei gleichzeitiger Zugabe von zusätzlichem Kohlenstoff ausgeglichen werden kann. Mit einer pH-gekoppelten Zufuhr von 10 M Essigsäure wurde nach 264 h ein maximaler Äpfelsäuretiter von 29,53 ± 1,82 g/L erreicht, was einer 5,3-fachen Steigerung gegenüber dem Batch-Prozess ohne pH-Kontrolle entspricht. Außerdem wurde die Ausbeute auf 0,29 ± 0,01 g/g erhöht und die bisher höchste Gesamtproduktionsrate von 0,112 ± 0,007 g/L/h erreicht. Da die Malatproduktion gegen Ende der Fermentation trotz Kohlenstoffverfügbarkeit stoppte, wurde außerdem eine mögliche Produkthemmung untersucht. Versuche mit Zugabe von bis zu 50 g/L Äpfel- und Bernsteinsäure zeigten, dass *A. oryzae* trotz Produktkonzentrationen in diesem Bereich in der Lage ist, weiter organische Säuren zu produzieren.

Zusammenfassend lässt sich sagen, dass diese Arbeit einen breiten Überblick über verschiedene Aspekte der L-Äpfelsäureproduktion aus Acetat mit dem Wildtyp *A. oryzae* DSM 1863 bietet. Während der Nutzung von Acetat als Kohlenstoffquelle wurden einige Herausforderungen identifiziert wie beispielsweise eine vom pH-Wert beeinflusste Substrathemmung, ein Einfluss von acetathaltigen Medien auf die Morphologie, und ein höherer Anteil an Nebenprodukten verglichen mit glucosebasierten Kultivierungen. Daher wurden Strategien untersucht und entwickelt, um eine effiziente Malatproduktion trotz dieser Anforderungen zu ermöglichen, und Optionen für eine weitere Optimierung aufgezeigt. Insbesondere die Entwicklung geeigneter Prozessführungsstrategien

wie die Nutzung von Essigsäure zur pH-Kontrolle und als gleichzeitige Substratzufuhr sind für eine Steigerung der Malatproduktion mit Acetat als Kohlenstoffquelle von Bedeutung.

Table of contents

1. Theoretical background and research proposal

The "Malic acid" subsection of this chapter is based in part on the publication stated below. The parts that are identical to the article belong to the author's contributions to this publication.

Malic acid production from renewables: a review
Aline Kövilein*, Christin Kubisch*, Liyin Cai, Katrin Ochsenreither (2020)
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Author contributions
Aline Kövilein: conceptualization, writing of first draft, manuscript revision.
Christin Kubisch: conceptualization, writing of first draft, manuscript revision.
Liyin Cai: constructively contributed to the content and review of the manuscript.
Katrin Ochsenreither: conceptualization, project supervision, writing of first draft, manuscript revision.

1.1. Introduction

The majority of chemicals is still produced from fossil resources to date. In a 2019 report from the European Union considering 10 key product categories, the share of bio-based chemicals produced in the EU was only about 3% (Spekreijse et al., 2019). Given the limitation of fossil resources, and the endeavor to reduce greenhouse gas emissions, renewable substrates will have to serve as raw materials for the manufacturing of these products in the near future. Organic acids are widely used chemicals and some of them, such as citric or lactic acid, are already produced microbially from renewable resources on large scale (Ciriminna et al., 2017; Singhvi et al., 2018). The dicarboxylic acid malic acid, however, is still synthesized from gas- or petroleum-based raw materials.

Especially in the last decade, research regarding microbial malic acid production intensified with most of this work focusing on glucose as carbon source (see Table 1 and Table 2 in Chapter 1.3.3). However, if the saccharide is not derived from non-edible lignocellulosic biomass, its utilization for the synthesis of chemicals is competing with food and feed production. Therefore, alternative carbon sources should be considered. Acetate is contained in many substrates derived from biomass such as lignocellulosic hydrolysates and condensates from fast pyrolysis which are promising feedstocks in a bio-based economy. Furthermore, it can be obtained through syngas fermentation using industrial off-gases or gasified waste streams. Despite being readily available from renewable feedstock, the number of processes evaluating acetate as sole carbon source is limited (Kiefer et al., 2021). The feasibility of malic acid production from acetate has been demonstrated, but maximum concentrations and production rates were low (Oswald et al., 2016), and the full potential of this carbon source for malate production has not yet been evaluated. Therefore, this study investigated malic acid production from acetate in more detail.

Substrates derived from biomass are often complex mixtures of different kinds of carbon sources and also contain degradation products which may be inhibiting to microorganisms. If the utilization of these substrates is targeted, robust production organisms are needed. In this regard, fungi are especially interesting, being natural saprophytes. The native malic acid producer *Aspergillus oryzae* was found capable of metabolizing a broad range of carbon sources and showed a high tolerance against pyrolysis oil (Dörsam et al., 2016; Dörsam et al., 2017). Therefore, it was used as production organism in this work.

1.2. The production organism *Aspergillus oryzae*

Aspergillus species, belonging to the phylum Ascomycota, are widely used in the industry, mainly for the production of organic acids and enzymes. Already more than 100 years ago, James Currie researched optimum cultivation conditions of citric acid production by *A. niger* which is now the microorganism used for large-scale production of the acid (Currie, 1917). *A. oryzae* has been used for centuries for the production of sake, miso and soy sauce in Asia. These processes make use of the high enzyme secretion ability of *A. oryzae* for hydrolysis of the starchy or protein-containing raw

materials (Ito and Matsuyama, 2021; Kusumoto et al., 2021). Enzymes produced by *A. oryzae* are furthermore used in various commercially available formulations such as Amaferm® (BioZyme Inc.), a feed additive, Amylofeed® (GNC Bioferm Inc.), a feed additive for piglets, or Nutrilife® FAA 102 (BASF), a baking additive, which all contain α-amylase.

Belonging to the *Aspergillus* section *Flavi*, *A. oryzae* is considered a domesticated form of *A. flavus* due to the high genetic similarity. The comparison of average nucleotide identity revealed a sequence homology of about 99% between *A. oryzae* RIB40 or *A. oryzae* A1560 and *A. flavus* NRRL3357 (Frisvad et al., 2018). It is therefore difficult to distinguish between the two species based on genomic analyses. However, a significant difference is the production of aflatoxins. Different than most *A. flavus* isolates, *A. oryzae* strains do not produce aflatoxins due to mutations in the aflatoxin biosynthesis gene cluster or a lack of expression of these genes (Kiyota et al., 2011; Tao and Chung, 2014; Tominaga et al., 2006). Therefore, *A. oryzae* can safely be used for food production whereas *A. flavus* is an undesired crop, human and animal pathogen. A comprehensive overview of literature regarding the safety of *A. oryzae* is provided by Frisvad et al. (2018).

A. oryzae is a filamentous fungus which propagates asexually through conidia. A sexual cycle has not been identified yet despite the presence of mating type genes (Wada et al., 2012). Other *Aspergilli*, including the closely related *A. flavus*, are capable of sexual reproduction (Horn et al., 2016). When inoculated into a submerged culture, the conidia first exhibit a period of swelling due to water uptake, followed by the formation of one germ tube per conidium which can then grow into a complex hyphal network (Kitamoto, 2015). Thereby, growth of the hyphae proceeds at the tip cells and different morphologies are formed through branching and agglomeration. The morphology of *Aspergillus* species can take a variety of different forms from completely dispersed mycelium to compact biomass pellets. Some examples which were obtained during the work on this thesis are displayed in Figure 1. A dispersed mycelial morphology can cause an increased viscosity of the fermentation broth, requiring a higher power input for mixing. Furthermore, biomass with a dispersed filamentous form is more difficult to separate from the fermentation broth (Böl et al., 2020). For these reasons, a pelleted morphology is often preferred. Pellet formation is traditionally described by either the coagulative or the non-coagulative model. Pellets of the coagulative type are formed by the initial coagulation of spores which then germinate and form a pellet. According to the non-coagulative model, biomass-pellets are formed after spore germination. Therefore, this type can originate from a single spore while the coagulative pellets are formed by a number of spores (Zhang and Zhang, 2016).

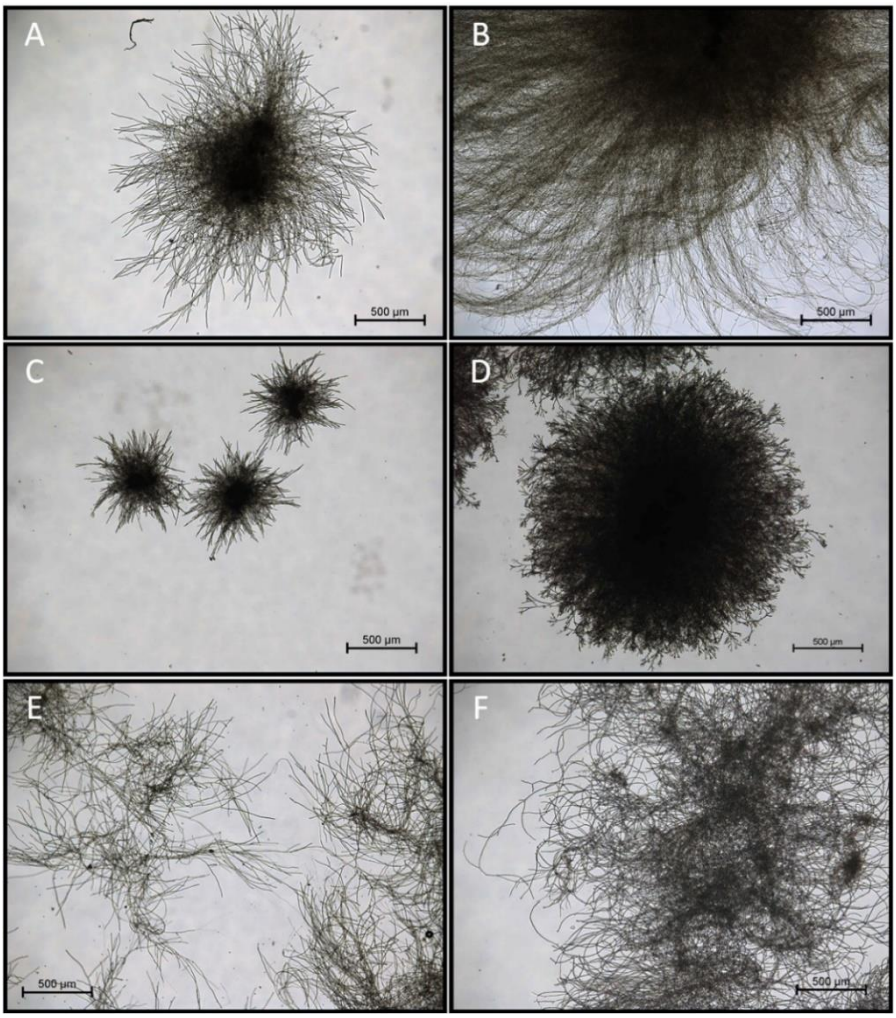

Figure 1. Microscopic images showing different morphologies of *A. oryzae*: small fluffy pellet (A), large fluffy pellet (B), small and more compact pellet (C), large compact pellet (D), dispersed-lumpy morphology (E, F).

Within the biomass pellet nutrient availability and gas exchange are limited. In large pellets, this may lead to autolysis and hollow particle cores. Hille et al. (2005) investigated the oxygen concentration in pellets of *A. niger*, demonstrating a steep decrease in oxygen supply in the outer 200 µm of large, dense pellets until the complete absence of oxygen towards the particle's core. The authors suggested small, dense biomass pellets to have the highest turnover activity. An advantage of small and compact pellets over fluffy and loose pellets was also observed for citric acid production with *A. niger*, resulting in higher product concentrations (Gómez et al., 1988). A correlation between a morphology change from mycelial to pelleted and an increased malic acid production due to a higher stirrer velocity was also observed in fermentations with *A. oryzae* (Chen et al., 2019). Regarding the production of the enzyme fructofuranosidase with *A. niger*, on the other side, a microparticle-induced mycelial morphology resulted in an increased enzyme yield and productivity was maintained for a significantly longer period. The authors pointed out that only the outer layer of the pellets showed metabolic activity, which decreased over time, whereas the mycelial structure was highly active until the end of the fermentation (Driouch et al., 2010). The morphology of filamentous

microorganisms is subject to various parameters such as the pH, medium composition, osmolality, inoculum concentration, stirrer velocity and aeration (Casas López et al., 2005; Chen et al., 2019; Gómez et al., 1988; Papagianni, Mattey, Kristiansen, 1999b; Papagianni and Mattey, 2006; Papagianni, Nokes, Filer, 1999; Wucherpfennig et al., 2011; Zhang et al., 2007). Furthermore, the presence of microparticles such as talc, titanate or aluminum oxide can have an effect (Driouch et al., 2012; Kowalska et al., 2019). Monitoring and controlling the morphology, although potentially challenging, is therefore important in fermentations with filamentous microorganisms and was also targeted in this thesis.

The genus *Aspergillus* is highly divers and widespread in many natural habitats (Varga et al., 2011). Generally, *Aspergillus* species are saprophytes (Pennerman et al., 2020) and therefore capable of coping with a variety of cultivation conditions and substrates. *A. oryzae* DSM 1863 is a potent natural malic acid producer which is capable of using a broad range of carbon sources for malate synthesis of which many are contained in lignocellulose-derived substrates or industrial side streams such as xylose, cellobiose, glycerol and levoglucosan (Dörsam et al., 2017). Furthermore, it is a robust microorganism with a high tolerance to biomass-derived inhibitors. In experiments evaluating the growth of different fungal species in medium with pyrolysis oil, *A. oryzae* tolerated 2% of this substrate which was a higher concentration than most other tested fungi were able to withstand (Dörsam et al., 2016). Moreover, its capability of using acetate derived from syngas fermentation was also demonstrated (Oswald et al., 2016). It is therefore a promising microorganism for bio-based malic acid production, for which high titers and production rates have been reported as described in the following chapter.

1.3. Malic acid

Malic acid is a C4-dicarboxylic acid and important intermediate of cell metabolism. Its name is derived from the Latin word ‘malum’, meaning apple, as malic acid was first isolated from this fruit in 1785 (Jensen, 2007). The current global production volume of malic acid is estimated to be around 150,000 tons/year (Jiang et al., 2021). The majority of it is used as acidulant and taste enhancer in the food and beverage industry for the manufacturing of various products such as candies, soft drinks, bakery products and jellies. Malic acid is also applied as buffering and chelating agent in personal care and cleaning formulations (Bellon, 2003; Sakurai and Sugimoto, 2005) and as animal feed additive (Stallcup, 1979; Stookey, 2000). Other areas include the pharmaceutical sector, e.g. as compound of the migraine drug almotriptan malate (Gore et al., 2010), or the fabrication of semiconductors where malic acid is used as polishing or cleaning formulation compound (Nishiwaki et al., 2010; Zhang et al., 2016). Furthermore, malic acid can be applied as component of low transition temperature mixtures (Radošević et al., 2016; Yiin et al., 2016) and for the manufacturing of polymers. Due to its dicarboxylic nature, malic acid is a suitable building block chemical for the synthesis of biodegradable homo- and heteropolymers in medical applications (Kajiyama et al., 2004; Shahruzzaman et al., 2015; Wang et al., 2010).

The first identification of malic acid as microbial product synthesized by *Saccharomyces cerevisiae* was reported in 1924 (Dakin, 1924). Since then, several more microorganisms were discovered as natural L-malic acid producers, among them species of *Aspergillus*, *Rhizopus* and *Penicillium*. Despite the availability of various production organisms, commercially available malic acid is mainly produced by chemical synthesis using fossil resources.

1.3.1. Chemical DL-malic acid production

Processes for chemical malic acid synthesis were developed more than half a century ago and are therefore well-established. They usually use maleic anhydride as starting material and yield racemic DL-malic acid. As raw materials for maleic anhydride production various hydrocarbons and hydrocarbon mixtures of at least four carbon atoms are suitable (Bergman and Frisch, 1966; Young et al., 1975). However, the preferred compound today is n-butane due to good availability, a relatively low price and fewer side reactions compared to more reactive or higher molecular weight hydrocarbons (Bremer and Dria, 1982; Hutchings and Higgins, 1981; Young et al., 1975). Originally, benzene was used predominantly for maleic anhydride production (Kerr, 1979; Udovich and Meyers, 1981) and is still used today, but to a significantly lower extent than butane. Hydrocarbons like n-butane and benzene are obtained from fossil resources such as natural gas and cracked petroleum. In the process of maleic anhydride synthesis through catalytic vapor phase oxidation, the reaction gas containing between 0.5 and 10% (v/v) of the hydrocarbon and an excess of an oxygen-containing gas like air is passed over vanadium phosphate catalysts at temperatures between 250 °C and 600 °C and often superatmospheric pressure. The maleic anhydride is recovered from the gas stream by condensation, followed by water-scrubbing of the residual gas resulting in a maleic acid solution. The maleic acid is then converted to maleic anhydride by thermal dehydration (Bremer and Dria, 1982; Dobner et al., 2015; Kerr, 1979; Wrobleski, 1984).

The production of DL-malic acid involves the hydration of maleic anhydride to maleic acid in an aqueous solution, followed by the isomerization of maleic acid to fumaric acid and the final hydration of fumaric acid to malic acid as displayed in Figure 2. For this reaction, elevated temperatures of 160-220 °C, superatmospheric pressure and reaction times of 3-6 h are favored. During the reaction an equilibrium is established, resulting in crude malic acid liquor containing fumaric acid and small amounts of maleic acid. Fumaric acid is separated from the reaction mixture by crystallization after cooling due to its low solubility, and the remaining crude malic acid usually contains 1-3% fumaric and maleic acid. Pure malic acid is obtained by liquid–liquid extraction, successive crystallization or chromatography. Besides maleic anhydride, maleic acid, fumaric acid and mixtures thereof can also be used for malic acid production. However, these substances are likewise produced chemically from maleic anhydride (see Figure 2) and thus from fossil resources. The process depictions referred to in this paragraph are described in various patents (Ahlgren, 1968; Ramsey and Schultz, 1992; Sumikawa et al., 1976; Sumikawa et al., 1977; Weinrotter et al., 1975; Winstrom and Frink, 1968).

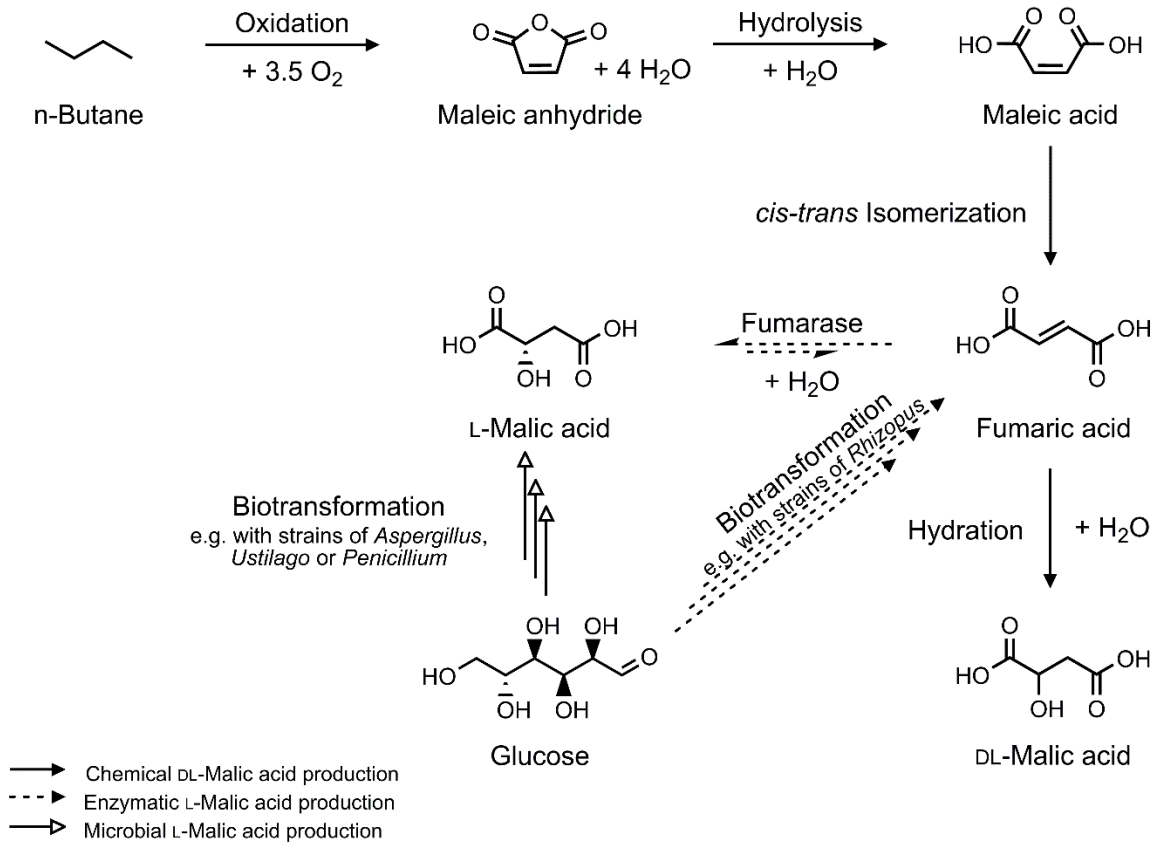

Figure 2. Chemical reaction route of DL-malic acid synthesis compared to enzymatic and microbial L-malic acid production.

1.3.2. Enzymatic L-malic acid production

The chemical synthesis yields racemic DL-malic acid. For specific applications such as in the pharmaceutical industry or for polymer production, enantiopure malic acid may be preferred. Resolution of the chemically produced racemate can be expensive and therefore seems not suitable for large-scale applications. Alternatively, enantiopure L-malic acid, which is the physiological form, can be selectively produced enzymatically under milder reaction conditions than applied in the chemical process. It is assumed that enzymatic L-malic acid production is also performed industrially but to a much lower extent than the chemical production method (Ortiz et al., 2017).

Enzymatic production of L-malic acid applies the enzyme fumarase which converts the substrate fumaric acid to malic acid through hydration. Processes use either the purified enzyme (Giorno et al., 2001; Presečki et al., 2007; Stojkovič et al., 2011), permeabilized cells of *Saccharomyces cerevisiae* (Pandurić et al., 2017; Presečki et al., 2007; Stojkovič and Žnidaršič-Plazl, 2012), or whole cells. Whole-cell catalysis using *S. cerevisiae*, *Brevibacterium flavus*, *Brevibacterium ammoniagenes* or *Rhizopus oryzae* has been reported, commonly reaching conversion rates of about 80% (Bressler et al., 2002; Chibata et al., 1975; Naude and Nicol, 2018; Oliveira et al., 1994; Terasawa et al., 1990). The cells may be immobilized in suitable materials such as polyacrylamide, alginate or carrageenan to allow for catalyst reutilization. However, the substrate fumaric acid applied in this enzymatic

method is produced by the isomerization of petroleum-derived maleic acid (Roa Engel et al., 2008). Fermentation processes for microbial fumaric acid production using *Rhizopus* spp. were established in the 1940s but have been replaced by the more economic chemical process over time (Kane et al., 1943; Lubowitz and La Roe, 1958). Therefore, instead of producing L-malic acid enzymatically from fumaric acid, the direct biotechnological synthesis of L-malic acid is a more promising alternative.

1.3.3. Microbial L-malic acid production

Microbial malic acid production offers the advantages that only the enantiopure L-form is produced and that a variety of microorganisms and renewable substrates can be employed. Depending on the microorganism or substrate, several metabolic routes can lead to malic acid overproduction. The three main pathways are the oxidative tricarboxylic acid (TCA) cycle, the reductive TCA (rTCA) cycle and the glyoxylate cycle (Figure 3). In the oxidative TCA cycle, citrate synthase (CS) first catalyzes the condensation of oxaloacetate and acetyl-CoA to citrate which is then further metabolized to malate in several enzymatic reactions. Due to the release of two molecules of CO_2 during the oxidation, the maximum theoretical yield of this pathway is 1 mol/mol glucose if pyruvate derived from glycolysis serves for the formation of both acetyl-CoA and oxaloacetate. In the rTCA cycle, oxaloacetate obtained through carboxylation of phosphoenolpyruvate or pyruvate is reduced to malate by malate dehydrogenase (MDH). The theoretical malate yield of this pathway is 2 mol/mol glucose, for which reason it is often targeted when aiming to improve malic acid production through metabolic engineering. Furthermore, malate can be produced via the glyoxylate pathway that involves the enzymes isocitrate lyase (ICL), which catalyzes the transformation of isocitrate to glyoxylate and succinate, and the subsequent formation of malate from glyoxylate and acetyl-CoA through malate synthase (MS). If a non-cyclic pathway is assumed during which oxaloacetate is replenished through carboxylation of pyruvate and the produced succinate is transformed to malate, the theoretical maximum yield is 1 1/3 mol/mol glucose. In case of a cyclic pathway, the metabolization of one molecule of glucose results in two molecules of acetyl-CoA which can then form one molecule of malate, yielding 1 mol/mol glucose (Zelle et al., 2008).

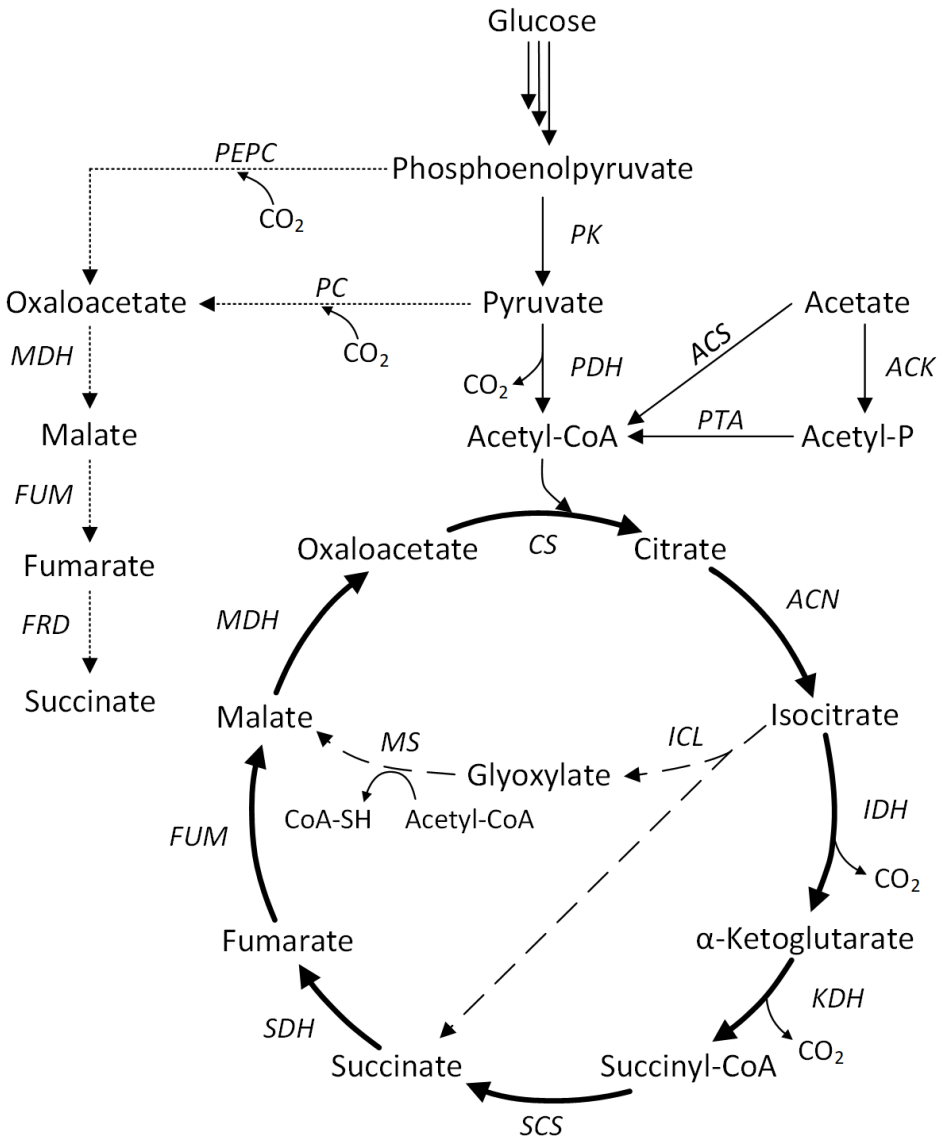

Figure 3. Metabolic pathways involved in L-malic acid production. Bold lines represent the TCA cycle, dotted lines the reductive TCA cycle and dashed lines the glyoxylate cycle. Enzymes are abbreviated as follows: ACK acetate kinase, ACN aconitase, ACS acetyl-CoA synthetase, CS citrate synthase, FRD fumarate reductase, FUM fumarase, ICL isocitrate lyase, IDH isocitrate dehydrogenase, KDH α-ketoglutarate dehydrogenase, MDH malate dehydrogenase, MS malate synthetase, PC pyruvate carboxylase, PDH pyruvate dehydrogenase, PEPC phosphoenolpyruvate carboxylase, PK pyruvate kinase, PTA phosphate acetyltransferase, SCS succinyl-CoA synthetase, SDH succinate dehydrogenase.

Especially filamentous fungi have been identified as excellent natural producers of malic acid with most research focusing on *Aspergillus* species (Table 1). Malic acid production with different strains of *A. flavus*, *A. parasiticus* and a strain of *A. oryzae* was first patented by Abe et al. in 1962. Using *A. flavus* ATCC 13697, the authors reported malic acid production of 60 g/L from 100 g/L glucose (Abe et al., 1962). Peleg et al. (1989) further studied malate production with this natural producer and suggested the rTCA cycle as main pathway leading to malic acid from glucose based on results

of ^{13}C nuclear magnetic resonance (NMR) analysis. Battat et al. (1991) optimized the process using *A. flavus* ATCC 13697 and obtained a malic acid titer of 113 g/L with a production rate of 0.59 g/L/h by adjusting process parameters such as the stirrer velocity and aeration rate as well as the medium composition. Although high concentrations of malic acid can be obtained with this natural producer, a disadvantage is the production of aflatoxins. Therefore, the closely related *A. oryzae* is better suited. As displayed in Table 1, the highest malic acid titer reported for wild type *A. oryzae* so far was 58.2 g/L. Higher titers were obtained with species of *Penicillium* isolated from marine environments. With *Penicillium viticola* 152, for instance, about 130 g/L malic acid were produced from glucose in a batch process with a high production rate of 1.36 g/L/h (Khan et al., 2014). So far, this value has only been exceeded with genetically modified strains.

Table 1. L-Malic acid production processes with wild type microorganisms.

Microorganism	Initial substrate	Process	$c_{malic\ acid}$ [g/L]	$Y_{P/S}$ [g/g]	r_P [g/L/h]	Reference
Aspergillus flavus ATCC 13697	100 g/L glucose	10-L bioreactor (25 d), batch	60	0.63	0.1	(Abe et al., 1962)
	100 g/L glucose	10-L bioreactor (144 h), batch	36.1	0.37	0.25	(Peleg et al., 1988)
	120 g/L glucose	16-L bioreactor (190 h), batch	113	0.94	0.59	(Battat et al., 1991)
Aspergillus niger ATCC 10577	100 g/L crude glycerol	125-mL shake flask (192 h), batch	20.3	0.20	0.11	(West, 2015, 2017)
	Thin stillage[1]	125-mL shake flask (192 h), batch	16.4	0.79[2]	0.09	(West, 2011, 2017)
Aspergillus niger ATCC 12846	100 g/L crude glycerol	125-mL shake flask (192 h), batch	23.5	0.24	0.12	(West, 2015, 2017)
Aspergillus niger ATCC 9142	100 g/L crude glycerol	125-mL shake flask (192 h), batch	16.5	0.17	0.09	(West, 2015, 2017)
	Thin stillage[1]	125-mL shake flask (192 h), batch	16.9	0.79[2]	0.09	(West, 2011, 2017)
Aspergillus ochraceus ATCC 18500	100 g/L glucose	1-L shake flasks (135 h), batch	17	-	0.13	(Bercovitz et al., 1990)
Aspergillus oryzae ATCC 56747	100 g/L glucose	1-L shake flasks (135 h), batch	35	-	0.26	(Bercovitz et al., 1990)
Aspergillus oryzae DSM 1863	120 g/L glucose monohydrate	500-mL shake flasks (353 h), batch	58.2	0.51	0.16	(Ochsenreither et al., 2014)

	109.1 g/L glycerol	500-mL shake flasks (353 h), batch	45.4	0.54	0.13	(Ochsenreither et al., 2014)
	111.5 g/L xylose	500-mL shake flasks (353 h), batch	39.4	0.44	0.11	(Ochsenreither et al., 2014)
	103.6 g/L cellobiose	500-mL shake flasks (168 h), batch	8.8	0.14	0.05	(Dörsam et al., 2017)
	109 g/L fructose	500-mL shake flasks (168 h), batch	24.8	0.63	0.15	(Dörsam et al., 2017)
	98.2 g/L levoglucosan	500-mL shake flasks (168 h), batch	17.2	0.34	0.10	(Dörsam et al., 2017)
	103.6 g/L maltose	500-mL shake flasks (168 h), batch	34.1	0.34	0.20	(Dörsam et al., 2017)
	109 g/L mannose	500-mL shake flasks (168 h), batch	32.8	0.69	0.19	(Dörsam et al., 2017)
	109 g/L ribose	500-mL shake flasks (168 h), batch	20.7	0.45	0.12	(Dörsam et al., 2017)
	Beech wood cellulose hydrolysate	2.5-L bioreactor, batch (168 h)	37.9	0.97	0.23	(Dörsam et al., 2017)
	Beech wood hemicellulose fraction	2.5-L bioreactor (168 h), batch	5.8	-	0.03	(Dörsam et al., 2017)
	Miscanthus cellulose hydrolysate	500-mL shake flask (168 h), batch	30.8	0.32	0.18	(Dörsam et al., 2017)
	Detoxified pyrolytic aqueous condensate	0.5-L bioreactor (144 h), batch	7.3	0.18	0.05	(Kubisch and Ochsenreither, 2022b)
Aspergillus oryzae DSM 1863/ *Clostridium ljungdahlii* DSM 13528	Syngas fermentation-derived acetic acid	2.5-L bioreactor (96 h), batch	1.1[3]	0.17	0.01	(Oswald et al., 2016)
Aspergillus oryzae NRRL 3488	50 g/L glucose monohydrate	2.7-L bioreactor (47.5 h), batch	30.3	0.73[4]	0.64	(Knuf et al., 2013)
	100 g/L glucose	2.7-L bioreactor (77 h), batch	26.8	0.62[4]	0.35	(Knuf et al., 2014)

Aspergillus sojae ATCC 46250	100 g/L glucose	1-L shake flasks (135 h), batch	30	-	0.22	(Bercovitz et al., 1990)
Penicillium viticola 152[5]	140 g/L glucose	10-L bioreactor (96 h), batch	130.1	0.99	1.36	(Khan et al., 2014)[6]
Penicillium sclerotiorum K302[5]	140 g/L glucose	10-L bioreactor (72 h), batch	71.2	0.68	0.99	(Wang et al., 2013)[7]
Rhizopus delemar ATCC20344	100 g/L glucose	250-mL shake flasks (80 h), batch	33.29	0.58	0.42	(Ronoh et al., 2022)
Schizophyllum commune IFO 4928	50 g/L glucose	8-L airlift column reactor (110 h), batch	ca. 20	ca. 0.4	0.18	(Kawagoe et al., 1997)
Zygosaccharomyces rouxii V19[8]	300 g/L glucose	Test tubes (15 d), batch	74.9	0.39	0.21	(Taing and Taing, 2007)

$Y_{P/S}$: substrate specific malic acid yield, r_p: volumetric malic acid production rate.

[1] Thin stillage contained 3.4 g/L glucose, 17.1 g/L glycerol and 15.8 g/L lactic acid.

[2] Yield refers to $g_{malic\ acid}/g_{glucose\ +\ glycerol}$.

[3] The average of three independent experiments (A: 1.83 g/L malic acid, 0.33 g/g; B: 1.42 g/L malic acid, 0.18 g/g; C: no acid production) was calculated.

[4] Values refer to the malic acid yield in the stationary phase only.

[5] Isolated from marine environment.

[6] Values indicated in publication refer to calcium malate (168.0 g/L, 1.28 g/g, 1.75 g/L/h) and were converted to malic acid in this table.

[7] Values indicated in publication refer to calcium malate (92.0 g/L, 0.88 g/g, 1.23 g/L/h) and were converted to malic acid in this table.

[8] Isolated from high-sugar fermented food.

Research on the generation of adapted or genetically modified organisms capable of producing high concentrations of malic acid has intensified especially in the past ten years (Table 2). Strategies focus on either enhancing the production capacity of wild type producers or modifying species that do not normally overproduce malic acid naturally. Among the latter, especially *E. coli* served as host. One strategy that enhanced the glyoxylate pathway by overexpression of genes encoding for PC, CS, ACN, ICL and MS and knocked out eight genes involved in byproduct formation or metabolization of malate resulted in the production of 36.1 g/L malic acid with a production rate of 0.60 g/L/h (Gao et al., 2018). In another study which reported one of the highest titers, yields and productivities obtained with *E. coli* so far, the construction of a CO_2-fixing bypass was employed by introducing the enzymes phosphoribulokinase, ribulose-1,5-bisphosphate carboxylase/oxygenase and carbonic anhydrase from *Synechocystis*, a cyanobacterium. Combined with the knockout of eleven genes and the introduction of phosphoenolpyruvate carboxykinase and malate dehydrogenase from *Actinobacillus succinogenes* to enforce malate synthesis, a titer of 51.9 g/L malic acid and a productivity of 0.72 g/L/h was obtained in a fed-batch process (Hu et al., 2018). However, these fermentation results cannot compare to those obtained with modified strains of natural malic acid producers, especially with species of *Aspergillus*. One of the earliest reports on malic acid production

with genetically modified *A. oryzae* enforced product synthesis through the rTCA pathway by overexpression of native pyruvate carboxylase and malate dehydrogenase as well as the native C4-dicarboxylate transporter C4T318. With this modified strain, 154 g/L malic acid were obtained in a fed-batch process from glucose with a productivity of 0.94 g/L/h (Brown et al., 2013). A similar approach was followed by Xu et al. (2019) in *A. niger*. By overexpressing pyruvate carboxylase and malate dehydrogenase, introducing the transporter C4T318 from *A. oryzae*, and deleting the gene encoding for oxaloacetate acetylhydrolase (catalyzes the hydrolytic cleavage of oxaloacetate to acetate and oxalate) to reduce byproduct formation, a potent strain was obtained which produced about 201 g/L malic acid, corresponding to a yield of 0.95 g/g and a volumetric production rate of 0.93 g/L/h (Xu et al., 2019). This strain was further optimized through deletion of a citrate exporter and the overexpression of a glucose transporter, hexokinase, 6-phosphofructo-2-kinase, and pyruvate kinase, which increased the malate yield to 1.22 g/g and the production rate reached 1.05 g/L/h (Xu et al., 2020). A higher production rate was for example obtained by Chen et al. (2019) who targeted the morphology of *A. oryzae* to increase malic acid production. The authors first performed random mutagenesis and screened for mutants exhibiting enhanced malate synthesis followed by metabolic engineering to overexpress tyrosine-protein phosphatase (CDC14). This resulted in a strain that formed smaller, more compact and smooth pellets with which 142.5 g/L malic acid were obtained in a fed-batch process after 132 h, resulting in a production rate of 1.08 g/L/h (Chen et al., 2019). The highest production rate of 1.38 g/L/h reported for *A. oryzae* so far was obtained by Liu, Xie et al. (2017) through a combination of various engineering strategies including the enforcement of the rTCA cycle through overexpression of MDH and PC, and introduction of genes encoding for phosphoenolpyruvate (PEP) carboxylase and PEP carboxykinase from *E. coli*. Furthermore, two transporters were overexpressed (C4T318) or introduced (maeI from *Schizosaccharomyces pombe*) and expression of 6-phosphofructokinase was increased, resulting in a malate titer of 165 g/L in a 120 h fed-batch process (Liu, Xie et al., 2017). Detailed descriptions of metabolic engineering strategies for malate production can be found in a recent review by Wei et al. (2021).

Table 2. L-Malic acid production processes with genetically modified or evolutionarily adapted microorganisms.

Microorganism	Initial substrate	Process	$c_{malic\ acid}$ [g/L]	$Y_{P/S}$ [g/g]	r_P [g/L/h]	Reference
Aspergillus carbonarius DCT	80 g/L glucose	100-mL shake flasks (9 d), batch	32	0.40	0.15	(Yang et al., 2017)
	Wheat straw hydrolysate (66 g/L glucose, 55 g/L xylose)	100-mL shake flasks (9 d), batch	20	0.18	0.09	(Yang et al., 2017)
Aspergillus niger PJR1	160 g/L crude glycerol	250-mL shake flask (192 h), batch	83.2	0.18[1]	0.43	(Iyyappan et al., 2018)

Aspergillus niger S1149	100 g/L glucose	2-L bioreactor (192 h), fed-batch	201.1	1.22	1.05	(Xu et al., 2020)
Aspergillus niger S575	100 g/L glucose	2-L bioreactor (216 h), fed-batch	201.2	0.95	0.93	(Xu et al., 2019)
Aspergillus oryzae 2103a-68.1	100 g/L glucose	2.7-L bioreactor (77 h), batch	66.3	1.11[2]	0.86	(Knuf et al., 2014)
Aspergillus oryzae CDC14(3)	120 g/L glucose	30-L bioreactor (132 h), fed-batch	142.5	0.75	1.08	(Chen et al., 2019)
Aspergillus oryzae CMPIMISN-3	130 g/L corn starch	250-mL shake flasks (100 h), batch	117.2	0.90	1.17	(Liu et al., 2018)
Aspergillus oryzae FMME218-37	146 g/L glucose	7.5-L bioreactor (168 h), fed-batch	95.2	0.54	0.57	(Ding et al., 2018)
Aspergillus oryzae FMME-S-38	130 g/L glucose	30-L bioreactor (144 h), fed-batch	164.9	0.77	1.14	(Ji et al., 2021)
Aspergillus oryzae GAAF41	30 g/L glucose, 70 g/L corn starch	250-mL shake flasks (70 h), batch	82.3	0.82	1.18	(Liu, Li et al., 2017)
Aspergillus oryzae SaMF2103a-68	160 g/L glucose	2-L bioreactor (164 h), fed-batch	154.0	1.03	0.94	(Brown et al., 2013)
Aspergillus oryzae WS-M-P-PP-C4-MA-PFK	110 g/L glucose	3-L bioreactor (120 h), fed-batch	165.0	0.68	1.38	(Liu, Xie et al., 2017)
Bacillus coagulans M03-M7-*pck*	50 g/L glucose	5-L bioreactor (84 h), fed-batch	25.5	0.3	0.30	(Sun et al., 2021)
Escherichia coli B0013-47	25 g/L glucose	3.6-L bioreactor (60 h), fed-batch	36.1	0.55	0.60	(Gao et al., 2018)
Escherichia coli BA063	50 g/L glucose	5-L bioreactor (67 h), batch	28.50	0.69	0.43	(Jiang et al., 2020)
Escherichia coli F0931	30 g/L glucose	5-L bioreactor (72 h), fed-batch	21.65	0.36	0.30	(Dong et al., 2017)
Escherichia coli FH389	50 g/L glucose	1-L bioreactor (72 h), fed-batch	51.9	1.09	0.72	(Hu et al., 2018)
Escherichia coli GL2306	20 g/L glucose	5-L bioreactor (72 h), fed-batch	25.9	0.39	0.36	(Guo et al., 2018)
Escherichia coli KJ071	100 g/L glucose	350-mL bioreactor[3], batch	69.2	1.07	-	(Jantama et al., 2008)
Escherichia coli WGS-10	20 g/L glucose	5-L bioreactor (12 h), batch	9.25	0.56	0.74	(Moon et al., 2008)
Escherichia coli XZ658	50 g/L glucose	3-L bioreactor (72 h[4]), batch	34	1.06	0.47	(Zhang et al., 2011)
Monascus araneosus ST91	100 g/L glucose	50-mL shake flask[3] (5 days), batch	27.9	0.37	0.23[5]	(Lumyong and Tomita, 1993)
Pichia pastoris Pp-PC-MDH1	100 g/L glucose	0.6-L bioreactor (96 h), batch	42.3	-	0.44	(Zhang et al., 2015)

Rhizopus delemar HF-121	Corn straw hydrolysate (125 g/L mixed sugar)	3-L bioreactor (60 h), batch	120.5	-	2.01	(Li et al., 2014)
Saccharomyces cerevisiae RWB525	200 g/L glucose	500-mL shake flasks (ca. 312 h), batch	59	0.31	0.19 or 0.29[6]	(Zelle et al., 2008)
Saccharomyces cerevisiae W4209	100 g/L glucose	500-mL shake flasks (96 h), batch	30.25	0.30	0.32	(Chen et al., 2017)
Thermobifida fusca muC-16	100 g/L cellulose (Avicel)	3-L bioreactor[3] (124 h), batch	62.8	-	0.51	(Deng et al., 2016)
	50 g/L milled corn stover	3-L bioreactor[3], (5 d), batch	21.47	-	0.18[5]	(Deng et al., 2016)
Torulopsis glabrata PMS	60 g/L glucose	500-mL shake flasks (60 h), batch	8.5	0.14	0.18	(Chen et al., 2013)
Ustilago trichophora TZ1	200 g/L glycerol	2.5-L bioreactor (264 h), fed-batch	195	0.43	0.74	(Zambanini et al., 2016)
	200 g/L crude glycerol	2.5-L bioreactor (ca. 160 h), fed-batch	ca. 120	0.26	0.75	(Zambanini et al., 2016)

$Y_{P/S}$: substrate specific malic acid yield, r_p: volumetric malic acid production rate.

[1] The yield of 0.18 g/g as indicated in the publication seems too low. From the data available the yield is estimated at about 0.6 g/g.

[2] Refers to the malic acid yield in the stationary phase only.

[3] Indicates working volume.

[4] Refers to the anaerobic malic acid production phase which was preceded by a 16-h aerobic phase for cell growth.

[5] Calculated using a time span of 120 h (= 5 days).

[6] Calculated over glucose consumption phase (= 192 h).

The processes described so far used glucose as carbon source. However, as displayed in Table 1 and Table 2, various other substrates have been evaluated for malic acid production. In microbial processes, the substrate costs represent a large part of the overall production expenses, for which reason the utilization of inexpensive raw materials is essential (Efe et al., 2013; Manandhar and Shah, 2020). Industrial side streams such as thin stillage or crude glycerol, or hydrolysates of lignocellulose are therefore promising carbon sources. Thin stillage, mainly containing glycerol and residual (poly-)saccharides (Kim et al., 2010), is a major byproduct of the dry-milling process for ethanol production. The amount of thin stillage produced during dry-milling can amount to volumes 15-fold higher than the produced volume of ethanol, making it a low-cost substrate (Reis et al., 2017). Thin stillage was evaluated for malic acid production with *A. niger*, resulting in a concentration of 16.9 g/L (West, 2011, 2017). In biodiesel production, crude glycerol represents the main side-product and amounts to about 10% (w/w) of the produced amount of biodiesel, and given the increasing worldwide production of biofuels, the market for crude glycerol is oversaturated,

making it a low-cost raw material (Yang et al., 2012). With a strain of *Ustilago trichophora* obtained through adaptive laboratory evolution, about 120 g/L malic acid were obtained using this substrate with a production rate of 0.75 g/L/h (Zambanini et al., 2016). Malic acid production from crude glycerol was also detected with *A. niger*, resulting in 23.5 g/L malic acid after 192 h (West, 2015, 2017). Besides industrial side streams, substrates derived from lignocellulose were also identified as suitable feedstocks for malic acid production. For instance, product formation was detected with hydrolysates of both the cellulose and the hemicellulose fraction of beech wood in fermentations with *A. oryzae*, although the titer using the cellulose fraction was considerably higher, amounting to 37.9 g/L after 168 h (Dörsam et al., 2017). With the cellulose hydrolysate of miscanthus, an energy crop with high biomass yields, a malic acid production rate of 0.18 g/L/h resulted in 30.8 g/L of the product after 168 h (Dörsam et al., 2017). Malic acid production was also recently reported during cultivation of *A. oryzae* on a detoxified pyrolytic aqueous condensate, reaching a concentration of 7.3 g/L and a production rate of 0.05 g/L/h (Kubisch and Ochsenreither, 2022b). Substrates derived from lignocellulosic biomass are usually a complex mixture containing various mono- and polysaccharides, phenolic compounds, and further potential inhibitors. They also often contain acetic acid, a substrate that was first demonstrated to be a suitable carbon source for malic acid production with *A. oryzae* by Oswald et al. (2016) and which will be presented in more detail in the next chapter.

1.4. The carbon source acetate

Considering an increasing world population and a decrease in cropland suitable for growing products intended for human or animal consumption due to climate change, alternatives need to be considered for the substrate glucose, which is commonly used in bioprocesses. In this regard, substrates derived from lignocellulosic biomass and C1 gases are especially interesting. These may either contain acetic acid due to biomass degradation or can be converted to acetate through microbial processes. An overview of resources from which acetate can be obtained and possible acetate-derived products is displayed in Figure 4.

Industrially, acetic acid is mainly obtained by chemical synthesis from fossil raw materials as summarized by Pal and Nayak (2017). Most commonly, the production is performed through carbonylation of methanol using the Monsanto process which applies a rhodium-based catalyst, or the Cativa process which uses a more economic iridium catalyst. Most of this chemically produced acetic acid is used for the synthesis of vinyl acetate monomer which is widely used in polymerized form in adhesives (Pal and Nayak, 2017). Microbial production of acetic acid intended for human consumption in the form of vinegar is also well established. In this aerobic process, ethanol, which can be produced from carbohydrates (e.g. fruits, grains) using yeasts, is converted to acetic acid by acetogenic bacteria of different genera such as *Acetobacter* or *Gluconacetobacter* (Fernández-Pérez et al., 2010; Gullo et al., 2014). Acetic acid production of 40-70 g/L is usually observed within 2-3 days of fermentation (Kondo and Kondo, 1996; Qi et al., 2014; Song et al., 2022; Zheng et al., 2017),

and maximum concentrations of 150-200 g/L are possible with adapted organisms and optimized processes (Ebner and Enenkel, 1978; Kunimatsu et al., 1983). However, the utilization of acetate derived from renewable but non-edible resources is more suitable for the production of chemicals in a bio-based economy.

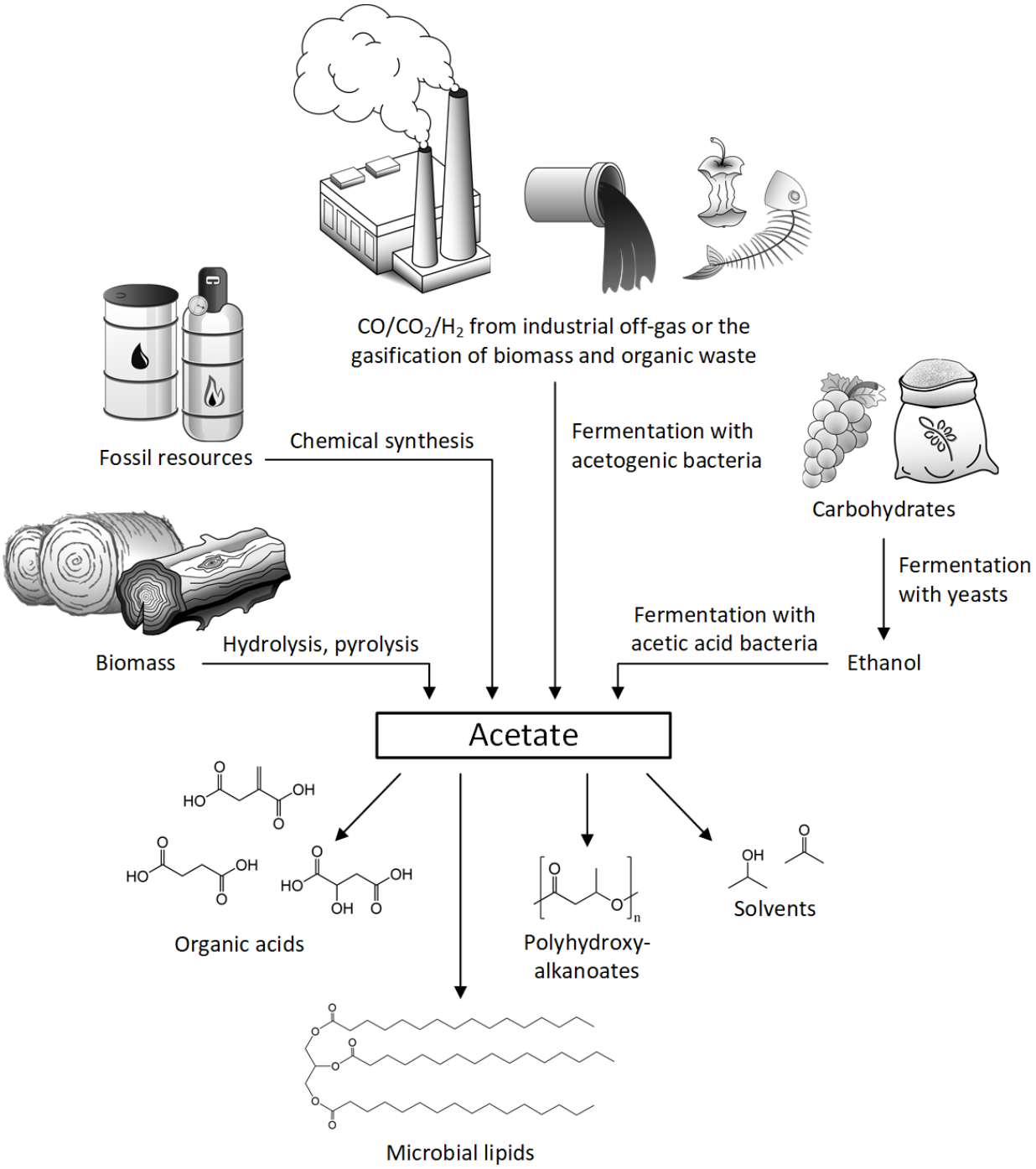

Figure 4. Resources from which acetate can be obtained and possible acetate-derived products. (Partly based on Kiefer et al. (2021)).

Acetic acid is a degradation product of lignocellulosic biomass, which describes a complex of polymers consisting of cellulose, hemicellulose and lignin. To make this feedstock accessible for

efficient chemical or microbial conversion, the polymer network needs to be pretreated. This can be achieved by various chemical, physical or biological methods. The acetic acid in the resulting liquid substrates originates from the cleavage of acetyl groups of hemicelluloses (Jönsson and Martín, 2016). Hence, the acetate content of biomass-derived products varies depending on the type of biomass, and feedstocks containing a high hemicellulose content are most suitable. A common thermal pretreatment method is fast pyrolysis. In this process, biomass of various origins, such as forestry or agricultural residues, is exposed to temperatures of about 500 °C in the absence of oxygen, producing solid char and pyrolysis vapors (Pfitzer et al., 2016). Condensation of these vapors results in an energy-rich bio-oil, a complex substrate containing a wide variety of organic compounds such as ketones, aldehydes, phenols, alcohols and acids, with acetic acid being one of them. Acetic acid contents of about 5-17% (wt. dry basis) are common in pyrolysis products, making it a main constituent in these substrates (Bertero et al., 2012; Demirbas, 2007; Oh et al., 2017). In some processes, such as the bioliq® process ('biomass-to-liquid'), established in the form of a pilot plant at the Karlsruhe Institute of Technology, the vapors are condensed in two steps, yielding the desired energy-rich bio-oil and an aqueous condensate with a low heating value (Pfitzer et al., 2016) which contains acetic acid in concentrations commonly ranging between 30-50 g/L (Arnold et al., 2019; Kubisch and Ochsenreither, 2022a). Although some of the aqueous condensate is further used in the process, a part of it remains unexploited, representing an inexpensive side stream, which features a carbon concentration suitable for microbial valorization. Besides pyrolysis, biomass can also be depolymerized through the treatment with solvents, hot water or steam, and/or acids and a subsequent enzymatic treatment if necessary. The resulting liquid substrates can contain about 10 g/L acetic acid depending on the type of biomass and pretreatment method (Costa et al., 2017; Taherzadeh et al., 1997).

Besides aerobic oxidation which is performed by acetic acid bacteria during vinegar production, acetic acid can also be obtained through anaerobic fermentation using acetogenic bacteria. These microorganisms are capable of using syngas for acetic acid production via the Wood-Ljungdahl or reductive acetyl-CoA pathway. Syngas is a mixture containing mainly CO, H_2 and CO_2 which can be obtained through industrial off-gases or the gasification of biomass and organic wastes (e.g. municipal solid waste, sewage sludge) (Gavala et al., 2021). Acetogens can utilize CO and/or CO_2 while H_2 or CO can function as electron donor. Hence, this pathway fixes CO_2 which is interesting regarding the reduction of greenhouse gas emissions. Suitable species are for example *Clostridium ljungdahlii*, *Acetobacterium woodii* or *Moorella thermoacetica*. Acetic acid production with these strains often results in concentrations of about 20-30 g/L (Hu et al., 2016; Infantes et al., 2020) but high titers of up to 59 g/L with a productivity of 0.77 g/L/h have been reported (Kantzow et al., 2015).

Another strategy for acetate production with acetogenic bacteria is microbial electrosynthesis (MES). In MES systems, microbial reduction of CO_2 to acetate takes place at a cathode when applying an electric current which is separated from the anode through an ion exchange membrane. The microorganisms can take up the electrons directly from the cathode or through intermediates

such as hydrogen produced in the MES cell (May et al., 2016). This is a rather new approach for microbial acetate production and optimization is required to increase maximum concentrations. In a biochemical cell consisting of three compartments (anode, cathode and an extraction compartment), about 13.5 g/L acetate were obtained in the extraction compartment after 43 days with a mixed microbial community (Gildemyn et al., 2015). In another study employing a mixed community, a space time yield of 0.74 g/L/h acetate was obtained after 36 days of operation (LaBelle and May, 2017), which is comparable to the production rate reported for gas fermentation with *A. woodii* in a batch process (Kantzow et al., 2015).

Instead of electricity, light irradiation can also be used for the generation of electrons for acetate production from CO_2 via artificial photosynthesis using a semiconductor. This was demonstrated using CdS nanoparticles encapsulating *Moorella thermoacetica* (Sakimoto et al., 2016) or *Sporomusa ovata* (He et al., 2022). When a photon is absorbed by CdS, the generated electron produces a reduction equivalent which is used for the synthesis of acetic acid from CO_2 via the Wood-Ljungdahl pathway. With this process, it is thus possible to directly use sunlight for organic acid production. However, this strategy is yet in the early stages of research and low acetate concentration of about 40 mM (ca. 2.4 g/L) were obtained within 4-5 days (He et al., 2022).

In lab scale, acetate has been evaluated as substrate for the production of several compounds including organic acids such as itaconic or succinic acid, microbial lipids, solvents such as acetone or isopropanol, polyhydroxyalkanoates (PHAs), or fine chemicals such as mevalonic acid or phloroglucinol. An overview of these most recent processes can be found in Kiefer et al. (2021). Acetate is generally metabolized via acetyl-CoA and the glyoxylate cycle. As displayed in Figure 3, acetate is transformed to acetyl-CoA either by acetate kinase (ACK) and phosphate acetyltransferase (PTA), which is a reversible reaction, serving both for production and consumption, or acetyl-CoA-synthetase (ACS), which only catalyzes the assimilation of acetate (Lim et al., 2018). The theoretical maximum molar yield for organic acid production from acetate is generally lower than that from glucose as the activation of acetate requires ATP. For itaconic acid production, 0.33 mol can be produced per mol of acetate (Noh et al., 2018) and 0.5 mol/mol of acetate were indicated for the synthesis of succinate (Li et al., 2016). For malic acid production through the glyoxylate cycle, a theoretical maximum yield of 0.5 mol per mol of acetate also seems possible (see Appendix A). However, this is only an estimate as several assumptions have been made regarding the regeneration of ATP and the actual yield may be lower due to energy requirements for other metabolic processes such as maintenance metabolism.

A challenge of using acetate as substrate is its pH-dependent shift between the protonated and anionic form. Acetic acid features a pK_a value of 4.75 and is therefore almost completely dissociated at neutral pH. The protonated form, dominating at low pH values, is assumed to be the main contributor to the inhibiting effect of the carbon source. Different than the acetate anion, acetic acid can enter the cytoplasm through diffusion through the lipid plasma membrane. It then deprotonates due to near-neutral pH values inside the cell, causing an intracellular acidification which can impede

the proton motive force (Stratford et al., 2009). An advantage of fungi is their ability to withstand low pH values, which reduces the risk of bacterial contamination in fermentation processes. During citric acid production with *A. niger*, for example, the pH may decrease to values of around 2 (Ikram-Ul et al., 2004; Omar et al., 1992; Sakurai et al., 1997). However, when acetate is used, low pH values are not suitable for production due to an increased amount of the free acid. Thus, the pH of the fermentation medium plays an important role regarding the substrate concentration a microorganism can tolerate in processes using acetate. Besides this potential challenge, the utilization of acetate also features some advantages. Acetic acid is highly miscible with water, is liquid in pure form, and can be used for pH control and as carbon feed simultaneously. These features allow to maintain high substrate concentrations with a low dilution of the fermentation medium. Together with the raw material flexibility from which acetate can be obtained, this makes it a promising substrate for the bio-based production of chemicals.

1.5. Research proposal

As presented in the previous chapter, acetate-containing substrates can be a promising feedstock in a bio-based economy. Therefore, the aim of this work was to explore the potential of the carbon source acetate for L-malic acid production, a chemical currently produced from fossil resources. For microbial malate synthesis, *Aspergillus oryzae* is a suitable production organism as it secretes the acid naturally and has a wide substrate spectrum. Although the feasibility of malic acid production from acetate has been demonstrated, no detailed study or optimization of the process has been reported.

The first question which needed to be addressed was the optimal acetate concentration for biomass and acid production, and the maximum malate concentration that can be achieved in a batch process. Therefore, shake flask experiments were performed and the growth behavior and production of organic acids with different initial acetate concentrations was evaluated. Since *Aspergillus* species are known to secrete not only one but usually several organic acids, it was of great interest to analyze the side product spectrum, as this has not been reported previously. Subsequently, the process should be characterized in more detail by investigating different cultivation temperatures during organic acid production, the influence of calcium carbonate and various nitrogen sources, and the effect of adding low concentrations of glucose to acetate medium.

Since the morphology plays a major role in processes involving filamentous microorganisms, this study aimed to address the question whether it can be controlled by entrapment in polymers. The morphology is dependent on various cultivation parameters and immobilization in a matrix of a defined size could allow for control of phenotypic growth largely independently of these influences. Therefore, conidia of *A. oryzae* were entrapped in the natural polymers alginate, κ-carrageenan, and agar for malic acid production in shake flasks and 2.5-L bioreactors.

After characterizing the process in batch operation, the possibility of improving the production by adapting the process management strategy should be evaluated. This was to take advantage of the dual function of acetic acid to simultaneously control the pH and provide additional carbon to avoid substrate inhibition. Therefore, a fed-batch and repeated-batch process was first investigated in shake flasks, followed by the transfer of the fed-batch process to 2.5-L bioreactors where the implementation of a pH-coupled feeding strategy was targeted.

2. Evaluation of growth and organic acid production with acetate as carbon source

This chapter is based on the publication:

Acetate as substrate for L-malic acid production with *Aspergillus oryzae* DSM 1863
Aline Kövilein, Julia Umpfenbach, Katrin Ochsenreither (2021)
Biotechnology for Biofuels 14, 48
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Author contributions

Aline Kövilein: conceptualization, investigation, data analysis, writing of first draft, manuscript revision.

Julia Umpfenbach: investigation, data analysis.

Katrin Ochsenreither: conceptual advice, project supervision, manuscript revision.

2.1. Introduction

At the beginning of the project, available information concerning malic acid production from acetate was scarce. Oswald et al. (2016) were the first to report malate production with *A. oryzae* using this carbon source but they did not further investigate the process. During the coupling of syngas fermentation with the subsequent production of malic acid, the authors obtained low malic acid concentrations of 1.8 g/L, 1.4 g/L and 0 g/L in a triplicate fermentation. This demonstrates that malic acid production based on acetate needs to be evaluated in more detail to eventually use complex biomass-based substrates efficiently. Therefore, several cultivation parameters of malic acid production from acetate were first investigated in shake flask fermentations. These include the acetate concentration during growth and organic acid production to find the optimum for each stage. Furthermore, the influence of adding low amounts of glucose to acetate medium was assessed. Additionally, the side product spectrum of *A. oryzae* depending on the substrate type and concentration was identified and the necessity of adding $CaCO_3$ for efficient acid production was demonstrated.

2.2. Materials and Methods

2.2.1. Microorganism and chemicals

All chemicals were purchased from Sigma-Aldrich (Germany) or Carl-Roth (Germany). *Aspergillus oryzae* DSM 1863 was obtained from DSMZ strain collection (German Collection of Microorganisms and Cell Cultures GmbH).

2.2.2. Spore propagation

For spore collection, *A. oryzae* was grown on a minimal medium for *Aspergillus* species containing 6 g/L $NaNO_3$, 0.52 g/L KCl, 0.52 g/L $MgSO_4 \cdot 7H_2O$, and 1.52 g/L KH_2PO_4 (Barratt et al., 1965). The pH was set to 6.5 with NaOH. 15 g/L glucose monohydrate, 2 mL/L of 1000x Hutner's trace elements, and 15 g/L agar were added afterwards and the medium was autoclaved for 20 min at 121 °C. 1000x Hutner's trace element solution is composed of 5 g/L $FeSO_4 \cdot 7H_2O$, 50 g/L EDTA-Na_2, 22 g/L $ZnSO_4 \cdot 7H2O$, 11 g/L H_3BO_3, 5 g/L $MnCl_2 \cdot 4H_2O$, 1.6 g/L $CoCl_2 \cdot 6H_2O$, 1.6 g/L $CuSO_4 \cdot 5H_2O$, and 1.1 g/L $(NH_4)_6Mo_7O_{24} \cdot 4H_2O$, pH 6.5 (Hill and Kafer, 2001). Conidia were harvested with 50% glycerol after 7-10 days of incubation at 30 °C by gently scraping the plate surface with a sterile inoculation loop. The conidia suspension was filtered through Miracloth (Merck KGaA, Darmstadt, Germany) and diluted to a concentration of 3×10^7 conidia per mL with 50% glycerol. Aliquots were stored at -80 °C.

2.2.3. Preculture for biomass production

Malic acid production was performed as a two-stage process consisting of a preculture for biomass production and a main culture for acid production. In the preculture an excess of nitrogen was present to promote biomass formation while the nitrogen supply in the main culture was reduced to

favor acid production over cell growth. The preculture medium consisted of 5-70 g/L sodium hydroxide-neutralized acetic acid (pH 6.5) or 40 g/L glucose monohydrate as carbon source, 4 g/L $(NH_4)_2SO_4$, 0.75 g/L KH_2PO_4, 0.98 g/L K_2HPO_4, 0.1 g/L $MgSO_4 \cdot 7H_2O$, 0.1 g/L $CaCl_2 \cdot 2H_2O$, 5 mg/L NaCl, and 5 mg/L $FeSO_4 \cdot 7H_2O$. While for the glucose medium the pH was not adjusted, it was adjusted to 6.5 for the preculture media containing acetate. All preculture media were sterilized by autoclaving for 20 min at 121 °C. For biomass formation, 100 mL of preculture medium in 500-mL baffled Erlenmeyer shake flasks was inoculated with 3 x 10^7 conidia and incubated for 48 h (acetate media) or 24 h (glucose media) at 30 °C and 100 rpm. After incubation, the biomass used for the inoculation of the main culture was separated from the preculture medium by filtration through Miracloth (Merck) and washed with double-distilled water. For the growth experiments in the preculture, samples of 1.9 mL were taken in the beginning of the experiment and after 48 h to determine substrate and ammonium consumption. Afterwards, the entire content of a flask was used for biomass determination via cell dry weight. All experiments were performed as biological triplicates.

2.2.4. Main culture for organic acid production

The main culture medium contained 10-55 g/L sodium hydroxide-neutralized acetic acid (pH 5.5) or 120 g/L glucose monohydrate as carbon source, 1.2 g/L $(NH_4)_2SO_4$, 0.1 g/L KH_2PO_4, 0.17 g/L K_2HPO_4, 0.1 g/L $MgSO_4 \cdot 7H_2O$, 0.1 g/L $CaCl_2 \cdot 2H_2O$, 5 mg/L NaCl, and 60 mg/L $FeSO_4 \cdot 7H_2O$. Additionally, 90 g/L $CaCO_3$ was added to the main culture media unless stated otherwise. While for the glucose medium the pH was not adjusted, it was adjusted to 5.5 for the main culture media containing acetate. The pH of the main culture media was set to a lower pH than the preculture media to moderate the pH increase caused by the $CaCO_3$ addition. For main cultures without $CaCO_3$, the pH was set to 6.5. All media were sterilized by autoclaving for 20 min at 121 °C. For the media containing 45 g/L acetic acid and 5-15 g/L glucose, the glucose solution was sterile filtered and added to the medium after autoclaving. For the experiments with 45 g/L acetic acid + 5-15 g/L glucose in the main culture medium, the preculture was grown in medium containing 45 g/L acetic acid. For acid production, 100 mL of the main culture medium was transferred to 500-mL baffled Erlenmeyer shake flasks, inoculated with 0.75 g of the washed biomass obtained during the preculture and incubated at 120 rpm and 32 °C. Samples of 4 mL were taken at the indicated time points for pH, organic acid and ammonium measurement. All experiments per performed as biological triplicates.

2.2.5. Analytics

In the preculture fungal morphology was characterized by light microscopy using a Nikon Eclipse E200 equipped with a DFK 23U274 camera (Imaging Source, Bremen, Germany) supported by the software NIS-Elements D ver. 4.50.

For quantification of biomass formation in the preculture, the dry biomass weight was determined. For this purpose, the culture broth of an entire flask was filtered through pre-dried and pre-weighed

Miracloth (Merck) after an incubation time of 48 h and washed thoroughly with double-distilled water. The biomass, together with the Miracloth, was dried at 70 °C for at least 48 h until weight constancy. The weight of the biomass-containing Miracloth was measured with precision scales and used for the calculation of the dry biomass weight in g/L.

Organic acids were quantified by HPLC. Samples were pretreated with sulfuric acid to release precipitated calcium malate as described by Ochsenreither et al. (2014). A sample volume of 1 mL was mixed with 3 mL of water and 1 mL of 3 M H_2SO_4, followed by an incubation period of 20 min at 80 °C. Subsequently, 1 mL of the suspension was centrifuged for 10 min at 20,000xg and the supernatant was used for HPLC analysis. The analysis was performed using a standard HPLC device (Agilent 1100 Series, Agilent, Germany) equipped with a Rezex ROA organic acid H+ (8%) column (300 by 7.8 mm, 8 µm, Phenomenex) and a Rezex ROA organic acid H+ (8%) guard column (50 by 7.8 mm). Samples were analyzed at 60 °C with 3 mM H_2SO_4 as mobile phase and a flow of 0.5 mL/min. The injection volume was 10 µL and detection was performed with a UV detector at 220 nm.

For glucose determination by HPLC, 1 mL of the culture broth was centrifuged for 10 min at 20,000xg and the supernatant was used for analysis using the same column set-up as for organic acid determination. Samples were analyzed at 50 °C with 5 mM H_2SO_4 and a flow of 0.5 mL/min. The injection volume was 10 µL and glucose was detected with a refractive index detector.

Ammonium concentration was quantified photometrically using the Spectroquant kit (114752, Merck KGaA, Darmstadt, Germany). The assay was scaled down to a volume of 200 µL and sample supernatants were measured in duplicate in microtiter plates according to the manufacturer's instructions.

2.2.6. Data Analysis

Product formation and substrate consumption for experiments using either acetate or glucose as sole carbon source were fitted using a four-parameter logistic equation with the data analysis and graphing software OriginPro 2020 (OriginLab Corporation, Northampton, USA). As iteration algorithm the Levenberg-Marquardt algorithm was used. The used equation was the following:

$$y(x) = \frac{A_1 - A_2}{1+(\frac{x}{x_0})^p} + A_2 \qquad \text{(Eq. 1)}$$

In Eq. (1), A_1 indicates the initial carbon source or product concentration, A_2 indicates the final carbon source or product concentration, x_0 indicates the process time when half of the amount of carbon source is consumed or half of the maximum product concentration is reached, and p is a shape parameter.

Product formation and substrate consumption for mixed substrate fermentations was fitted using a modified Gompertz model (Erkmen and Alben, 2002):

$$y(x) = A \exp(-\exp(\frac{ve}{A}(d - x) + 1) \quad \text{(Eq. 2)}$$

with y being the carbon source or product concentration, A the upper asymptote value, v the acid formation or product consumption rate and d the lag phase duration. For both equations, the first derivative of the fitted curve was used for the calculation of the maximum production rates and corresponding time points.

2.3. Results

2.3.1. Acetate as substrate for growth of *A. oryzae*

To determine the optimum acetate concentration for biomass production with *A. oryzae*, substrate concentrations of 5-70 g/L were evaluated. As presented in Table 3, biomass formation was detected for all conditions. The highest dry biomass titers were obtained with acetic acid concentrations of 40-55 g/L with values ranging from 1.08 ± 0.14 g/L to 1.14 ± 0.11 g/L. Below 40 g/L and above 55 g/L the dry biomass concentration decreased, and the lowest value of 0.12 ± 0.02 g/L was measured for cultures with 70 g/L acetic acid. The substrate and ammonium consumption showed a correlating behavior. The highest consumption values were measured for cultures with 40-55 g/L acetic acid and ranged from 3.47 ± 0.19 g/L to 5.68 ± 0.31 g/L for acetate and 0.17 ± 0.06 g/L to 0.22 ± 0.03 g/L for ammonium. Thus, the overall substrate and ammonium consumption within 48 h was low. The biomass yields decreased with increasing substrate concentration and ranged from 0.17 ± 0.08 g/g (70 g/L acetic acid) to 0.76 ± 0.19 g/g (5 g/L acetic acid).

Table 3. Comparison of growth of *A. oryzae* after 48 h of cultivation with different acetic acid concentrations. Data are the means of triplicate experiments with respective standard deviations.

Initial acetic acid [g/L]	Consumed substrate [g/L]	Consumed ammonium [g/L][a]	Dry biomass [g/L]	$Y_{X/S}$ [g/g]
5	0.76 ± 0.14	0.05 ± 0.02	0.56 ± 0.09	0.76 ± 0.19
10	1.54 ± 0.24	0.16 ± 0.03	0.79 ± 0.08	0.52 ± 0.05
20	1.65 ± 0.11	0.16 ± 0.02	0.80 ± 0.10	0.48 ± 0.03
30	2.05 ± 1.09	0.15 ± 0.07	0.92 ± 0.11	0.54 ± 0.29
40	3.47 ± 0.19	0.18 ± 0.04	1.14 ± 0.11	0.33 ± 0.04
45	5.68 ± 0.31	0.21 ± 0.04	1.11 ± 0.10	0.18 ± 0.03
50	3.48 ± 0.42	0.17 ± 0.06	1.11 ± 0.15	0.33 ± 0.09
55	4.25 ± 0.49	0.22 ± 0.03	1.08 ± 0.14	0.25 ± 0.03
60	2.86 ± 0.67	0.15 ± 0.03	0.74 ± 0.12	0.26 ± 0.04
65	1.97 ± 0.54	0.12 ± 0.01	0.53 ± 0.11	0.27 ± 0.02
70	0.55 ± 0.56	0.03 ± 0.02	0.12 ± 0.02	0.17 ± 0.08

$Y_{X/S}$ = substrate specific dry biomass yield calculated as g(dry biomass)/g(consumed acetic acid).

[a] Initial ammonium concentration was 1.09 g/L.

The morphology of *A. oryzae* was influenced by the concentration of acetate as depicted in Figure 5. Microscopic analysis showed a pellet-like morphology with acetic acid concentrations up to 30 g/L while the size of the pellet core decreased with increasing acetate concentration. With 5 g/L acetic acid, the pellet core size was about 2 mm while it was only about 1.0-1.5 mm with 30 g/L of the substrate. Concentrations above 30 g/L acetic acid led to a filamentous-lumpy structure and no pellets were formed.

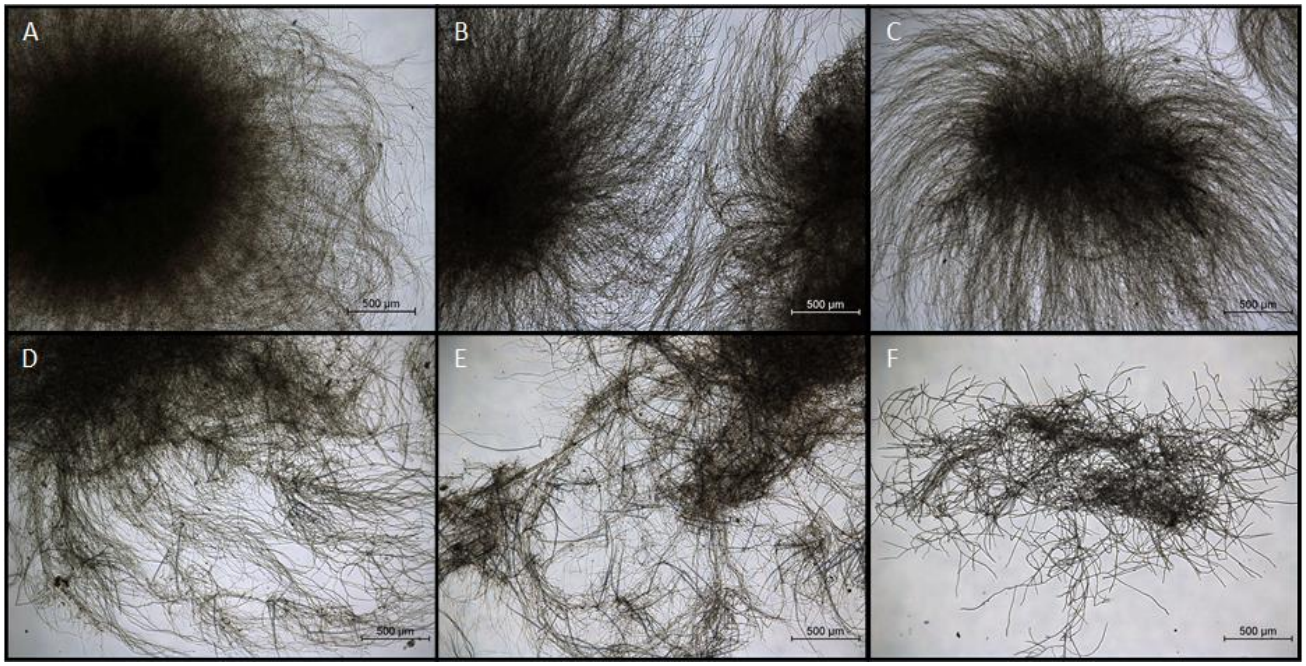

Figure 5. Morphology of *A. oryzae* after 48 h of growth with 5 g/L (A), 20 g/L (B), 30 g/L (C), 45 g/L (D), 55 g/L (E) and 65 g/L (F) acetic acid.

2.3.2. Acetate as substrate for L-malic acid production with *A. oryzae*

For the production of malic acid, acetic acid concentrations of 10 to 55 g/L were tested. In the acid production stage, the ammonium concentration was reduced to 0.33 g/L and 90 g/L $CaCO_3$ was added. The pH of all main culture media was set to 5.5, thus lower than that of the preculture media (pH 6.5) to level out the pH increase caused by the addition of $CaCO_3$. In preliminary experiments, initial medium pH values of 4.5-6.5 were tested with 45 g/L acetic acid and the highest malic acid production was observed for an initial medium pH of 5.5 (Appendix B, Figure B1). Concentration curves for malic and acetic acid are displayed in Figure 6 and calculated values are summarized in Table 4. After 48 h, malic acid was first detected in cultures with 45 g/L acetic acid (0.33 ± 0.13 g/L). The majority of malic acid production was completed within 144 h and only a slight further increase was monitored between 144 and 192 h. After 192 h, no significant increase of malic acid concentration was observed. For this reason, all calculations of Table 4 are based on the measurements at 192 h. The highest malic acid concentrations after 192 h were determined for cultures with 45 g/L (8.44 ± 0.42 g/L) and 50 g/L acetic acid (8.45 ± 0.49 g/L). Lower malic acid titers were obtained with decreasing substrate concentration and no malate synthesis was detected in

cultures with 10 g/L acetic acid. For the cultures with 10-45 g/L initial acetic acid, the carbon source was either depleted or very close to depletion after 192 h whereas for the two highest substrate concentrations about 14% (50 g/L acetic acid) and 30% (55 g/L acetic acid) remained. Maximum productivities and respective time points were calculated as the maximum of the first derivative of the fitted curve (Equation 1). Maximum productivities ranged from 0.020 g/L/h (20 g/L acetic acid) to 0.123 g/L/h (40 g/L acetic acid). The time point of maximum productivity was lowest for cultures with 45 g/L acetic acid (85 h) and highest using 20 g/L acetic acid (107 h). As for the overall productivities, the highest value of 0.044 g/L/h was obtained for cultures with 45 and 50 g/L acetic acid. Regarding the nitrogen consumption, no ammonium could be detected after 96 h of cultivation with acetic acid concentration of 30-55 g/L while with 20 g/L acetic acid about 5% of the nitrogen was left. In the cultures with 10 g/L acetic acid nitrogen limitation was only reached after 192 h (Appendix B, Figure B2).

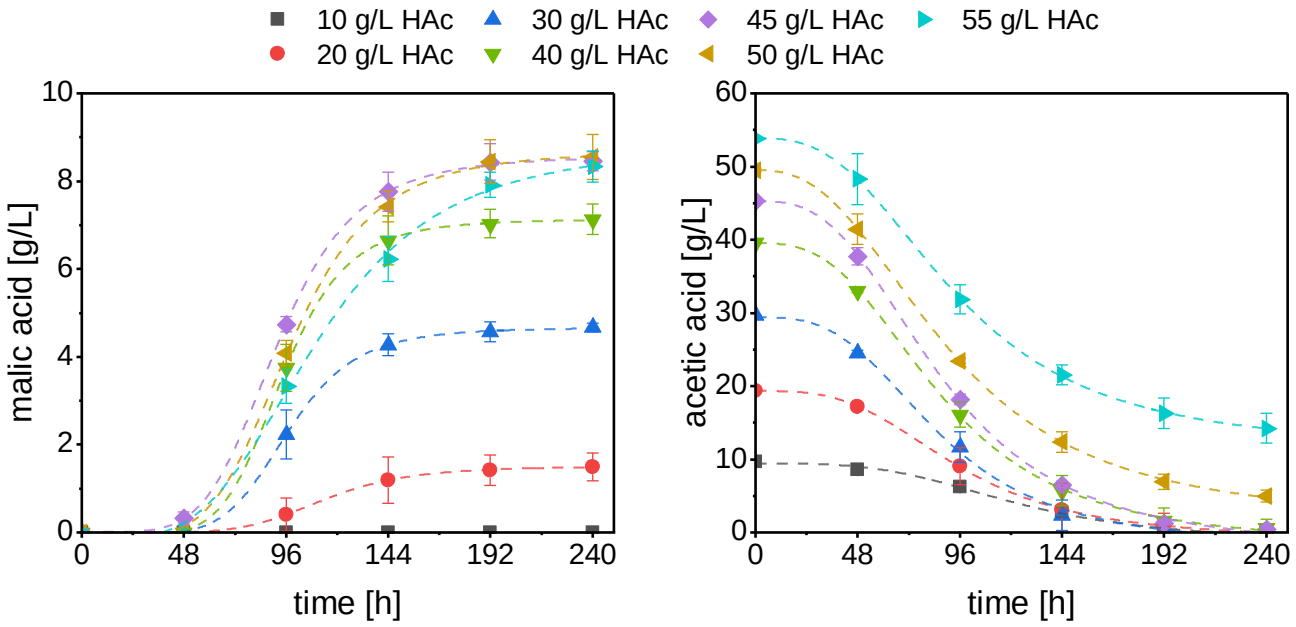

Figure 6. Malic acid production with initial acetic acid concentrations of 10-55 g/L. Data are the means of triplicate experiments with respective standard deviations. HAc = acetic acid.

Besides acetate as sole carbon source, mixtures of 45 g/L acetic acid and 5, 10 and 15 g/L glucose were evaluated for malic acid production. By adding low concentrations of glucose to a medium with acetate as the main carbon source, the possibility of accelerating the production should be assessed. As displayed in Figure 7, glucose was depleted within 48 h for cultures with 5 and 10 g/L and within 96 h for an initial concentration of 15 g/L. In the cultures with 15 g/L glucose, about 12 g/L of the substrate was consumed during the first 48 h while at the same time about 10 g/L acetic acid was consumed, showing simultaneous consumption of the two carbon sources. Acetic acid was close to depletion after 144 h which is faster compared to the cultures using it as sole carbon source in which still about 7 g/L acetic acid remained after 144 h. A maximum malic acid concentration of 14.18 ± 0.09 g/L was quantified using 45 g/L acetic acid + 15 g/L glucose which corresponds to a

yield of 0.24 ± 0.00 g/g total substrate. An increase in overall and maximum productivities as well as a decrease in the time of maximum productivity was associated with the presence of glucose in the medium. The lowest time point of maximum productivity (59 h) was calculated for cultures with 10 g/L glucose which represents a decrease of 31% compared to the cultures with 45 g/L acetic acid only. Regarding the nitrogen consumption, ammonium was consumed faster in all cultures with glucose addition and was depleted after 48 h (Appendix B, Figure B2).

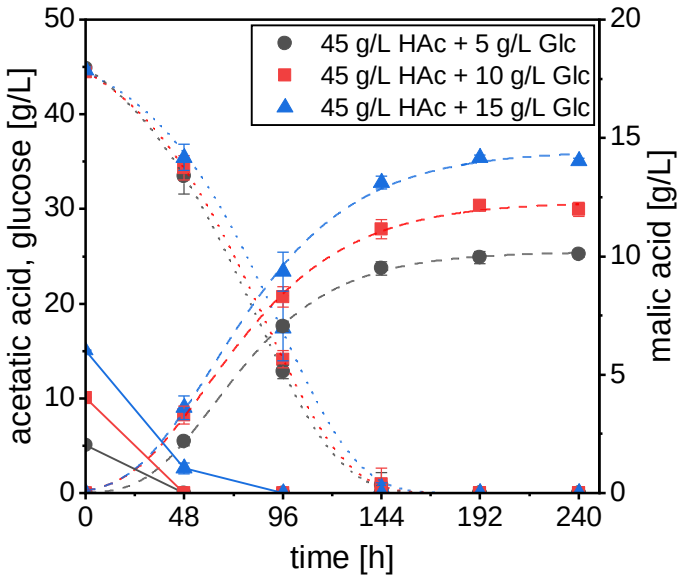

Figure 7. Malic acid production with 45 g/L acetic acid and 5-15 g/L glucose. Acetic acid: dotted lines, malic acid: dashed lines, glucose: solid lines. Data are the means of triplicate experiments with respective standard deviations. HAc = acetic acid, Glc = glucose.

Table 4 furthermore displays values for malic acid production based on glucose as sole carbon source. As the process with glucose is generally faster than the one with acetate, all calculations were performed with the data obtained after 168 h of cultivation. With an initial glucose concentration of 109 g/L, 38.75 ± 0.29 g/L malic acid was produced, corresponding to a yield of 0.66 ± 0.00 g/g and an overall productivity of 0.231 ± 0.002 g/L/h.

The pH value in cultures with acetate showed a strong increase during fermentation. After 192 h, the pH was between 9.5 and 9.8 for all tested acetic acid concentrations and for the cultures with mixed substrate. With glucose, by contrast, the pH decreased to a value of about 6.6 within 168 h (Appendix B, Figure B3).

Table 4. Fermentation results for malic acid production using different substrate types and concentrations after 192 h. Data are the means of triplicate experiments with respective standard deviations.

Substrate type	Initial substrate [g/L]	Consumed substrate [g/L]	Malic acid [g/L]	$Y_{P/S,\ malic\ acid}$ [g/g]	Overall productivity [g/L/h]	Maximum productivity [g/L/h]	Time of maximum productivity [h]
HAc	10	9.72 ± 0.15	0.00	0.00	0.000	0.000	0.00
	20	18.43 ± 1.54	1.42 ± 0.34	0.08 ± 0.01	0.007 ± 0.002	0.020	107.15
	30	29.64 ± 0.06	4.56 ± 0.23	0.15 ± 0.01	0.024 ± 0.001	0.076	92.25
	40	38.01 ± 1.74	7.04 ± 0.33	0.19 ± 0.00	0.037 ± 0.002	0.123	89.61
	45	44.14 ± 0.53	8.44 ± 0.42	0.19 ± 0.01	0.044 ± 0.002	0.122	85.05
	50	42.58 ± 0.80	8.45 ± 0.49	0.20 ± 0.01	0.044 ± 0.003	0.115	90.33
	55	37.55 ± 2.27	7.92 ± 0.29	0.21 ± 0.01	0.041 ± 0.002	0.081	95.38
Mixed	HAc: 45 Glc: 5	HAc: 44.87 ± 0.30 Glc: 5.06 ± 0.04	9.95 ± 0.25	0.20 ± 0.01	0.052 ± 0.001	0.114	61.98
	HAc: 45 Glc: 10	HAc: 44.46 ± 0.30 Glc: 10.07 ± 0.03	12.15 ± 0.21	0.22 ± 0.00	0.063 ± 0.001	0.119	58.62
	HAc: 45 Glc: 15	HAc: 44.66 ± 0.33 Glc: 15.06 ± 0.04	14.18 ± 0.09	0.24 ± 0.00	0.074 ± 0.000	0.138	61.02
Glc[a]	109	58.90 ± 0.86	38.75 ± 0.29	0.66 ± 0.00	0.231 ± 0.002	0.292	87.61

HAc = acetic acid, Glc = glucose, $Y_{P/S,\ malic\ acid}$ = substrate specific malic acid yield calculated as g(malic acid)/g(total consumed substrate(s))

[a] The values for the fermentation with glucose as sole carbon source were determined for a cultivation time of 168 h.

2.3.3. Side products of malic acid production

During the acid production stage, not only malic acid but also several other organic acids were produced by *A. oryzae* which are summarized in Table 5. The concentration curves for these products were similar to the one of malic acid and are therefore not displayed individually. As indicated in the table, the acid composition was influenced by the substrate type and concentration.

In cultures with acetate as the sole carbon source, the total acid concentration increased from 0.58 ± 0.14 g/L for cultures with 10 g/L acetic acid to 15.11 ± 0.92 g/L with 50 g/L acetic acid. Interestingly, acid production was also detected in cultures with a substrate concentration of 10 g/L for which malate could not be quantified as described in the previous section. With this acetate concentration, oxalic acid was the main product accounting for approximately 99% of the total acid concentration. The oxalic acid percentage decreased with increasing substrate concentration and was lowest in cultures with 50 g/L acetic acid (0.7 ± 0.1%). The highest malic acid proportion was quantified with 45 g/L acetic acid (56.8 ± 0.2%). Succinic acid represented the main side product in cultures with acetic acid concentrations of 30 g/L and above featuring values of 35.0% to 37.1%. While with an acetate concentration of 30 g/L, oxalic acid was the third largest byproduct, this position was taken by fumaric acid in cultures with 40-55 g/L acetic acid, ranging from 4.1% to 4.7%. Citric acid was only determined with acetic acid concentrations of 40-55 g/L and did not exceed 1.1 ± 0.2%. Pyruvic and α-ketoglutaric acid were produced in minor concentrations below 1.0% of the totally produced acid.

Regarding the mixtures of 45 g/L acetic acid and glucose, the total acid concentration reached values between 20.54 ± 0.42 g/L with 5 g/L glucose and 29.75 ± 0.23 g/L with 15 g/L glucose which is a doubling of the concentration compared to cultures without glucose. Malic acid also represented the main product followed by succinic acid. However, the distribution of the proportions shifted to the disadvantage of malic acid compared to the cultures with 45 g/L acetic acid only. The percentage of malic acid was about 9% lower while that of succinic acid was up to 6% higher. Furthermore, the addition of glucose was accompanied by an increased secretion of pyruvic acid which represented 4.9 ± 0.5% of the total acid production for cultures with 15 g/L glucose compared to 0.8 ± 0.1% when glucose was absent. The percentage of oxalic acid, on the other hand, was decreased and accounted for only 0.2-0.4%.

Regarding the cultures with 109 g/L glucose, a total acid concentration of 51.88 ± 1.15 g/L was obtained. With 74.7 ± 1.1% malic acid and 14.6 ± 0.7% succinic acid, the ratio between the two products was clearly shifted to the side of malic acid compared to the cultures containing acetate. With glucose, however, more citric acid was produced which represented 8.1 ± 1.1% of the total acid concentration. Oxalic, fumaric, pyruvic and α-ketoglutaric acid were produced in minor amounts which did not exceed 1.5%.

Table 5. Organic acid distribution for cultivation with different substrate types and concentrations after 192 h. Data are the means of triplicate experiments with respective standard deviations.

Substrate type	Initial substrate [g/L]	Total acids [g/L]	Organic acid distribution [%]						
			Malate	Succinate	Fumarate	Pyruvate	α-Keto-glutarate	Oxalate	Citrate
HAc	10	0.58 ± 0.14	0.0	0.0	1.4 ± 0.2	0.0	0.0	98.6 ± 0.2	0.0
	20	3.95 ± 0.54	35.6 ± 4.4	23.3 ± 6.2	2.5 ± 0.7	0.6 ± 0.1	0.9 ± 0.0	37.1 ± 11.3	0.0
	30	8.37 ± 0.20	54.5 ± 2.3	35.0 ± 1.1	3.4 ± 0.2	0.6 ± 0.1	0.6 ± 0.1	5.8 ± 2.4	0.0
	40	12.69 ± 0.82	55.5 ± 1.1	37.1 ± 0.8	4.1 ± 0.4	0.6 ± 0.0	0.5 ± 0.1	1.2 ± 0.1	1.0 ± 0.9
	45	14.86 ± 0.77	56.8 ± 0.2	35.9 ± 0.3	4.2 ± 0.1	0.8 ± 0.1	0.4 ± 0.0	0.8 ± 0.1	1.1 ± 0.2
	50	15.11 ± 0.92	55.9 ± 0.3	37.1 ± 1.2	4.2 ± 0.1	0.6 ± 0.0	0.5 ± 0.1	0.7 ± 0.1	1.0 ± 1.0
	55	14.32 ± 0.32	55.3 ± 0.9	37.1 ± 0.7	4.7 ± 0.3	0.6 ± 0.0	0.4 ± 0.1	0.9 ± 0.4	0.9 ± 0.8
Mixed	HAc: 45 Glucose: 5	20.54 ± 0.42	48.5 ± 1.3	41.5 ± 1.6	5.3 ± 0.4	2.7 ± 0.3	0.3 ± 0.1	0.4 ± 0.2	1.4 ± 0.1
	HAc: 45 Glucose: 10	25.28 ± 0.39	48.1 ± 1.3	39.3 ± 1.2	5.9 ± 0.2	4.8 ± 0.5	0.3 ± 0.0	0.4 ± 0.2	1.2 ± 0.2
	HAc: 45 Glucose: 15	29.75 ± 0.23	47.7 ± 0.3	39.7 ± 1.4	5.9 ± 0.6	4.9 ± 0.5	0.3 ± 0.1	0.2 ± 0.2	1.3 ± 0.4
Glucose[a]	109	51.88 ± 1.15	74.7 ± 1.1	14.6 ± 0.7	1.5 ± 0.1	0.4 ± 0.1	0.3 ± 0.1	0.3 ± 0.2	8.1 ± 1.1

HAc = acetic acid; Percentages were calculated based on the acid concentration in g/L as fraction of the total acid concentration in g/L

[a] The values for the fermentation with glucose as sole carbon source were determined for a cultivation time of 168 h.

2.3.4. Influence of $CaCO_3$ on malic acid production

For malic acid production using glucose as carbon source the presence of high $CaCO_3$ concentrations has proven necessary for efficient acid synthesis (Geyer et al., 2018). Since glucose and acetate metabolism for malic acid production are different, it was of interest to investigate the relationship between the availability of $CaCO_3$ and malate synthesis with acetate as substrate. Therefore, $CaCO_3$ was added to the medium in the range of 0-90 g/L and organic acid production was monitored. As displayed in Figure 8 and Table 6, a relationship between malate synthesis and the $CaCO_3$ concentration was observed. The utilization of 90 g/L $CaCO_3$ resulted in the highest malic acid titer of 8.77 ± 0.23 g/L. Furthermore, the highest maximum productivity of 0.121 g/L/h at the lowest corresponding time point of about 85 h was calculated for the highest $CaCO_3$ concentration. With lower $CaCO_3$ concentrations, malic acid titers and productivities were reduced. While 6.60 ± 0.48 g/L malic acid was still detected in cultures with 10 g/L $CaCO_3$ after 192 h, the titer decreased to 0.88 ± 0.22 g/L when $CaCO_3$ was absent. Furthermore, substrate consumption was greatly reduced. In the cultures with 10 g/L $CaCO_3$, 41% of the substrate was still present after 192 h, whereas as much as 91% remained without $CaCO_3$ addition. With 90 g/L $CaCO_3$, by contrast, all substrate was consumed within this period which suggests the necessity of $CaCO_3$ for malic acid production from acetate.

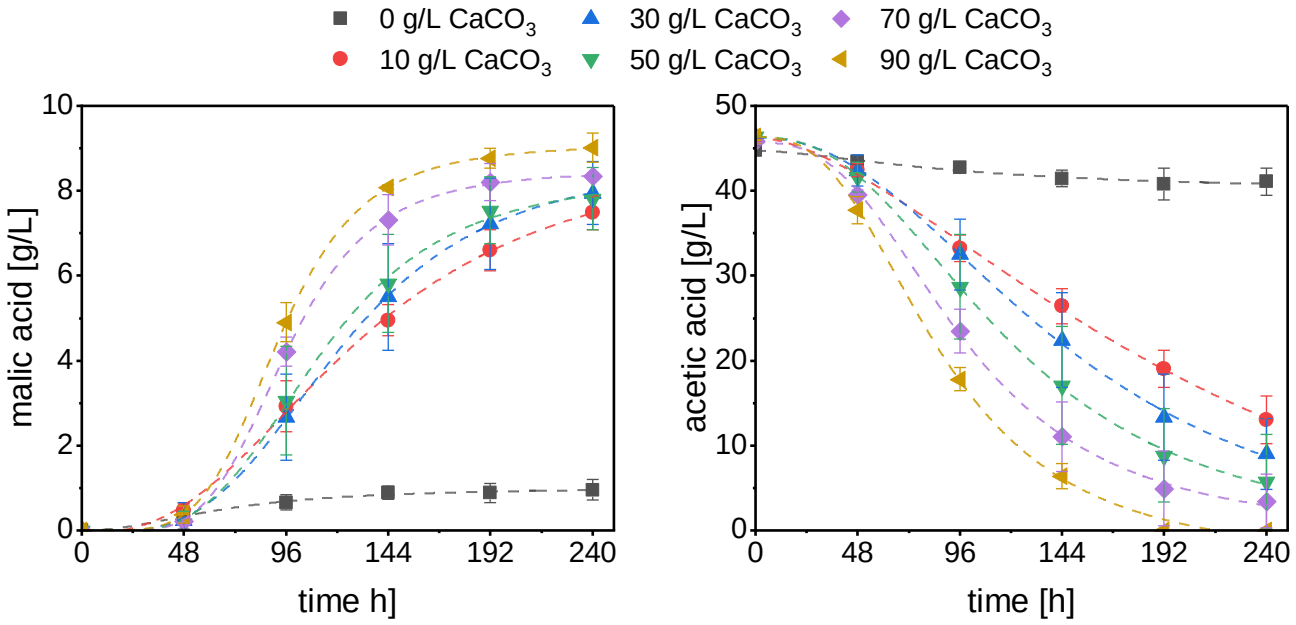

Figure 8. Malic acid production and acetate consumption with different initial $CaCO_3$ concentrations. Data are the means of triplicate experiments with respective standard deviations.

The distribution of the side products did not change considerably depending on the $CaCO_3$ concentration and was not displayed for this reason. Regarding the pH values, a similar progression was observed for all $CaCO_3$ concentrations with starting values of 6.4-6.7 and final values of 9.4-9.9 (Appendix B, Figure B4). The nitrogen source was depleted after 96 h of incubation for cultures with

70 and 90 g/L $CaCO_3$ and after 144 h with 10-50 g/L $CaCO_3$. Without $CaCO_3$ about 40% of the initial ammonium was still detected after 240 h (Appendix B, Figure B5).

Table 6. Fermentation results for malic acid production with different $CaCO_3$ concentrations after 192 h. Data are the means of triplicate experiments with respective standard deviations.

$CaCO_3$ [g/L]	Consumed substrate [g/L]	Malic acid [g/L]	$Y_{P/S,\ malic\ acid}$ [g/g]	Overall Productivity [g/L/h]	Maximum productivity [g/L/h]	Time of maximum productivity [h]
0	3.95 ± 2.15	0.88 ± 0.22	0.28 ± 0.18	0.005 ± 0.001	0.010	41.32
10	27.07 ± 1.95	6.60 ± 0.48	0.24 ± 0.00	0.034 ± 0.003	0.052	95.14
30	33.01 ± 5.07	7.22 ± 1.08	0.22 ± 0.02	0.038 ± 0.006	0.067	102.82
50	37.57 ± 5.46	7.54 ± 0.78	0.20 ± 0.01	0.037 ± 0.004	0.075	96.58
70	40.82 ± 4.19	8.21 ± 0.44	0.20 ± 0.01	0.043 ± 0.002	0.109	87.93
90	46.40 ± 0.15	8.77 ± 0.23	0.19 ± 0.00	0.046 ± 0.001	0.121	84.80

$Y_{P/S,\ malic\ acid}$ = substrate specific malic acid yield calculated as g(malic acid)/g(consumed acetic acid)

2.4. Discussion

2.4.1. Influence of acetate concentration on morphology and biomass formation

In the preculture, the growth behavior of *A. oryzae* up to a substrate concentration of 70 g/L acetic acid was evaluated. While the presence of 40-55 g/L of the substrate yielded similar dry biomass concentrations, lower or higher concentrations of the carbon source resulted in reduced growth (Table 3). The reason for the lower biomass concentrations with 30 g/L acetic acid and below is probably the pellet-like morphology of *A. oryzae*. Filamentous microorganisms are known to display a high variation in morphological phenotypes ranging from dispersed mycelia to spherical particles termed “pellets”. The morphology is influenced by different cultivation parameters including the temperature, pH value, medium composition, inoculum concentration or mechanical stress (Böl et al., 2020). A pellet morphology is advantageous due to a lower viscosity of the culture broth compared to media containing dispersed mycelia (Miyazawa et al., 2020). However, the oxygen and nutrient supply is limited within biomass pellets which can lead to cell lysis in the pellet core. Experiments with *A. niger* showed that in dense pellets with a radius of about 600 µm, the oxygen supply declines to zero within the outer 200 µm of the pellet (Hille et al., 2005). The largest pellet cores in the study presented here were obtained with 5 g/L acetic acid (Figure 5), which is probably associated with the low biomass production. An increase in the acetate concentration is accompanied by an increase in osmotic pressure which was likely the reason for the decrease in pellet size until a mycelial morphology was reaching at acetic acid concentrations of 40 g/L and above (Wucherpfennig et al., 2011).

The reduced biomass formation observed with acetic acid concentrations above 55 g/L likely originates from an increased concentration of the undissociated acetic acid. The toxicity of acetic

acid can be explained by the "weak-acid theory" (Stratford et al., 2009). The undissociated acid is lipid soluble and thus able to diffuse into the cytosol. It then dissociates due to the higher cytosolic pH which has been reported to be between 7.0 and 7.5 for fungal mycelia (Stratford et al., 2009; Yang et al., 2021). The resulting ions are not able to dissociate back and therefore accumulate in the cytoplasm. The proton release causes a cytosolic pH drop which inhibits enzyme activity (Stratford et al., 2009). The medium pH for all tested acetic acid concentrations in the preculture was set to pH 6.5. As the pK_a of acetic acid is 4.75, only a small portion of the substrate is present in its protonated form at this pH value. However, with an increasing acetic acid concentration, the amount of undissociated acid rises. With 45 g/L acetic acid, about 0.8 g/L (0.013 mol/L) are present in the protonated form which increases to 1.3 g/L (0.021 mol/L) at 70 g/L. Alcano et al. (2016) showed that spore germination stops when the undissociated acetic acid concentration reaches values between 0.018 and 0.022 mol/L for *Aspergillus flavus*, *Aspergillus carbonarius* and *Aspergillus parasiticus*. Thus, with acetic acid concentrations above 55 g/L and a pH of 6.5, the amount of the protonated acid becomes inhibiting for *A. oryzae*. Another possible inhibition factor in fermentations with high acetic acid concentrations is the increased ion concentration accompanying the adjustment of the medium pH. The amount of NaOH required for the pH adjustment with 45 g/L acetic acid to a value of 6.5 is about 0.72 mol/L while it amounts to 1.12 mol/L for media with 70 g/L acetic acid. Other studies found that the addition of low NaCl concentrations was beneficial for the growth of *Aspergillus* species, whereas high concentrations of 1 mol/L and above inhibited it (O'Mahony et al., 2002; Wang et al., 2020). As consequence, the presence of an increased concentration of sodium and acetate ions with initial acetic acid concentrations above 55 g/L could have contributed to the reduced biomass production.

The overall consumption of ammonium during the growth phase was low. Investigating the possibility of a nitrogen reduction in the preculture to save process costs should therefore be considered. Evaluating further nitrogen sources besides ammonium sulfate could be interesting to enhance biomass formation and reduce the process time. Germination in acetate media only started between 8 and 24 h. As demonstrated for *A. niger*, L-amino acids and complex nitrogen sources such as yeast extract and peptone were better suited to initiate germination (Hayer et al., 2014).

2.4.2. Influence of substrate type and concentration on malic acid production

For an efficient malic acid production with *A. oryzae* using glucose as substrate a high C:N ratio is necessary (Ochsenreither et al., 2014). The objective was therefore to identify the highest applicable acetate concentration before observing an inhibition of malate synthesis. The highest malic acid titers were obtained with 45 g/L and 50 g/L acetic acid (Figure 6, Table 4). Since a slightly higher productivity, a lower time point of maximum productivity and substrate depletion after 192 h was observed with 45 g/L acetic acid, this concentration is considered to be optimal for malic acid production with *A. oryzae*. In the cultures with 55 g/L of the substrate, malic acid synthesis seemed to be inhibited. This is pointed out by a slower acid production and the finding that about a third of

the substrate remained unused. Malic acid production diminished with acetic acid concentrations below 45 g/L and was absent in cultures with 10 g/L acetic acid. With the lowest carbon source concentration, ammonium was detected as long as acetic acid was present in the medium. It is therefore assumed that most of the substrate was channeled towards biomass formation instead of organic acid production and malate synthesis was probably not observed due to substrate limitation.

The maximum titer obtained with acetate amounted to only about 22% of the concentration produced with glucose. With the sugar, a much higher initial carbon concentration can be applied in a batch process. The key to achieving higher malate concentrations could be in a fed-batch or a continuous process with acetic acid as pH regulator and substrate supply, which should therefore be evaluated in further experiments.

Considering that efficient malic acid production with acetate only started after 48 h of cultivation, the possibility of adding low amounts of glucose to shorten the lag phase was evaluated. Indeed, the glucose supplementation proved beneficial by decreasing the time of maximum productivity and accelerating acetate consumption. Supplementing acetate media with glucose probably serves as growth stimulant, leading to a shorter lag phase and faster depletion of the nitrogen source. *A. oryzae* metabolized glucose and acetate simultaneously, suggesting that the acetate metabolism is not suppressed when small amounts of glucose are present. Similar observations were made by Armitt et al. (1971) who reported that the enzymes involved in acetate metabolism in *A. nidulans* were as active in mixtures of glucose and acetate as with acetate alone and that acetate was even the preferred carbon source. It must be mentioned that these authors only tested low acetate and glucose concentrations of 0.1 and 0.02 mol/L, respectively. Lucas et al. (1994) used a medium with 0.1 mol/L acetate and added 3% (w/v) glucose and observed that isocitrate lyase activity, a key enzyme involved in acetate metabolization, decreased upon the addition of glucose in *A. nidulans*. Thus, the positive effect of glucose supplementation might be limited to media with high acetate and low glucose concentrations.

2.4.3. Influence of substrate type and concentration on the side product spectrum

With acetic acid concentrations of 30 g/L and above, malic acid represented the main product and succinic acid the main side product (Table 5). Compared to the process using glucose as sole carbon source, the malic acid percentage with acetate was decreased in favor of an increased succinic acid production. This observation can be explained by the different production routes with the two substrates. There are three microbial pathways known for malic acid synthesis which are the oxidative tricarboxylic acid (TCA) cycle, the reductive TCA (rTCA) cycle and the glyoxylate cycle (Figure 3). With glucose as carbon source the rTCA was identified as the main pathway leading to malic acid in *Aspergillus* species (Knuf et al., 2013; Peleg et al., 1989). This pathway involves the carboxylation of pyruvate originating from glycolysis to oxaloacetate by pyruvate carboxylase and the subsequent production of malate by malate dehydrogenase. Further experiments suggested that the succinic acid produced with glucose as substrate is synthesized via the oxidative TCA only and

that the so-produced succinic acid is not the main precursor for the secreted malic acid (Peleg et al., 1989). Acetate metabolism generally involves the glyoxylate pathway, a bypass of the oxidative TCA cycle. First, acetate is converted to acetyl-CoA which then enters the TCA cycle where it is used for the synthesis of isocitrate. This transformation involves the action of citrate synthase and aconitase. Isocitrate lyase then catalyzes the conversion of isocitrate to succinate and glyoxylate while malate synthase converts glyoxylate with acetyl-CoA to malate. The existence of the glyoxylate pathway in *A. nidulans* was demonstrated by the induction of the two key enzymes isocitrate lyase and malate synthase in the presence of acetate while no induction was observed when only glucose was present (Armitt et al., 1970; Armitt et al., 1971). This finding was further supported by the observation that mutants lacking either of the two enzymes did not grow on acetate (Armitt et al., 1976). It has not been demonstrated whether the glyoxylate cycle is the only pathway leading to the overproduction of malic acid from acetate in *A. oryzae*, but it explains the higher succinic acid yield compared to the process using glucose. Stoichiometrically, one molecule of isocitrate should yield one molecule of malate and succinate, respectively. The reason for the lower succinate concentration (with 45 g/L acetic acid: 63 mmol/L malic acid and 45 mmol/L succinic acid) in the results presented here might be due to further conversion of succinate to malate by the enzymes succinate dehydrogenase and fumarase. Genetic modification of *A. oryzae* to enforce the oxidative path from succinate to malate could therefore be a tool to increase the accumulation of malate and reduce byproduct formation.

With an initial concentration of 10 g/L acetic acid no malic acid production was detected, although about 0.6 g/L total acid was produced, most of which was oxalic acid (Table 5). Oxalic acid production has been reported in various fungal species and there are two main hypotheses for the reason of its secretion. Oxalic acid is a rather strong acid ($pK_a,1 = 1.23$) and therefore might be produced to outcompete other species by medium acidification. This theory is supported by the observation that oxalic acid is mainly accumulated if the pH of the culture broth is at a value of 6 or above (Kobayashi et al., 2014; Kubicek et al., 1988; Walaszczyk et al., 2018). Furthermore, oxalic acid acts as efficient chelator which supports fungal growth by mobilizing metal ions (Gadd et al., 2014). The mechanism of oxalate production with acetate in *A. oryzae* has not yet been described. In *A. niger* with glucose as carbon source, oxalic acid is produced by the conversion of oxaloacetate to oxalate and acetate in the cytosol through oxaloacetate acetylhydrolase (Kubicek et al., 1988). Other organisms such as the wood rotting basidiomycete *Gloeophyllum trabeum* feature a second enzyme, glyoxylate dehydrogenase, which is able to synthesize oxalate from glyoxylate in the peroxisome (Zhuang et al., 2015). Why oxalic acid is mainly produced at low acetate concentrations remains unclear. One could speculate that this is due to the equilibrium of the enzyme reaction catalyzed by oxaloacetate acetylhydrolase. With a high acetate concentration in the medium, the equilibrium is strongly shifted to the side of oxaloacetate while with a low concentration, some of the produced oxaloacetate might react to acetate and oxalate. However, further experiments are required to answer this question.

With glucose as sole carbon source, a noticeable amount of citric acid was secreted which was not the case in cultures with acetate. The cause for this observation might be the different pH development. The acid spectrum produced by filamentous fungi is influenced by the pH and citric acid is produced most efficiently at low pH values (Walaszczyk et al., 2018). While the pH value decreased during malic acid production with glucose, it strongly increased when acetate was used as carbon source. Thus, the high pH value in the cultures with acetate probably suppressed citric acid synthesis.

2.4.4. Influence of $CaCO_3$ addition on malic acid production

During malic acid production using glucose, there are three factors influencing the pH which are the ammonium consumption, the acid production (both leading to a decrease in pH), and the presence of $CaCO_3$. To mitigate against the pH decrease, $CaCO_3$ is often added as buffering substance during organic acid production. As the step from pyruvate to oxaloacetate fixes CO_2, it is assumed that $CaCO_3$ furthermore functions as CO_2 supply (Geyer et al., 2018; Zambanini et al., 2017). The third reason for adding $CaCO_3$ is the formation of calcium malate precipitates, allowing for the production of higher malic acid concentrations (Zambanini et al., 2016). The utilization of acetate, however, adds another influencing factor as the consumption of the carbon source leads to an increase of pH (Appendix B, Figure B3). This observation raised the question of whether the presence of $CaCO_3$ is necessary for malic acid production using acetate. The results presented here suggest that this is indeed the case, as less than 1 g/L malic acid was produced when $CaCO_3$ was not present (Figure 8, Table 6). In acetate media, $CaCO_3$ does not contribute to the pH buffering in the manner it does for media with glucose since the pH increases. Furthermore, if malate is not produced with the involvement of the enzyme pyruvate carboxylase, $CaCO_3$ neither fulfills the role of a CO_2-donor. This could lead to the conclusion that the main function of $CaCO_3$ is the formation of calcium malate. However, the results presented here cannot answer this question conclusively. $CaCO_3$ could furthermore positively influence the fermentation by the presence of insoluble solids or by providing calcium ions. Evaluating the effect of different carbonates such as Na_2CO_3 or K_2CO_3 could be interesting as these are soluble and can be handled more easily than the poorly soluble $CaCO_3$.

2.4.5. Considerations regarding the utilization of acetate derived from lignocellulose

The results presented so far show that acetic acid concentrations in the range of 40-55 g/L are optimal for biomass production with *A. oryzae*, while about 45 g/L are ideal for malic acid production in a batch process. In the context of bioeconomy, the utilization of acetate-containing waste or side streams derived from lignocellulose is targeted. Acetic acid is for example contained in lignocellulosic hydrolysates, in side streams of fast pyrolysis or produced during syngas fermentation. The acetic acid content in these products encompasses a broad range. Lignocellulosic hydrolysates usually contain acetic acid up to a concentration of about 10 g/L (Costa et al., 2017; Kim, 2018; Sulzenbacher et al., 2021). According to the results presented in this work, these concentrations are too low for malic acid synthesis with *A. oryzae*, however, they can be used for

biomass production. Within an integrated bioeconomy, it is also conceivable that the sugars in these hydrolysates are first used in other microbial processes and the remaining acetate is then used for biomass production with *Aspergillus*. Higher acetate concentrations are contained in pyrolysis products or in the culture broth after syngas fermentation. During fast pyrolysis, biomass is subjected to high temperatures for a few seconds while oxygen is absent. The remaining vapors are condensed subsequently and yield a bio-oil and, in some processes using ash-rich biomasses, an aqueous condensate. The acetic acid concentration in these condensates can range between 45 and 80 g/L acetic acid, which is a suitable range for malic acid production with *A. oryzae* (Arnold et al., 2019; Oh et al., 2017). Through further processing of fast pyrolysis products, biomass-derived syngas is obtained. With acetogenic microorganisms, this gas, mainly containing CO, CO_2 and H_2, can be converted to acetic acid. With *Clostridium ljungdahlii* acetic acid concentrations of about 21 g/L were reported (Infantes et al., 2020) while with *Acetobacterium woodii* titers of over 50 g/L were measured (Kantzow et al., 2015; Straub et al., 2014). Malic acid production with acetate obtained from *C. ljungdahlii* has already been reported by Oswald et al. (2016). During their fermentations, 14-18 g/L acetic acid was produced and only low malic acid concentrations up to 1.8 g/L were measured. As demonstrated by the results obtained here, the acetic acid concentrations produced during syngas fermentation of Oswald et al. was just on the merge of the malate production limit. Therefore, choosing a different acetogenic microorganism which is capable of a higher acetate production is advisable. However, compared to directly using pyrolysis products like the bio-oil or the aqueous condensate, another fermentation step is required. In this sense, the direct utilization of the pyrolytic aqueous condensate for malic acid production could be particularly interesting, since it is a low-value side stream that can only be partially used in the subsequent gasification process due to its low energy content (Arnold et al., 2017). Therefore, further exploration of this possibility is of great interest in the context of a bio-based economy.

2.5. Conclusions

This chapter presents results on the use of acetate as substrate for growth and malic acid production with *A. oryzae*. While for biomass formation acetic acid concentrations of 40-55 g/L were best, the highest malic acid titers were observed with a narrower range of 45-50 g/L. Considering that the time of maximum productivity occurred earlier in cultures with 45 g/L acetic acid and no substrate remained unused this concentration was identified optimal for malic acid production. The side product spectrum was found to be dependent on the initial acetate concentration. While oxalic acid was the main product at low concentrations of 10-20 g/L acetic acid, this place was taken by malic acid at substrate concentrations of 30 g/L and above. Succinic acid was found to be the major byproduct in malate production from acetate. Reducing its proportion by enhancing the metabolic pathway from succinic to malic acid, e.g. by metabolic engineering, could help to improve malate production. Addition of small amounts of glucose to acetate media increased the acid yield and enhanced the production rate. Besides glucose, the presence of $CaCO_3$, a buffering agent commonly

applied in malic acid production from glucose, also enhanced malate production. Substrate inhibition limited the maximum malate concentration to values of less than 10 g/L in a batch process. Therefore, the production of malic acid as a fed-batch process with the addition of acetic acid to maintain both pH and substrate concentration in a stable range should be evaluated in further studies.

3. Effect of process mode, nitrogen source and temperature on L-malic acid production

This chapter is based on the publication:

Effect of process mode, nitrogen source and temperature on L-malic acid production with *Aspergillus oryzae* DSM 1863 using acetate as carbon source

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Katrin Ochsenreither: conceptual advice, project supervision, manuscript revision.

3.1. Introduction

As described in the previous chapter, malic acid production from acetate in batch fermentations is limited to maximum concentrations of less than 10 g/L. A batch process is especially unfavorable when acetate is the carbon source due to substrate inhibition observed at rather low concentrations. Therefore, malic acid production in fed-batch or repeated-batch mode is likely to enhance the fermentation outcome. Through the periodic exchange of medium, a repeated-batch process furthermore prevents the occurrence of high product titers, thus minimizing product inhibition. With glucose as substrate, a repeated-batch process has already been shown to extend the production time and enhance malic acid productivity with *A. oryzae* (Schmitt et al., 2021).

Apart from the process mode, the cultivation temperature and type of nitrogen source can also have a significant effect on microbial production. Some nitrogen sources have already been evaluated for L-malic acid production with *A. oryzae*, but with different strains and glucose as substrate. In these studies, the complex nitrogen sources peptone and tryptone were found to result in considerably higher malic acid concentrations than ammonium compounds (Ding et al., 2018; Knuf et al., 2013). The optimum cultivation temperature varies depending on the microorganism and target product. Using *A. oryzae* DSM 1863 and acetate as carbon source, the effects of temperature on organic acid synthesis have not yet been studied. For the production of succinic, itaconic or malic acid with other *Aspergillus* species from sugars, temperatures between 30 and 35 °C were reported most suitable (Chen et al., 2019; Saha et al., 2019; Yang et al., 2020).

This chapter evaluates strategies for improving L-malic acid production using acetate as carbon source. First, organic acid production is investigated at different cultivation temperatures and the side product spectrum is analyzed. Furthermore, different defined and complex nitrogen sources are evaluated for growth and organic acid production. Eventually, batch, fed-batch and repeated-batch fermentations are compared with the aim to increase the maximum malic acid concentration and enhance the productivity.

3.2. Materials and methods

3.2.1. Microorganism and media

Aspergillus oryzae DSM 1863 was obtained from DSMZ strain collection (German Collection of Microorganisms and Cell Cultures GmbH) and spore propagation was performed as described in Chapter 2.2.2.

The basis preculture medium was composed of 45 g/L acetic acid, 0.75 g/L KH_2PO_4, 0.98 g/L K_2HPO_4, 0.1 g/L $MgSO_4 \cdot 7H_2O$, 0.1 g/L $CaCl_2 \cdot 2H_2O$, 5 mg/L NaCl, and 5 mg/L $FeSO_4 \cdot 7H_2O$. The basis main culture medium for organic acid production consisted of 45 g/L acetic acid, 0.1 g/L KH_2PO_4, 0.17 g/L K_2HPO_4, 0.1 g/L $MgSO_4 \cdot 7H_2O$, 0.1 g/L $CaCl_2 \cdot 2H_2O$, 5 mg/L NaCl, 60 mg/L $FeSO_4 \cdot 7H_2O$, and 90 g/L $CaCO_3$. The nitrogen source and further modifications of these media specific for each experiment are indicated in the following sections. The pH of all preculture media

containing acetate was set to 6.5 with NaOH. For the main culture media containing acetate, the pH was adjusted to a value of 5.5 with NaOH.

The experiments comparing different cultivations temperatures and the effect of Hutner's trace elements solution with acetate as carbon source were compared to cultivations using glucose. Instead of acetic acid, the basis pre- and main culture medium then contained 40 g/L (preculture) or 120 g/L (main culture) glucose monohydrate. The pH of the glucose media was not adjusted.

Hutner's trace element solution (1000x) was added to all preculture media except for those used in the experiments comparing different nitrogen sources. This solution consists of 5 g/L $FeSO_4 \cdot 7H_2O$, 50 g/L EDTA-Na_2, 22 g/L $ZnSO_4 \cdot 7H_2O$, 11 g/L H_3BO_3, 5 g/L $MnCl_2 \cdot 4H_2O$, 1.6 g/L $CoCl_2 \cdot 6H_2O$, 1.6 g/L $CuSO_4 \cdot 5H_2O$, and 1.1 g/L $(NH_4)_6Mo_7O_{24} \cdot 4H_2O$ with a pH of 6.5 (Hill and Kafer, 2001).

All pre- and main culture media were sterilized by autoclaving for 20 min at 121 °C.

3.2.2. Cultivation conditions

The following describes the general cultivation conditions that apply to all experiments presented in this study. Details regarding the conditions for individual experiments are given in the following sections.

100 mL of preculture medium in 500-mL baffled shake flasks was inoculated with 3 x 10^7 conidia and incubated at 30 °C and 100 rpm. After 48 h (media containing acetate) or 24 h (media containing glucose) of incubation, the biomass was separated from the medium by filtration through Miracloth (Merck KGaA, Darmstadt, Germany) and washed thoroughly with distilled water.

Organic acid production was performed in 500-mL baffled shake flasks containing 100 mL main culture medium. In the experiments comparing the different nitrogen sources, 0.75 g of washed biomass was used for the inoculation of one main culture flask. For all other experiments, the biomass of two precultures was resuspended in 200 mL main culture medium and 10 mL were used for the inoculation of one shake flask. The main culture was incubated at 120 rpm and 32 °C unless stated otherwise.

3.2.3. Evaluation of temperature effects on organic acid production

Organic acid production was evaluated at 29, 32, 35 and 38 °C. In addition to acetate, experiments were also run with glucose as carbon source. For the main cultures with acetate, precultures grown on acetate were used, while for the main cultures containing glucose, biomass formed with glucose was used. The precultures with both carbon sources were grown with 4.00 g/L $(NH_4)_2SO_4$ and 2 mL/L 1000x Hutner's trace elements solution. All temperatures were tested as biological quadruplicates.

3.2.4. Evaluation of nitrogen sources for growth and organic acid production

The concentration of all nitrogen sources added to the basis pre- and main culture media was equivalent to the nitrogen concentration of 4.00 g/L $(NH_4)_2SO_4$ (preculture) or 1.20 g/L $(NH_4)_2SO_4$ (main culture). This refers to a nitrogen concentration of about 0.85 g/L in the preculture and 0.25 g/L the main culture. For the complex nitrogen sources, the total nitrogen concentration as indicated by the manufacturer was used for calculating their respective amounts. The following concentrations were therefore applied in the preculture: 4.00 g/L $(NH_4)_2SO_4$, 5.15 g/L $NaNO_3$, 1.82 g/L urea, 12.31 g/L L-glutamic acid monopotassium salt monohydrate, 8.00 g/L yeast extract, 10.87 g/L casein hydrolysate, and 6.95 g/L peptone from soy. In the main culture the concentrations were the following: 1.20 g/L $(NH_4)_2SO_4$, 0.55 g/L urea, 3.69 g/L L-glutamic acid monopotassium salt monohydrate, 2.40 g/L yeast extract, 3.26 g/L casein hydrolysate, and 2.09 g/L peptone from soy. The total nitrogen (TN) and amino nitrogen (AN) values for the complex N-sources were the following: yeast extract (10.6% TN, 5.4% AN), casein hydrolysate (acid-hydrolyzed, 7.8% TN, 5.8% AN), peptone from soy (papainic digested, 12.2% TN, 3.7% AN). All nitrogen compounds were purchased from Carl Roth GmbH + Co. KG except for L-glutamic acid monopotassium salt monohydrate which was obtained from Alfa Aesar. Urea was not autoclaved with the medium but added in the form of a sterile filtered stock solution afterwards. The biomass used for the inoculation of all main culture conditions was grown in the basis preculture medium containing 4.00 g/L $(NH_4)_2SO_4$. All experiments for evaluating the effect of the nitrogen source in pre- or main culture were performed as biological triplicates.

3.2.5. Evaluation of process modes

In 500-mL baffled shake flasks the batch process was compared to a fed-batch and repeated-batch process using acetate as carbon source. Each process mode was tested with either 1.20 g/L $(NH_4)_2SO_4$ or 2.40 g/L yeast extract as nitrogen source in the main culture while all precultures were grown using the basis medium containing 4.00 g/L $(NH_4)_2SO_4$ and 2 mL/L 1000x Hutner's solution. The yeast extract was the same as in the experiments evaluating different nitrogen sources.

In the fed-batch process, the acetic acid concentration in each shake flask was measured every 48 h, and 5 M acetic acid was added to obtain a concentration of about 45 g/L acetic acid. Hence, "fed-batch" in this work refers to a process with feeds of acetic acid only.

In the repeated-batch process, the medium was first exchanged after 96 h and then every 72 h. Thereby, the biomass was filtered through Miracloth, washed with distilled water, and transferred to a new flask containing 100 mL of fresh main culture medium, including 90 g/L $CaCO_3$. Hence, the term "repeated-batch" in this work refers to a process with complete medium replacement and reutilization of the entire amount of biomass in each cycle.

All experiments for the evaluation of process modes were performed as biological triplicates.

3.2.6. Analytics

For the determination of the biomass concentration at the end of the preculture, pre-weighed Miracloth was used for the filtration of the entire content of one flask. The biomass-containing Miracloth was dried at 70 °C until the weight remained constant. Afterwards, the weight of the Miracloth with the biomass was measured with precision scales to calculate the dry biomass concentration.

Organic acid and glucose concentration was quantified by HPLC (Agilent 1100 Series) using a Rezex ROA organic acid H+ (8%) column (300 by 7.8 mm, 8 µm, Phenomenex) with a UV detector at 220 nm for organic acid and an RI detector for glucose determination. Conditions for analysis and sample preparation were the same as in Chapter 2.

Ammonium was measured photometrically using a Spectroquant test kit (114752, Merck KGaA). The reaction volume of the assay was scaled down to 200 µL and samples were measured in duplicate in microtiter plates according to the instructions of the manufacturer.

3.3. Results

3.3.1. Effect of temperature on organic acid production

Four cultivation temperatures (29, 32, 35 and 38 °C) were tested for organic acid production with *A. oryzae*. In addition to cultures with acetate as sole carbon source, the temperature effects were also investigated using glucose. Differences in organic acid productivity and composition of the acid spectrum as a function of the cultivation temperature were observed for both substrates.

In the cultures with acetate, organic acid production started earlier when incubated at 35 or 38 °C compared to the two lower temperatures (Figure 9). Regarding the production of malic acid, a cultivation temperature of 35 °C resulted in the fastest product synthesis. At this temperature, production was already finished after about 120 h while an increase in malic acid concentration was detected until 192 h in cultures incubated at 29 °C. Despite the differences in production velocity, the malic acid concentration obtained after 240 h of cultivation was similar for all temperatures. This was different for succinic acid production, which is the major side product of malic acid synthesis with *A. oryzae* DSM 1863. The succinic acid concentration after 48 h was similar at 35 and 38 °C (about 1.3 g/L) while no succinate was detected in cultures incubated at 29 or 32 °C at this sampling time. From 72 h, the succinate concentration was proportional to the cultivation temperature, with the highest value obtained at 38 °C. This concentration difference was observed until the end of the cultivation and resulted in a higher succinic than malic acid titer in cultures incubated at 38 °C. The substrate was almost completely consumed in cultures incubated at 32-38 °C, whereas about 6.5 g/L acetic acid remained after 240 h of cultivation at 29 °C. Since the consumption of acetate is accompanied by an alkalinization of the medium, the pH increased to values of about 9.9-10.3 after 240 h. Table 7 summarizes the cultivation outcomes after 144 h. At this sampling point, malic acid production was completed in most cultures. The lowest acetate consumption and substrate

production was observed in cultures incubated at 29 °C, with a total acid concentration of 10.91 ± 0.77 g/L. In cultures incubated at 38 °C, the total acid concentration was highest (16.36 ± 1.03 g/L) but about half of this was succinic acid. Besides malate and succinate, also citric, pyruvic, fumaric, α-ketoglutaric and oxalic acid were detected. Figure 10 shows the percentages of these products related to the total acid concentration quantified after 144 h. In Figure 10A, the left axis displays the main products malate and succinate while the right axis covers the remaining acids. The cultivation temperature affected the oxalate concentration considerably, as 6.2 ± 1.6% of this acid were produced in cultures incubated at 29 °C compared to 0.3 ± 0.1% at 38 °C. Furthermore, the pyruvate and α-ketoglutarate percentages showed a slight increase and the fumarate percentage a slight decrease with increasing temperature. For citrate, no temperature dependent trend was found with acetate as carbon source. The numerical values which are displayed in Figure 10 can be found in Table C1 (Appendix C).

The same temperatures were also evaluated in cultivations using glucose. With this carbon source as well, malic acid production was fastest at 35 °C in the beginning which was surpassed by the cultures incubated at 38 °C (Figure 9). Different than with acetate, the malic acid concentration of the cultures incubated at 29 °C did not level up to the three higher temperatures in later stages of fermentation. Regarding the succinate concentration, on the other hand, similar observations were made as for acetate, with the highest concentration found at 38 °C and the lowest at 29 °C. The substrate consumption matches these observations, and the highest carbon utilization was quantified for cultures incubated at 35 and 38 °C (Table 7). Except for cultures incubated at 29 °C, the malic acid concentration after 144 h was above 30 g/L, and the highest total acid concentration of 54.76 ± 5.57 g/L was obtained at a temperature of 38 °C. Due to the buffering capacity of the added $CaCO_3$, the pH was rather stable at values of about 6.5-7.0 after an initial decline (Figure 9). The temperature also affected the organic acid distribution, largely in a similar manner to the findings described for acetate. In Figure 10B, the left axis displays the malic acid percentage while the right axis covers the remaining acids, including succinate. The oxalic and fumaric acid percentages decreased while the succinic, α-ketoglutaric, and pyruvic acid percentages increased with increasing temperature. Different than with acetate, the proportion of citric acid was affected by the cultivation temperature, decreasing from 14.4 ± 1.7% at 29 °C to 4.7 ± 0.2% at 38 °C.

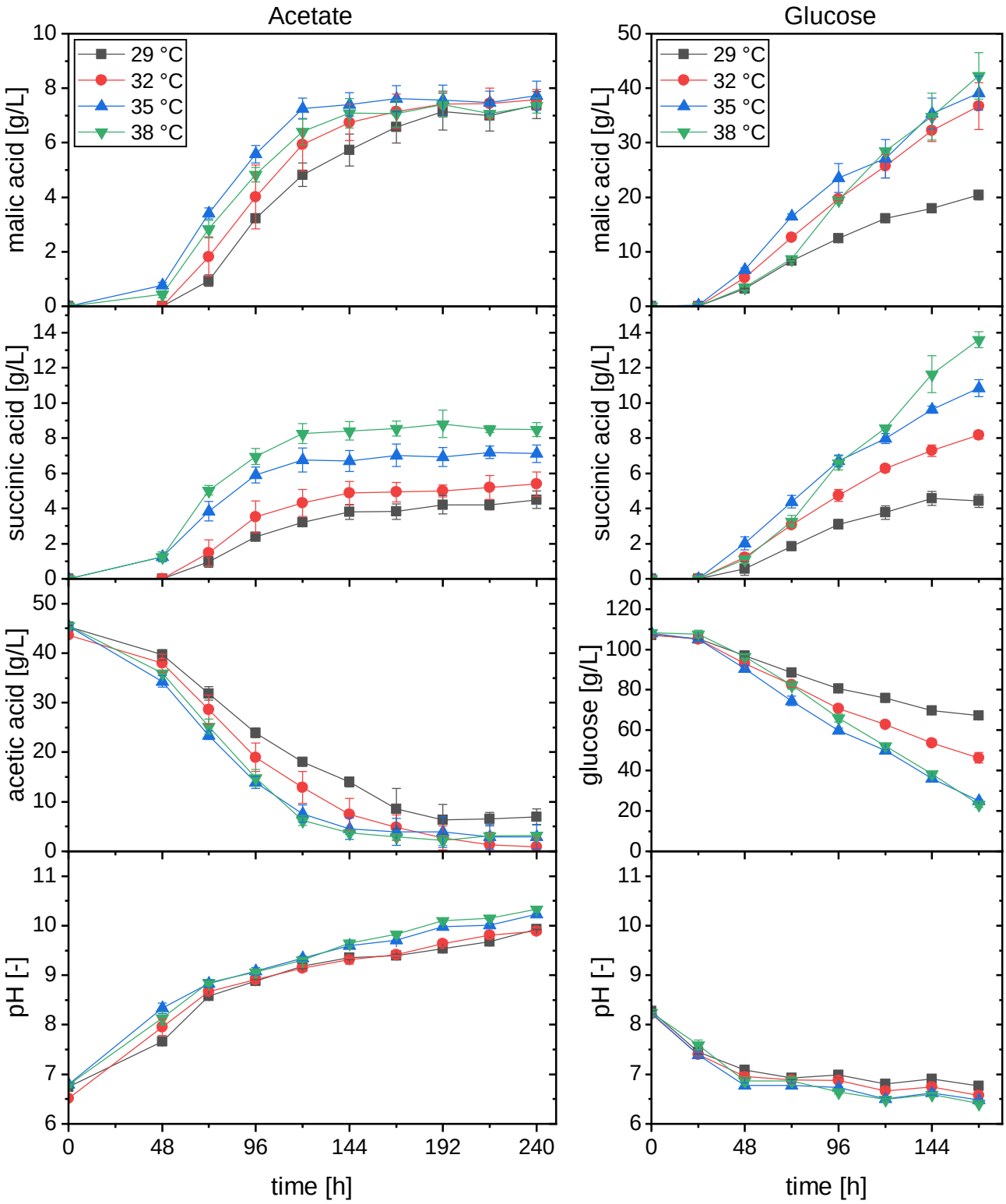

Figure 9. Malic and succinic acid production, substrate consumption, and pH development at different cultivation temperatures with acetate or glucose as carbon source. Data are the means of quadruplicate experiments with respective standard deviations.

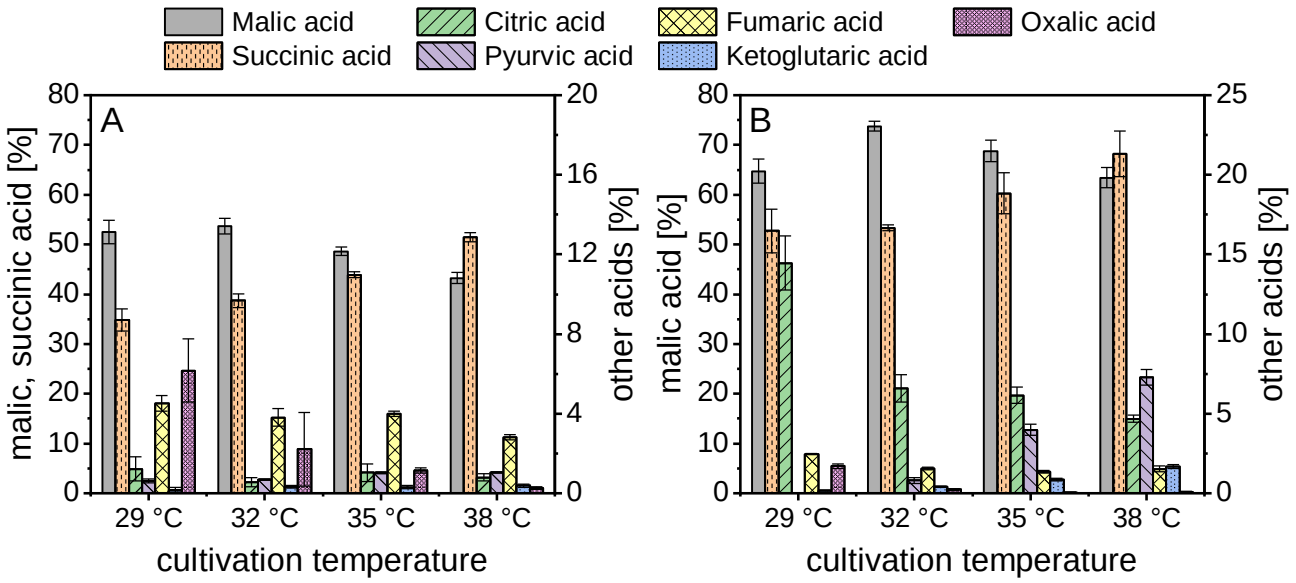

Figure 10. Organic acid composition after 144 h of cultivation at different temperatures with acetate (A) or glucose (B) as carbon source. In (A) the left axis covers the malate and succinate percentages, while the right axis displays the data for the other products. In (B), the left axis presents only the malic acid percentages, while the right axis covers all side products, including succinate. Data are the means of quadruplicate experiments with respective standard deviations.

For both substrates, the malic acid yield was highest in cultures incubated at 32 °C, amounting to 0.19 ± 0.00 g/g with acetate and 0.60 ± 0.06 g/g with glucose (Table 7). Hence, the relation of the product to the side products was most suitable for malic acid production at this cultivation temperature which was therefore used in the following experiments.

Table 7. Fermentation results for cultivations of *A. oryzae* at different temperatures after 144 h. Data are the means of quadruplicate experiments with respective standard deviations.

Substrate	T [°C]	Consumed substrate [g/L]	Malic acid [g/L]	$Y_{P/S,\ malic\ acid}$ [g/g][a]	Malic acid productivity [g/L/h]	Succinic acid [g/L]	Total acid [g/L][b]	$Y_{P/S,\ total\ acid}$ [g/g]	Total acid productivity [g/L/h]
Acetic acid	29	31.32 ± 1.17	5.73 ± 0.59	0.18 ± 0.01	0.040 ± 0.004	3.80 ± 0.41	10.91 ± 0.77	0.35 ± 0.01	0.076 ± 0.005
	32	36.25 ± 3.53	6.74 ± 0.67	0.19 ± 0.00	0.047 ± 0.005	4.88 ± 0.65	12.60 ± 1.55	0.35 ± 0.02	0.087 ± 0.011
	35	40.90 ± 2.11	7.41 ± 0.44	0.18 ± 0.00	0.051 ± 0.003	6.70 ± 0.60	15.25 ± 1.17	0.37 ± 0.01	0.106 ± 0.008
	38	41.79 ± 0.34	7.08 ± 0.54	0.17 ± 0.01	0.049 ± 0.004	8.42 ± 0.52	16.36 ± 1.03	0.39 ± 0.03	0.114 ± 0.007
Glucose	29	37.46 ± 0.57	17.95 ± 0.68	0.48 ± 0.01	0.125 ± 0.005	4.57 ± 0.41	27.73 ± 0.48	0.74 ± 0.02	0.193 ± 0.003
	32	53.47 ± 1.73	32.25 ± 2.07	0.60 ± 0.06	0.224 ± 0.014	7.28 ± 0.33	43.71 ± 2.20	0.82 ± 0.06	0.304 ± 0.015
	35	72.02 ± 0.94	35.32 ± 2.83	0.49 ± 0.04	0.245 ± 0.020	9.63 ± 0.20	51.29 ± 2.60	0.71 ± 0.04	0.356 ± 0.018
	38	70.10 ± 0.95	34.78 ± 4.30	0.50 ± 0.05	0.242 ± 0.030	11.64 ± 1.06	54.76 ± 5.57	0.78 ± 0.07	0.380 ± 0.039

[a] $Y_{P/S,\ malic\ acid}$ = substrate specific malic acid yield calculated as g(malic acid)/g(consumed substrate).

[b] Refers to all organic acids quantified (see Figure 10 or Table C1, Appendix C).

3.3.2. Effect of the nitrogen source on growth of *A. oryzae*

Growth of *A. oryzae* was evaluated with different defined and complex nitrogen sources using acetate as substrate. The nitrogen concentration in these experiments was adjusted to 0.85 g/L in all media. Regarding the defined nitrogen sources, biomass production was similar with $(NH_4)_2SO_4$, urea and glutamate (Table 8). In these cultures, a dry biomass concentration of about 0.9 g/L was determined after 48 h of growth, whereas the lowest concentration of 0.48 ± 0.06 g/L was obtained with $NaNO_3$. The utilization of complex nitrogen sources considerably enhanced the growth of *A. oryzae*. While similar dry biomass concentrations were determined with yeast extract and peptone (3.59 ± 0.14 and 3.58 ± 0.05 g/L, respectively), a slightly lower value of 3.11 ± 0.08 g/L was obtained with casein hydrolysate. The biomass yield was lowest for cultures containing $NaNO_3$ (0.12 ± 0.01 g/g), followed by $(NH_4)_2SO_4$ and urea (Table 8). Yields with the complex nitrogen sources were considerably increased and the highest value of 0.79 ± 0.05 g/g was obtained with peptone.

Table 8. Comparison of growth of *A. oryzae* after 48 h of cultivation in preculture medium with different nitrogen sources. Data are the means of triplicate experiments with respective standard deviations.

Nitrogen source	Consumed substrate [g/L]	Dry biomass [g/L]	$Y_{X/S}$ [g/g]
$(NH_4)_2SO_4$	5.63 ± 0.20	0.92 ± 0.13	0.16 ± 0.02
$(NH_4)_2SO_4$ + 2 mL/L Hutner's solution	5.59 ± 0.40	3.10 ± 0.25	0.56 ± 0.04
$NaNO_3$	4.11 ± 0.09	0.48 ± 0.06	0.12 ± 0.01
Urea	5.18 ± 0.57	0.90 ± 0.23	0.17 ± 0.02
Glutamate	4.47 ± 0.24	0.92 ± 0.03	0.25 ± 0.06
Yeast extract	7.83 ± 0.90	3.59 ± 0.14	0.46 ± 0.05
Casein hydrolysate	7.65 ± 0.26	3.11 ± 0.08	0.41 ± 0.02
Peptone	4.47 ± 0.29	3.58 ± 0.05	0.79 ± 0.05

$Y_{X/S}$ = substrate specific dry biomass yield calculated as g(dry biomass)/g(consumed acetic acid).

The nitrogen source also affected the onset of germination. When $NaNO_3$ was used, growth was delayed as germination was only detected after 24 h of cultivation (Appendix C, Figure C1A). With the other defined nitrogen sources urea, glutamate and $(NH_4)_2SO_4$, biomass formation was already observed at this time. This is exemplarily shown for cultures with $(NH_4)_2SO_4$ in Figure C1A (Appendix C). With all complex nitrogen sources germination was accelerated, as exemplarily shown for yeast extract in Figure C1A (Appendix C). In these cultures, the majority of conidia had already started to form germ tubes after 8 h, whereas this was only sporadically observed with $(NH_4)_2SO_4$ at this point.

The addition of Hutner's trace element solution to cultures with $(NH_4)_2SO_4$ also considerably enhanced biomass production, resulting in a concentration of 3.10 ± 0.25 g/L, which is a 3.4-fold

increase compared to the cultures without trace elements. Thus, a similar dry biomass concentration was obtained as with casein hydrolysate, and the second highest biomass yield of 0.56 ± 0.04 g/g was determined. The trace element addition resulted in the formation of shorter biomass branches but did not accelerate the onset of germination (Appendix C, Figure C1A). The effect of the trace element solution was also studied with glucose as carbon source, and similar observations were made. The addition of 2 mL/L of the trace element solution increased the dry biomass concentration by a factor of 2.9 (Appendix C, Figure C2) and shorter and more branched filaments were formed (Appendix C, Figure C1B). Increasing the trace element solution's concentration to 4 or 6 mL/L had no considerable effect on biomass production for either carbon source (Appendix C, Figure C2).

3.3.3. Effect of the nitrogen source on L-malic acid production

The effect of different nitrogen sources was also evaluated during organic acid production. Due to the poor results obtained with $NaNO_3$ in the preculture, this nitrogen source was not further considered for acid production. The course of malic acid concentration with different nitrogen sources is shown in Figure 11. Malic acid was detected in all cultures after 48 h. However, yeast extract considerably accelerated organic acid production especially in the beginning of the fermentation. Within the first 48 h, the highest malic acid concentration of 3.56 ± 0.32 g/L was detected with this nitrogen source, while the cultures with ammonium sulfate produced only 1.36 ± 0.13 g/L within the same time. The steepest concentration increase for all cultures was observed between 48 and 96 h. After 144 h of cultivation, only minor changes in acid concentration were observed, for which reason the calculations in Table 9 are based on this measurement point. After 144 h, the highest malic acid concentrations were quantified in cultures with glutamate (9.10 ± 0.56 g/L) and yeast extract (9.05 ± 0.98 g/L), resulting in a volumetric productivity of 0.063 g/L/h. The lowest acid production of 6.27 ± 0.51 g/L was detected in cultures with urea which also resulted in the lowest yield of 0.16 ± 0.00 g/g. The total acid concentration followed the same pattern, with the highest concentrations obtained with yeast extract and glutamate. In cultures containing peptone, the lowest substrate consumption was observed with about 10 g/L acetic acid remaining after 144 h. In these cultures, 7.89 ± 0.24 g/L acetate was still detected at the end of the cultivation after 240 h while in most other cultures the carbon source was depleted or close to depletion at this time (Appendix C, Figure C3). The pH development showed only little differences between the nitrogen sources, with all cultures starting at a value of about 6.5 and increasing to pH 9.8-10.0 after 240 h (Appendix C, Figure C3).

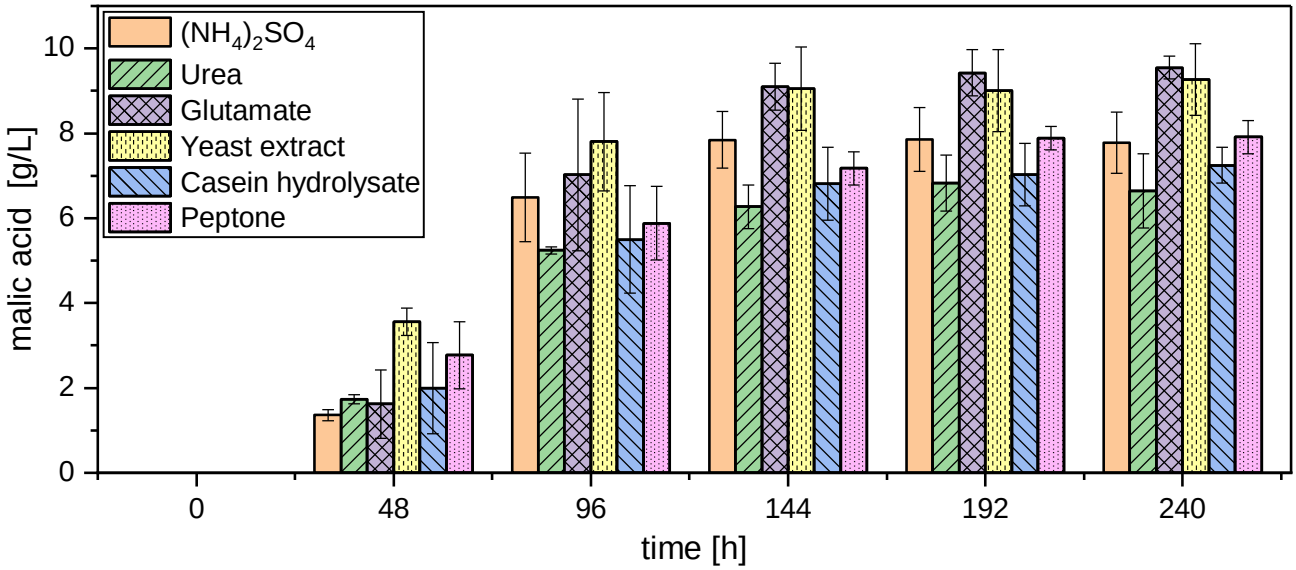

Figure 11. Malic acid production with *A. oryzae* using different nitrogen sources. Data are the means of triplicate experiments with respective standard deviations.

Regarding the total acid composition, the malate share was lowest (49.3 ± 1.5%) and the oxalate percentage was highest (4.5 ± 1.2%) with urea, while malate was highest (54.8 ± 2.3%) and oxalate lowest (1.8 ± 0.4%) in the cultures with ammonium sulfate (Appendix C, Table C2). Overall, however, the type of nitrogen source does not appear to have a pronounced effect on the proportions of byproducts.

Table 9. Fermentation results for cultivations of *A. oryzae* with different nitrogen sources after 144 h. Data are the means of triplicate experiments with respective standard deviations.

Nitrogen source	Consumed substrate [g/L]	Malic acid [g/L]	$Y_{P/S,\ malic\ acid}$ [g/g][a]	Malic acid productivity [g/L/h]	Total acid [g/L][b]	$Y_{P/S,\ total\ acid}$ [g/g]	Total acid productivity [g/L/h]
$(NH_4)_2SO_4$	43.31 ± 0.72	7.84 ± 0.67	0.18 ± 0.01	0.054 ± 0.005	14.30 ± 0.85	0.33 ± 0.02	0.099 ± 0.006
Urea	38.11 ± 2.75	6.27 ± 0.51	0.16 ± 0.00	0.044 ± 0.004	12.74 ± 1.22	0.33 ± 0.01	0.088 ± 0.009
Glutamate	41.76 ± 3.28	9.10 ± 0.56	0.22 ± 0.00	0.063 ± 0.004	16.89 ± 1.30	0.40 ± 0.01	0.117 ± 0.009
Yeast extract	42.84 ± 1.87	9.05 ± 0.98	0.21 ± 0.01	0.063 ± 0.007	17.21 ± 1.51	0.40 ± 0.02	0.120 ± 0.010
Casein hydrolysate	39.16 ± 4.12	6.81 ± 0.86	0.17 ± 0.00	0.047 ± 0.006	13.09 ± 1.44	0.33 ± 0.00	0.091 ± 0.010
Peptone	34.11 ± 0.65	7.18 ± 0.39	0.21 ± 0.01	0.050 ± 0.003	14.16 ± 0.67	0.42 ± 0.02	0.098 ± 0.005

[a] $Y_{P/S,\ malic\ acid}$ = substrate specific malic acid yield calculated as g(malic acid)/g(consumed acetic acid).

[b] Refers to all organic acids quantified (see Appendix C, Table C2).

3.3.4. Comparison of process modes for L-malic acid production

Fed-batch and repeated-batch processes with yeast extract or ammonium sulfate as nitrogen source were performed in shake flasks and compared to the respective batch process. The time course of malic acid production, substrate consumption and pH is displayed in Figure 12.

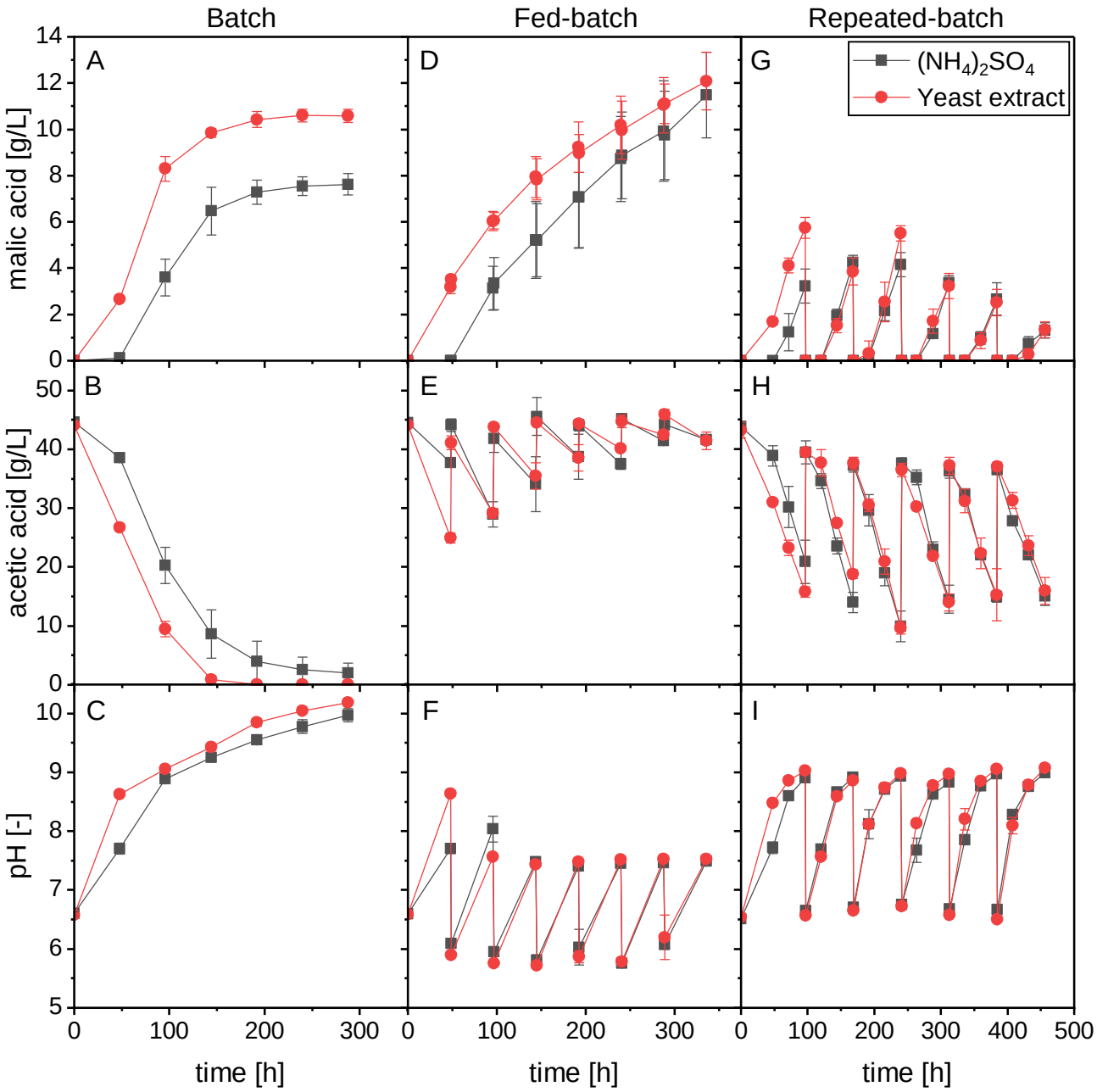

Figure 12. Batch, fed-batch and repeated-batch process for L-malic acid production with *A. oryzae* and $(NH_4)_2SO_4$ or yeast extract as nitrogen source. Data are the means of triplicate experiments with respective standard deviations.

In the fed-batch process, 5 M acetic acid was added every 48 h to restore a substrate concentration of about 45 g/L (Figure 12E). No further medium components were added besides the substrate. With both nitrogen sources, the period of malic acid accumulation was extended compared to the batch process as production was observed until the end of the cultivation at 336 h. The final malic acid concentrations were therefore higher than in the batch process, amounting to 11.49 ± 1.84 g/L with $(NH_4)_2SO_4$ and 12.08 ± 1.25 g/L with yeast extract (Figure 12D). However, the substrate consumption and acid production rates decreased in later stages of the fermentation despite adequate carbon availability (Figure 13). Through the repeated addition of acetic acid, the pH was prevented from surpassing a value of 9.0 and was mostly below 7.5 except for the first 96 h of fermentation (Figure 12F). Immediately after addition of the acid, pH values between 5.5 and 6.0 were measured. In growth experiments performed with *A. oryzae,* the germination of conidia was inhibited at a pH of 6.0 with 45 g/L acetic acid as substrate was observed (Appendix C, Figure C4). It is therefore possible that the low pH obtained directly after feeding negatively affected *A. oryzae* although further acid production was not prevented.

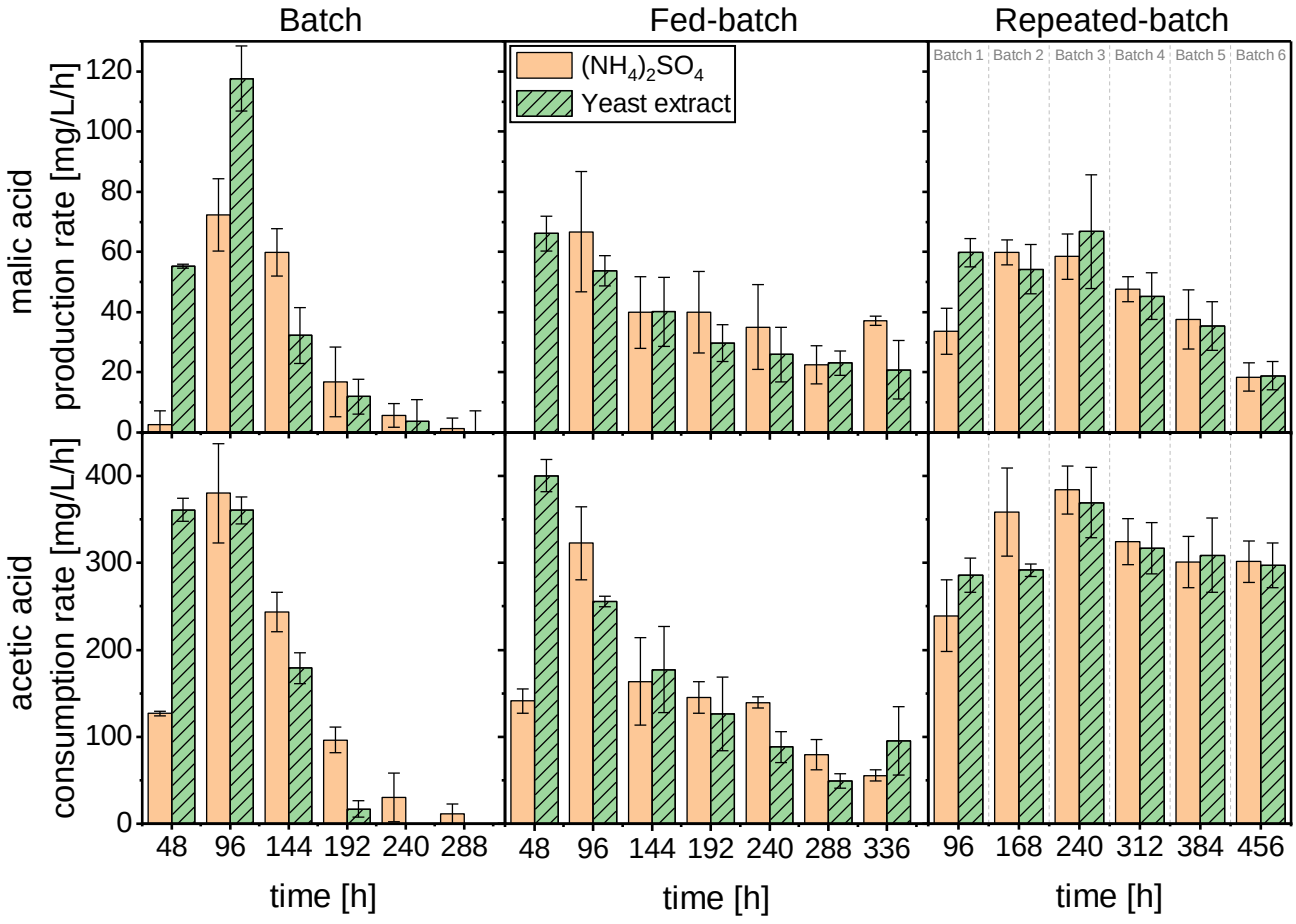

Figure 13. Malic acid production and acetic acid consumption rates for different process modes. Values were calculated between measurements every 48 h for the batch and fed-batch process. For the repeated-batch process, values were calculated for each batch. Data are the means of triplicate experiments with respective standard deviations.

Malic acid productivity also declined in later stages of the repeated-batch process. In these experiments, the medium was completely exchanged after the first 96 h and then every further 72 h. Through the repeated medium exchange, the pH was kept between values of about 6.5 and 9.0 (Figure 12I). In the first batch, 3.23 ± 0.74 g/L malic acid was obtained in cultures with $(NH_4)_2SO_4$ compared to 5.74 ± 0.45 g/L with yeast extract. Starting with the fourth medium exchange, malic acid production declined for both nitrogen sources while the substrate consumption remained similar (Figure 12G,H). In the sixth and last batch, 1.30 ± 0.33 g/L or 1.34 ± 0.34 g/L malic acid were measured with ammonium sulfate or yeast extract, respectively. Interestingly, a difference between the two nitrogen sources was observed mainly in the first batch, while both substrate consumption and malic acid production were mostly similar in later cycles. A lag phase occurred after each medium exchange, which is illustrated by the absence or very low production of malate during the first 24 h of cycles no. 2-6, and a lower acetate consumption rate than between the following two samplings (Appendix C, Figure C5). The ammonium concentration was monitored for the cultures containing $(NH_4)_2SO_4$, and the nitrogen source was consumed during the first 24 h of each cycle, indicating biomass formation (Appendix C, Figure C6). Ammonium was only depleted at the end of each batch phase, meaning that nitrogen was not limiting until later stages in the cycles. Since each batch started with a new growth phase, cultures with yeast extract were expected to exhibit a shortened lag phase not only in the first but also in later cycles, but this was not observed.

Table 10 summarizes the calculated values of the three process modes for a cultivation time of 240 h, which comprises the first four feeds of the fed-batch and the first three medium changes of the repeated-batch process. For the batch process, the values in parentheses represent calculations performed for a cultivation time of 144 h, since the acid production was nearly completed within this period. As already displayed in the previous chapter, the batch process with yeast extract performed better than that with $(NH_4)_2SO_4$, resulting in malic acid yields of 0.24 ± 0.01 g/g and 0.18 ± 0.00 g/g, respectively. Regarding the fed-batch process, the yield for cultures with $(NH_4)_2SO_4$ was slightly higher than in the batch process (0.20 ± 0.02 g/g) and slightly lower in the cultures with yeast extract (0.21 ± 0.02 g/g). The lower yield of the fed-batch process with yeast extract could be due to a higher biomass accumulation in the beginning of the main culture, as demonstrated in the growth experiments, which then required more carbon for maintenance metabolism throughout the fermentation. The amount of malic acid produced within 240 h was highest in the repeated-batch process with 1.28 ± 0.09 g ($(NH_4)_2SO_4$) and 1.58 ± 0.25 g (yeast extract). The yield of the repeated-batch processes, however, was lowest. The productivities of the repeated-batch process after 240 h were similar compared to those of the batch process considering a cultivation time of 144 h. Thus, a high productivity could be maintained at least in the first three cycles of the repeated-batch process.

The total acid composition after 240 h was largely similar between the process modes with a malic acid ratio of 52-57% and succinic acid representing 35-40% (Appendix C, Table C3).

Table 10. Fermentation results for cultivations of *A. oryzae* using different process modes after 240 h. Data are the means of triplicate experiments with respective standard deviations.

Process mode	Nitrogen source	Consumed substrate [g]	Malic acid [g]	$Y_{P/S,\ malic\ acid}$ [g/g][b]	Malic acid productivity [g/L/h]	Total acid [g][c]	$Y_{P/S,\ total\ acid}$ [g/g]	Total acid productivity [g/L/h]
Batch[a]	$(NH_4)_2SO_4$	4.63 ± 0.22 (3.96 ± 0.42)	0.83 ± 0.04 (0.71 ± 0.11)	0.18 ± 0.00 (0.18 ± 0.01)	0.031 ± 0.002 (0.045 ± 0.007)	1.52 ± 0.11 (1.36 ± 019)	0.33 ± 0.01 (0.34 ± 0.01)	0.058 ± 0.004 (0.086 ± 0.012)
	Yeast extract	4.84 ± 0.01 (4.76 ± 0.06)	1.17 ± 0.03 (1.08 ± 0.02)	0.24 ± 0.01 (0.23 ± 0.00)	0.044 ± 0.001 (0.068 ± 0.001)	2.06 ± 0.03 (1.97 ± 0.03)	0.42 ± 0.01 (0.41 ± 0.01)	0.078 ± 0.001 (0.124 ± 0.002)
Fed-batch	$(NH_4)_2SO_4$	5.02 ± 0.51	1.01 ± 0.21	0.20 ± 0.02	0.036 ± 0.008	2.03 ± 0.40	0.40 ± 0.06	0.067 ± 0.013
	Yeast extract	5.83 ± 0.63	1.21 ± 0.18	0.21 ± 0.02	0.042 ± 0.005	2.07 ± 0.53	0.38 ± 0.03	0.071± 0.017
Repeated-batch	$(NH_4)_2SO_4$	7.80 ± 0.50	1.28 ± 0.09	0.16 ± 0.00	0.048 ± 0.003	2.43 ± 0.20	0.31 ± 0.01	0.098 ± 0.008
	Yeast extract	7.71 ± 0.52	1.58 ± 0.25	0.20 ± 0.02	0.060 ± 0.010	2.91 ± 0.42	0.38 ± 0.03	0.116 ± 0.017

[a] Values in parentheses show the results after 144 hours of cultivation, during which most of the product formation was detected in batch processes.

[b] $Y_{P/S,\ malic\ acid}$ = substrate specific malic acid yield calculated as g(malic acid)/g(consumed acetic acid).

[c] Refers to all organic acids quantified (see Appendix C, Table C3).

3.4. Discussion

3.4.1. Effect of temperature on organic acid production

This study evaluated the effect of four cultivation temperatures in the range of 29-38 °C on organic acid production with the carbon sources acetate and glucose. Generally, the accumulation of organic acids was accelerated with increasing temperature and total acid concentration was highest in cultures incubated at 38 °C (Figure 9, Table 7). However, the ratio of malic acid to byproducts was best at 32 °C (Figure 10).

The difference in percentages of the main products malate and succinate between the two carbon sources can be explained by the different pathways for substrate metabolization and L-malic acid production. With glucose, the reductive tricarboxylic acid cycle taking place in the cytosol was identified as main pathway for malate synthesis in *Aspergillus flavus* (Peleg et al., 1989). In this pathway, pyruvate carboxylase first catalyzes the carboxylation of pyruvate to oxaloacetate while fixing CO_2. Then, malate dehydrogenase reduces oxaloacetate to malate. With acetate, on the other hand, the glyoxylate cycle is probably mainly involved in malate production. This bypass of the citric acid cycle involves the enzymes isocitrate lyase which converts isocitrate to glyoxylate and succinate, and malate synthase which uses glyoxylate and acetyl-CoA for the synthesis of malate (Armitt et al., 1970; Armitt et al., 1971; Ries et al., 2021). Thus, with the carbon source acetate, the production of one mole of malate is accompanied by the synthesis of one mole of succinate.

The temperature effect was especially pronounced regarding the ratio between malate and succinate in cultures with acetate, as succinate became the main product at the highest temperature tested. An increased succinate concentration at higher temperatures may indicate an elevated activity of enzymes involved in the production of succinic acid, or an inhibition of enzymes involved in its further metabolization. It is also conceivable that the temperature affects the transport of the compounds. However, this cannot be conclusively determined until the effects of temperature on individual enzymes of *A. oryzae* are studied. For *Aspergillus kawachii* (now *A. luchuensis*), for example, down-regulation of fumarase and up-regulation of malate synthase were observed by Futagami et al. (2015) when the temperature was lowered from 40 °C to 30 °C. Furthermore, these authors obtained a higher citric acid concentration during cultivation of the same strain at 30 °C compared to 40 °C, which is consistent with the results obtained with glucose in the current study.

Finding the process-specific ideal temperature is thus of great importance for optimizing product yields which was also demonstrated by other authors. For succinic acid production with *A. niger*, a temperature of 35 °C was found optimum (Yang et al., 2020), while 33 °C was best for itaconic acid production with *A. terreus* (Saha et al., 2019). Regarding malic acid production with an engineered strain of *A. oryzae* FMME 338, a slightly higher titer was quantified in cultures incubated at 30 °C compared to 32 and 34 °C, while less glucose was consumed (Chen et al., 2019). In these studies, the temperature resulting in the highest product concentration differed from the one yielding the highest

biomass concentration. Therefore, it might be interesting to evaluate the effect of a temperature shift between the initial growth phase and the production phase in the main culture. The reported studies did not investigate the effect of cultivation temperature on the production of possible side products. Regarding the byproduct spectrum in the current study, a temperature of 32 °C was most suitable for malic acid production with both glucose and acetate.

3.4.2. Effect of nitrogen sources on growth and L-malic acid production

Several nitrogen sources were evaluated both for growth and organic acid production. With the defined nitrogen sources $(NH_4)_2SO_4$, urea and glutamate, similar biomass concentrations were obtained (Table 8). With glutamate, however, the biomass yield related to the consumed acetate was higher compared to cultures containing $(NH_4)_2SO_4$ and urea. This was also observed during organic acid production (Table 9) and is probably due to glutamate not only functioning as nitrogen but also as carbon source. Although urea theoretically provides one carbon atom per molecule, it does not appear to be utilized as carbon source since no considerable effect on biomass yield was observed. Urea is likely metabolized by urease in *A. oryzae*, converting urea to CO_2 and ammonia (Strope et al., 2011).

The lowest biomass concentration and yield was determined with sodium nitrate. Like urea, this nitrogen source is metabolized via ammonium in *Aspergillus* species. First, NO_3^- is reduced to NO_2^- by nitrate reductase followed by a further reduction to NH_4^+ by nitrite reductase (Amaar and Moore, 1998; Schinko et al., 2010). This process is energy-consuming which could explain the lower biomass concentration and delayed germination.

Key enzymes involved in ammonium assimilation are glutamate dehydrogenase, glutamine synthetase and glutamate synthase. For *Aspergillus* species it is assumed that the pathway via glutamate dehydrogenase is the main pathway for the metabolization of ammonium and therefore also urea and nitrate. This was demonstrated by the observation that *gdhA*-lacking mutants of *A. nidulans* displayed lagged or poor growth when ammonium, nitrate or urea was used as nitrogen source (Arst and MacDonald, 1973; Kinghorn and Pateman, 1973). The NADP-dependent glutamate dehydrogenase (encoded by *gdhA*) catalyzes the amination of α-ketoglutarate, yielding glutamate which is then further used in anabolic reactions. The NAD-linked glutamate dehydrogenase (*gdhB*), however, is assumed to mainly be involved in glutamate catabolism, hence the deamination of glutamate, as mutants deficient in this enzyme did not grow on glutamate as the sole carbon source (Kinghorn and Pateman, 1973). The observation that growth of a double mutant deficient in both glutamate dehydrogenase genes on ammonium, nitrate and urea was delayed but not completely inhibited, suggested another pathway to be involved in ammonium assimilation (Kinghorn and Pateman, 1976). Glutamine synthetase uses ammonia to aminate glutamate, forming glutamine. Together with α-ketoglutarate, the glutamine is then used by glutamate synthase to form two molecules of glutamate. The involvement of glutamate synthase (*gltA*) in the nitrogen metabolism of *A. nidulans* was demonstrated by observing the growth of mutants deficient in this enzyme. When

both *gdhA* and *gltA* were lacking, no growth was observed with the nitrogen sources ammonium and nitrate, whereas biomass was formed with glutamate or glutamine (Macheda et al., 1999). As urea and nitrate first need to be converted to ammonia and then glutamate, it is generally assumed that the latter two are preferred nitrogen sources. This was confirmed for $NaNO_3$ in the growth experiments and urea during organic acid production presented here.

The utilization of complex nitrogen sources considerably increased biomass production. Besides nitrogen, these compounds also provide carbohydrates, amino acids, vitamins and trace elements (Klotz et al., 2017). The advantage of trace element availability during growth was demonstrated by an increase in dry biomass concentration with the addition of Hutner's trace element solution. The trace element supplementation was sufficient to enhance biomass production to levels similar to those obtained with the complex nitrogen sources (Table 8). Different than the trace element solution, however, the complex nitrogen sources also caused an earlier onset of germination (Appendix C, Figure C1). The enhanced biomass production and earlier germination could have been caused by the availability of carbohydrates or the supply of free amino acids as direct precursors for protein synthesis provided by the complex nitrogen sources. For *A. niger*, some amino acids were found to trigger germination as opposed to other nitrogen sources such as sodium nitrate, urea, or ammonium sulfate (Hayer et al., 2014). In addition, phosphate availability, which was likely increased in cultures with complex nitrogen sources, may have played a role. Regarding the organic acid production in the main culture, especially yeast extract had a positive influence as it increased the productivity in the beginning of the fermentation. The composition and therefore the effect of complex nitrogen sources strongly depend on their manufacturing process. The type or brand can therefore have a significant impact on growth and production (Klotz et al., 2017; Sørensen and Sondergaard, 2014). A detailed analysis of the nitrogen source's composition is thus necessary for determining the effects conclusively.

The concentration of the nitrogen sources applied in the media was determined according to their total nitrogen concentration, with the aim of keeping it at 0.85 g/L in the preculture and 0.25 g/L in the main culture. This resulted in rather high concentrations of the complex nitrogen sources especially in the preculture. Further experiments should evaluate the minimum concentration of these compounds required to obtain a positive effect on growth or malic acid production. It would also be interesting to study malic acid production in media with $(NH_4)_2SO_4$ as the main nitrogen source, supplemented with low concentrations of yeast extract.

Using glucose, several nitrogen sources have already been evaluated for malic acid production. With *A. oryzae* NRRL3488 the utilization of peptone resulted in a 34% higher malic acid productivity during the stationary phase compared to cultures with ammonium sulfate, yielding a final titer of 30.27 ± 1.05 g/L compared to 22.27 ± 0.46 g/L, respectively (Knuf et al., 2013). Given that the yields were similar with the two nitrogen sources, Knuf et al. (2013) suggested a difference in biomass production as a reason for this observation. Ding et al. (2018) compared malic acid production with *A. oryzae* CCTCC M 2016401 using different ammonium compounds as well as urea and

tryptone. Among the defined nitrogen sources, they found the highest malic acid titer of 30.7 ± 0.9 g/L with $(NH_4)_2SO_4$ but a significantly higher concentration of 70.2 ± 0.7 with tryptone. In the current study, enhanced organic acid production was mainly observed in the beginning of the fermentation with the complex nitrogen sources but smaller differences towards the end. This is likely due to the depletion of acetate which prevented further acid production. Ding et al. (2018) found optimizing both the C:N ratio as well as the absolute concentrations of both the carbon and nitrogen source effective for enhancing the L-malate titer. The C:N ratio was also identified as an important parameter for cultivation of *A. oryzae* DSM 1863 with glucose, as the concentration of the side product fumaric acid was greatly affected and ranged from 0.70 ± 0.07 g/L (C:N of 100:1) to 8.44 ± 1.55 g/L (C:N of 300:1) while the malic acid concentration was about 52-55 g/L (Ochsenreither et al., 2014). Therefore, it may be interesting to investigate the effects of different C:N ratios on the organic acid spectrum produced from acetate in future studies.

3.4.3. Comparison of process modes for L-malic acid production

Substrate inhibition is a disadvantage of batch processes which is especially relevant using acetate as carbon source. Malic acid production with *A. oryzae* was already inhibited at concentrations above 50 g/L acetic acid, resulting in a maximum product concentration of less than 10 g/L in batch mode as discussed in Chapter 2. Therefore, a fed-batch and repeated-batch process was evaluated, aiming to enhance product synthesis. Given that yeast extract accelerated malic acid production, all experiments were also run with this nitrogen source in addition to cultures with ammonium sulfate.

Whereas the production rate in the batch process with both nitrogen sources considerably decreased after 144 h of cultivation due to substrate depletion or high pH values, the period of malic acid production was prolonged in both fed-batch and repeated-batch mode (Figure 12). In the fed-batch process, furthermore, higher maximum malic acid concentrations were obtained. However, the productivity decreased during the cultivation (Figure 13). This resulted in similar volumetric production rates for the fed-batch and batch processes considering a cultivation time of 240 h, and even decreased production rates for the fed-batch when comparing it to the first 144 h of the batch process (Table 10). A different observation was made in fed-batch cultivations for L-malic acid production with a modified strain of *A. oryzae* performed by Liu, Xie et al. (2017). They reported a 13% increase in productivity in the fed-batch process (1.38 g/L/h) compared to the batch process (1.22 g/L/h) with glucose as substrate. A reason for the decrease in productivity in the current study could be the inhibitory effect of acetic acid which is pH dependent. The pH of the fermentation medium was temporarily brought to values between 5.5 and 6.0 by the addition of acetic acid (Figure 12F). It is assumed that acetic acid enters the cytoplasm through diffusion, dissociates and thereby leads to an intracellular acidification (Stratford et al., 2009). The lower the pH of the medium, the more acetate is present as acetic acid. Although the low pH values reached due to the feeding did not prevent further acid production, repeated exposure to higher concentrations of free acid could have had an adverse effect on *A. oryzae* over time. In experiments evaluating the effect of pH on growth,

no biomass formation or germination was observed at a pH of 6.0 when 45 g/L acetic acid was used as substrate (Appendix C, Figure C4), demonstrating a negative effect of the pH range temporarily reached during the fed-batch. Further experiments should therefore investigate whether maintaining a constant pH in the controlled environment of a bioreactor is beneficial for fed-batch malic acid production. Another reason for the decline in productivity during the fed-batch process might be the gradual dissolution of the $CaCO_3$ through the repeated feeds of acetic acid. A major function of $CaCO_3$ during malic acid production with glucose is the buffering of the pH (Schmitt et al., 2021). Moreover, $CaCO_3$ could supply CO_2 required by pyruvate carboxylase, or have a positive effect on malate production by providing calcium ions which can form poorly soluble calcium malate (Peleg et al., 1989; Zambanini et al., 2016). Although the function of $CaCO_3$ for malic acid production from acetate with *A. oryzae* has yet to be determined conclusively, elevated concentrations seem to be favorable as demonstrated in Chapter 2. The addition of $CaCO_3$ during a fed-batch process could therefore be considered in future research.

In the repeated-batch process, the volumetric productivity was maintained in the first three cycles, but then gradually decreased from the fourth cycle onwards (Figure 13). A productivity decline in later cycles during repeated-batch or repeated fed-batch cultivation was also reported for other processes such as L-tyrosine production with *E. coli* (Li et al., 2020), lipid production with *Rhodosporidium toruloides* (Zhao et al., 2011), kojic acid production with *A. oryzae* (Wan et al., 2005), or L-malic acid production with *A. oryzae* and glucose as carbon source (Schmitt et al., 2021). The authors indicated a decrease in cell viability, the accumulation of undesired byproducts or temporary substrate limitation as possible explanations. Yu et al. (2018) reported a decrease in productivity with increasing batch number during citric acid production with *A. niger* when free cells were used, while a high productivity was maintained also at later process stages with mycelium immobilized on a porous foam. Although the productivity declined in the results presented here, the substrate consumption remained comparable, resulting in lower yields. This is most likely attributed to an increased biomass concentration, although the biomass content was not determined. In each cycle, the medium was completely replaced with medium containing 0.25 g/L nitrogen. Within the first 24 h after each medium replacement no or very little malate production was detected but acetate and ammonium were consumed (Appendix C, Figure C5 and Figure C6). The nitrogen consumption was measured for the cultures with $(NH_4)_2SO_4$ and a depletion of nitrogen was only observed between 48 and 72 h in batches 2-6. As long as nitrogen was available, some carbon was most likely used for the formation of new biomass instead of malic acid production. By reducing or omitting the nitrogen source in the replacement medium, it may be possible to direct more acetate towards malate production, which could allow for higher productivities in later batches. However, Schmitt et al. (2021) showed that in repeated-batch experiments with *A. oryzae* and glucose as carbon source, the complete omission of the nitrogen source in the replacement medium caused a gradual reduction of malic acid production between cycles. Since malic acid production based on acetate differs from the process using glucose in aspects such as the metabolic pathway towards malate formation or the

morphology, evaluating the effect of nitrogen availability in repeated-batch experiments with acetate could be considered. The lag phase observed in each cycle of the repeated-batch experiments could also be associated with stress due to the pH drop from values of about 9.0 to 6.5 caused by the medium exchange (Figure 12I), or the filtration and washing of the biomass between the cycles. Although this approach was successful in removing all products and allowed for the reutilization of the entire amount of biomass, it might be better to replace the fermentation medium only partly. During itaconic acid production with *Ustilago cynodontis* in a repeated-batch process, a lag phase was also observed which the authors attributed to the centrifugation step performed between the cycles to recover the biomass (Hosseinpour Tehrani et al., 2019). Determining the optimum replacement ratio can be beneficial for repeated-batch processes. Regarding repeated fed-batch production of cellulase with *Penicillium oxalicum*, for example, a replacement percentage of 50% resulted in the highest volumetric productivity after 144 h (Han et al., 2017). Furthermore, during isocitric acid production with *Yarrowia lipolytica*, a medium removal scheme replacing 20-80% of the medium after 24-72 h resulted in high product titers even after more than 700 h of cultivation (Morgunov et al., 2019).

In summary, higher maximum malate titers were achieved in the fed-batch process, and the repeated-batch process allowed to maintain productivity at least in the first three cycles. However, neither the fed-batch nor the repeated-batch process showed clearly positive results regarding yield and volumetric productivity when compared to the 144 h-values of the batch process. This might be owed to the limitations of the shake flask environment in which it is difficult to control the pH. To prevent the productivity from declining in later fermentation stages in the fed-batch and repeated-batch process, factors like the nutrient availability, the strategy for biomass separation or the replacement ratio likely need to be optimized.

3.5. Conclusion

In this chapter, the cultivation temperature was identified as an important process parameter for controlling the side product spectrum. With increasing temperature, malic acid production accelerated but at the same time, the proportion of succinic acid increased. The highest share of malic acid from acetate was quantified at 32 °C, which was also found in comparative experiments performed with glucose. The choice of nitrogen source largely affected fungal growth and the highest biomass concentrations were obtained with yeast extract or peptone. Possibly, trace elements provided by the complex nitrogen sources contributed to this effect, since the utilization of ammonium sulfate together with a trace element solution resulted in similar biomass concentrations. Regarding malic acid synthesis, the utilization of yeast extract proved to be particularly advantageous, as production was enhanced at the beginning of cultivation. Since organic acid production with acetate is limited due to substrate inhibition in a batch process, fed-batch and repeated-batch strategies were evaluated. Although the maximum malic acid concentration was

enhanced and the production period was extended compared to the batch process, optimization is required to increase yields and maintain high productivities.

4. Immobilization of *Aspergillus oryzae* for L-malic acid production

This chapter is based on the publication:

Immobilization of *Aspergillus oryzae* DSM 1863 for L-Malic Acid Production
Aline Kövilein, Vera Aschmann, Silja Hohmann, Katrin Ochsenreither (2022)
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Author contributions

Aline Kövilein: conceptualization, investigation, data analysis, writing of first draft, manuscript revision.

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Katrin Ochsenreither: conceptual advice, project supervision, manuscript revision.

4.1. Introduction

As illustrated in Chapter 2, cultivations with acetate concentrations of around 45 g/L result in the highest malic acid titers. However, at such substrate levels, *A. oryzae* forms a filamentous-lumpy morphology instead of biomass pellets which is the morphology commonly obtained during cultivation in glucose media. As a pelleted morphology ensures a lower viscosity of the fermentation broth, leading to a better heat, oxygen, and nutrient transfer, it is often preferred. Furthermore, it facilitates the separation and retention of the biomass which allows for a continuous process management. One possibility for morphological control could be immobilization which allows for physical confinement of the biomass in a defined morphology. As malic acid production from acetate with *A. oryzae* is limited to concentrations below 10 g/L in a batch process due to substrate inhibition, immobilization could enable a continuous operation of bioreactors.

A common method for whole-cell immobilization is entrapment in a polymer matrix. Solutions of polymers such as agar, carrageenan and alginate, or the monomer acrylamide are mixed with biomass and solidified by cooling, ionic gelation, or polymerization. Immobilization by entrapment of *Aspergillus* species for malic acid production has not been described so far, but for the production of other organic acids and enzymes. For citric acid production, *Aspergillus niger* was immobilized in alginate (Bayraktar and Mehmetoglu, 2000; Eikmeier et al., 1984; Eikmeier and Rehm, 1984, 1987; Garg and Sharma, 1992; Omar et al., 1992; Tsay and To, 1987; Vaija et al., 1982), agarose (Khare et al., 1994), κ-carrageenan (Eikmeier et al., 1984), and polyacrylamide (Garg and Sharma, 1992; Horitsu et al., 1985). Further reports describe the immobilization of *Aspergillus terreus* in alginate, agar, and polyacrylamide for itaconic acid production (Horitsu et al., 1983; Kautola et al., 1985). With *A. oryzae*, immobilization was performed in a sol-gel matrix consisting of tetraethyl-orthosilicate, starch and alginate for α-amylase production (Evstatieva et al., 2014), alginate for the biotransformation of L-tyrosine to dopamine (Ali and Nawaz, 2016), or agar as well as alginate for phytase production (Sapna and Singh, 2017). Several studies have found an entrapment of *Aspergillus* species to improve the organic acid production process. Compared to free biomass, immobilized cells of *A. niger* performed better in repeated-batch cultures for citric acid production as the productivity was maintained at a high level for an increased number of cycles (Horitsu et al., 1985; Khare et al., 1994; Tsay and To, 1987). Similar observations were made for *A. terreus* and itaconic acid production (Kautola et al., 1985). It was furthermore shown that entrapment can decrease byproduct formation (Alexandri et al., 2017) and increase the tolerance of microorganisms to certain inhibitors such as ethanol, acetic acid, and phenol (Hanaki et al., 1994; Heipieper et al., 1991; Krisch and Szajani, 1997), which is especially interesting when side or waste streams are used as substrates.

Reports on the production of malic acid by immobilized microorganisms are scarce. The fumarase activity of *Brevibacterium flavum* and *Brevibacterium ammoniagenes* immobilized in κ-carrageenan and polyacrylamide was utilized for the conversion of fumaric to malic acid (Takata et al., 1980; Takata et al., 1984; Yamamoto et al., 1976). Fumarate was also used as substrate for malic acid

production with *Saccharomyces cerevisiae* immobilized in polyacrylamide (Figueiredo and Carvalho, 1991; Oliveira et al., 1994) and a copolymeric hydrogel consisting of alginate and polyvinyl alcohol (Menegatti and Žnidaršič-Plazl, 2019). However, fumaric acid is currently produced from fossil resources and therefore not a renewable carbon source.

The aim of this chapter was to evaluate L-malic acid production with immobilized *A. oryzae* in batch cultivations. Immobilization was performed by entrapment of conidia in the natural polymers alginate, agar, and κ-carrageenan. The performance of the immobilized particles was assessed in shake flask and 2.5-L bioreactor cultivations and compared to that of fermentations with free biomass. As immobilization of *A. oryzae* for malic acid production has not been described before, experiments were also performed with glucose besides cultivations with the substrate acetate.

4.2. Materials and methods

4.2.1. Microorganism and media

Aspergillus oryzae DSM 1863 was obtained from DSMZ (German Collection of Microorganisms and Cell Cultures GmbH, Braunschweig, Germany) and the propagation of spores was performed as described in Chapter 2.2.2.

The preculture medium contained either 45 g/L acetic acid or 40 g/L glucose monohydrate as carbon source, 4 g/L $(NH_4)_2SO_4$, 0.75 g/L KH_2PO_4, 0.98 g/L K_2HPO_4, 0.1 g/L $MgSO_4 \cdot 7H_2O$, 0.1 g/L $CaCl_2 \cdot 2H_2O$, 5 mg/L NaCl, and 5 mg/L $FeSO_4 \cdot 7H_2O$. For the cultivation of free biomass with acetate both in shake flasks and bioreactors, furthermore 2 mL/L 1000x Hutner's Trace Elements solution was added which consists of 5 g/L $FeSO_4 \cdot 7H_2O$, 50 g/L EDTA-Na_2, 22 g/L $ZnSO_4 \cdot 7H_2O$, 11 g/L H_3BO_3, 5 g/L $MnCl_2 \cdot 4H_2O$, 1.6 g/L $CoCl_2 \cdot 6H_2O$, 1.6 g/L $CuSO_4 \cdot 5H_2O$, and 1.1 g/L $(NH_4)_6Mo_7O_{24} \cdot 4H_2O$ with a pH of 6.5 (Hill and Kafer, 2001). The main culture medium was composed of 45 g/L acetic acid or 120 g/L glucose monohydrate, 1.2 g/L $(NH_4)_2SO_4$, 0.1 g/L KH_2PO_4, 0.17 g/L K_2HPO_4, 0.1 g/L $MgSO_4 \cdot 7H_2O$, 0.1 g/L $CaCl_2 \cdot 2H_2O$, 5 mg/L NaCl, 60 mg/L $FeSO_4 \cdot 7H_2O$ and 90 g/L $CaCO_3$. The pH of the preculture and main culture medium containing acetic acid was adjusted to a value of 6.5 and 5.5 with NaOH, respectively, while the pH of the glucose-containing media remained unadjusted. The preculture medium without carbon source was adjusted to a pH value of 6.5. All pre- and main culture media containing either glucose or acetate were sterilized by autoclaving for 20 min at 121 °C.

4.2.2. Immobilization

Immobilization was performed by entrapment of *A. oryzae* conidia in the natural polymers sodium alginate (Carl Roth GmbH & Co. KG, Karlsruhe, Germany), agar (European agar Difco TM, BD, Germany), and κ-carrageenan (Carl Roth GmbH & Co. KG, Karlsruhe, Germany). Polymer solutions were prepared by dissolving 0.75–6.00 g polymer powder in a final volume of 100 g double distilled water and autoclaved for 20 min at 121 °C. For the alginate beads 4 mL spore suspension

(c = 2 x 10^7/mL) was mixed with 16 mL alginate solution and extruded through a canula with a diameter of 0.80 mm into a gently stirred 0.2 M $CaCl_2$ solution which was cooled in an ice bath. After 2 h of solidification in the cooled 0.2 M $CaCl_2$ solution the beads were washed with double distilled water and stored at 4 °C overnight until the inoculation of the preculture on the next day. The 0.75% κ-carrageenan beads were prepared in the same way as the alginate beads, but solidification was performed in a 0.1 M KCl solution. The agar and 1.5% κ-carrageenan solutions were brought to a temperature of 45 °C, then 16 mL of the respective solution was mixed with 4 mL spore suspension (c = 2 x 10^7/mL). Subsequently, the mixtures were poured in petri dishes and solidified at room temperature. The petri dishes were stored at 4 °C overnight. Before inoculation of the precultures the films were cut in cubes of about 2-3 mm in length.

4.2.3. Preculture conditions

For the cultivations with free biomass, 100 mL preculture medium in 500-mL baffled shake flasks was inoculated with 1 mL of a spore suspension containing 3 x 10^7 conidia/mL. For the precultures with immobilized biomass, 20 mL of immobilized material was distributed equally by weight to three shake flasks containing 100 mL of preculture medium. Precultures with glucose were incubated for 24 h at 30 °C and 100 rpm and those with acetate for 48 h under the same conditions. The carbon source remained the same in pre- and main culture, hence biomass cultivated in preculture medium containing glucose was used for the inoculation of main cultures with glucose medium, the precultures with acetate were used for the main cultures with acetate as carbon source.

4.2.4. Main culture for acid production in shake flasks

Main culture cultivations were performed in 500-mL baffled shake flasks containing 100 mL main culture medium and 90 g/L $CaCO_3$. The free biomass and immobilized particles grown in the preculture were separated from the preculture medium by filtration and thoroughly washed with distilled water to remove medium residues. For the cultures with free *A. oryzae*, 0.75 g biomass was used for the inoculation of one shake flask. For all immobilized cultures, the content of one preculture triplicate was distributed equally by weight to three shake flasks. Main cultures were incubated at 120 rpm and 32 °C for 168 h (glucose media) or 240 h (acetate media). All experiments were performed as biological triplicates. Samples were taken at the indicated time points and analyzed for pH and glucose, organic acid, and ammonium concentration.

4.2.5. Main culture for acid production in bioreactors

For bioreactor cultivations 2.5-L stirred tank reactors (Minifors, Infors HT, Bottmingen, Switzerland) filled with 1.4 L main culture medium were used. Before inoculation 0.1 mL of the antifoaming agent Contraspum A 4050 HAc (Zschimmer und Schwarz GmbH und Co KG, Lahnstein, Germany) was added. To the bioreactors containing acetate medium, 17.5 mL of a sterile glucose solution (c = 400 g/L) was added before inoculation to reach a concentration of 5 g/L glucose. This additional carbon source was used to decrease the duration of the lag phase. One

bioreactor was inoculated with four precultures. The temperature was controlled at 32 °C and the aeration rate was 0.5 vvm. Two Rushton turbines with a distance of 8 cm were used at a stirrer velocity of 300 rpm. All experiments were performed as biological duplicates. The pH value was recorded via online measurements. Samples were taken every 24 h for offline determination of glucose, organic acid, and ammonium concentration.

4.2.6. Analytics

For organic acid and glucose quantification a standard HPLC device (Agilent 1100 Series, Agilent, Germany) equipped with a Rezex ROA organic acid H+ (8%) column (300 by 7.8 mm, 8 µm, Phenomenex) and a Rezex ROA organic acid H+ (8%) guard column (50 by 7.8 mm) was used. For glucose analysis, 1 mL of the sample was centrifuged for 10 min at 20,000xg. The supernatant was diluted appropriately and analyzed under isocratic conditions using 5 mM H_2SO_4 as eluent with a flow rate of 0.5 mL/min at 50 °C. The injection volume was 10 µL. Detection was performed with a refractive index detector. For organic acid determination 1 mL of the sample was added to 3 mL of distilled water and 1 mL of 3 M H_2SO_4 according to Ochsenreither et al. (2014). The suspension was incubated for 20 min at 80 °C and subsequently centrifuged for 10 min at 20,000xg. The supernatant was diluted appropriately and analyzed under isocratic conditions using 3 mM H_2SO_4 as eluent with a flow rate of 0.5 mL/min at 60 °C. The injection volume was 10 µL. Organic acids were detected with a UV detector at 220 nm.

Ammonium concentration was quantified photometrically using a Spectroquant assay kit (114752, Merck KGaA, Darmstadt, Germany). The assay was scaled down to a volume of 200 µL and sample supernatants were measured in duplicate in microtiter plates according to the manufacturer's instructions.

Fungal morphology was characterized by light microscopy using a Nikon Eclipse E200 equipped with a DFK 23U274 camera (Imaging Source, Bremen, Germany) and the software NIS-Elements D ver. 4.50.

4.3. Results

4.3.1. Malic acid production in shake flask cultivations

Malic acid production with immobilized *A. oryzae* cells was first studied in shake flasks with acetate or glucose as substrate. When grown in medium with 45 g/L acetic acid, *A. oryzae* develops a dispersed filamentous structure whereas in glucose medium biomass pellets are formed naturally. Therefore, the term "free biomass" refers to either of these morphological forms depending on the carbon source. In the cultures with free biomass, the acid production was mostly completed within 144 h. For this reason, all values summarized in Table 11 are based on the measurements of this sampling point.

With acetate as carbon source, four alginate concentrations (3-6%), two agar concentrations (1.5% and 3%) and two κ-carrageenan concentrations (0.75% and 1.5%) were tested. Compared to the malic acid concentration obtained in cultures with free biomass (7.96 ± 0.26 g/L), the production was lower in all cultures with immobilized *A. oryzae* (Figure 14). With matrices made of alginate and agar, an increase of the polymer concentration resulted in a lower malic acid titer. While *A. oryzae* entrapped in beads prepared with 3% alginate produced 6.34 ± 0.72 g/L malic acid within 144 h, the utilization of 6% alginate resulted in 1.30 ± 0.43 g/L which was the lowest concentration measured of all tested conditions (Table 11). With 1.5% agar the second highest concentration of 5.53 ± 0.52 g/L malic acid was detected which was reduced to 3.68 ± 0.21 g/L with 3% agar. With κ-carrageenan, on the other hand, beads produced with 0.75% of the polymer reached a malic acid concentration of 2.98 ± 0.34 g/L compared to 4.72 ± 0.39 g/L obtained with 1.5% κ-carrageenan. Yields obtained after 144 h ranged from 0.08 ± 0.02 g/g with 6% alginate to 0.20 ± 0.01 g/g with free biomass. Regarding the productivity, the highest value was obtained with free biomass (0.055 ± 0.002 g/L/h) and the lowest with 6% alginate beads (0.009 ± 0.003 g/L/h). For all conditions, ammonium was depleted or close to depletion within 96 h of cultivation and the pH increased to values of around 9.0 within 144 h (Appendix D, Figure D1). Besides malic acid, *A. oryzae* produces several other organic acids. The total acid concentration with the substrate acetate ranged from 3.71 ± 0.61 g/L (6% alginate) to 15.69 ± 0.67 g/L (free biomass). Malic acid is usually the main product accounting for about 50-55% of the total acid concentration when acetate is used as carbon source (Appendix D, Table D1). Malic and succinic acid percentages decreased in favor of oxalic acid with increasing density of the immobilization matrix. This is especially illustrated with 6% alginate (26.4 ± 8.1% oxalic acid) but cultures with 5% alginate, 3% agar, and 0.75% κ-carrageenan also showed a considerably higher oxalic acid concentration than the control.

With glucose as carbon source, five alginate concentrations (1.5-6%), two agar concentrations (1.5% and 3%) and 1.5% κ-carrageenan were tested as immobilization matrices. Different than with acetate, the majority of cultures with entrapped *A. oryzae* performed slightly better or similar compared to the control with free biomass (Figure 14). Only the cultures with 5% and 6% alginate showed lower acid concentrations after 144 h. With 1.5% alginate the production was accelerated compared to that of the control, reaching its peak concentration at 120 h with 32.06 ± 2.44 g/L malic acid compared to 24.49 ± 0.10 g/L with free biomass. At the beginning of cultivation, the 1.5% alginate beads featured the smallest diameter of about 0.3 cm, whereas the beads prepared with higher alginate concentrations had a diameter of approximately 0.4 cm. Productivities calculated for a cultivation time of 144 h ranged from 0.166 ± 0.030 g/L/h obtained with 6% alginate to 0.220 ± 0.012 g/L/h for cultures with 1.5% κ-carrageenan (Table 11). The highest yield was calculated for the cultures with free biomass (0.59 ± 0.05 g/g) and the lowest for the cultures with 6% alginate (0.40 ± 0.03 g/g). Ammonium was depleted for all conditions after 48 h and the pH decreased to values of around 6.7 during cultivation (Appendix D, Figure D2). Total acid concentrations ranged from 33.34 ± 6.81 g/L (6% alginate) to 44.63 ± 3.10 g/L (free biomass). The

proportion of malic acid was not considerably affected by the immobilization and ranged from about 70-75% (Appendix D, Table D1). Generally, the differences amongst the performance of *A. oryzae* in the tested matrices compared to that of the free biomass was less pronounced than in cultures with acetate.

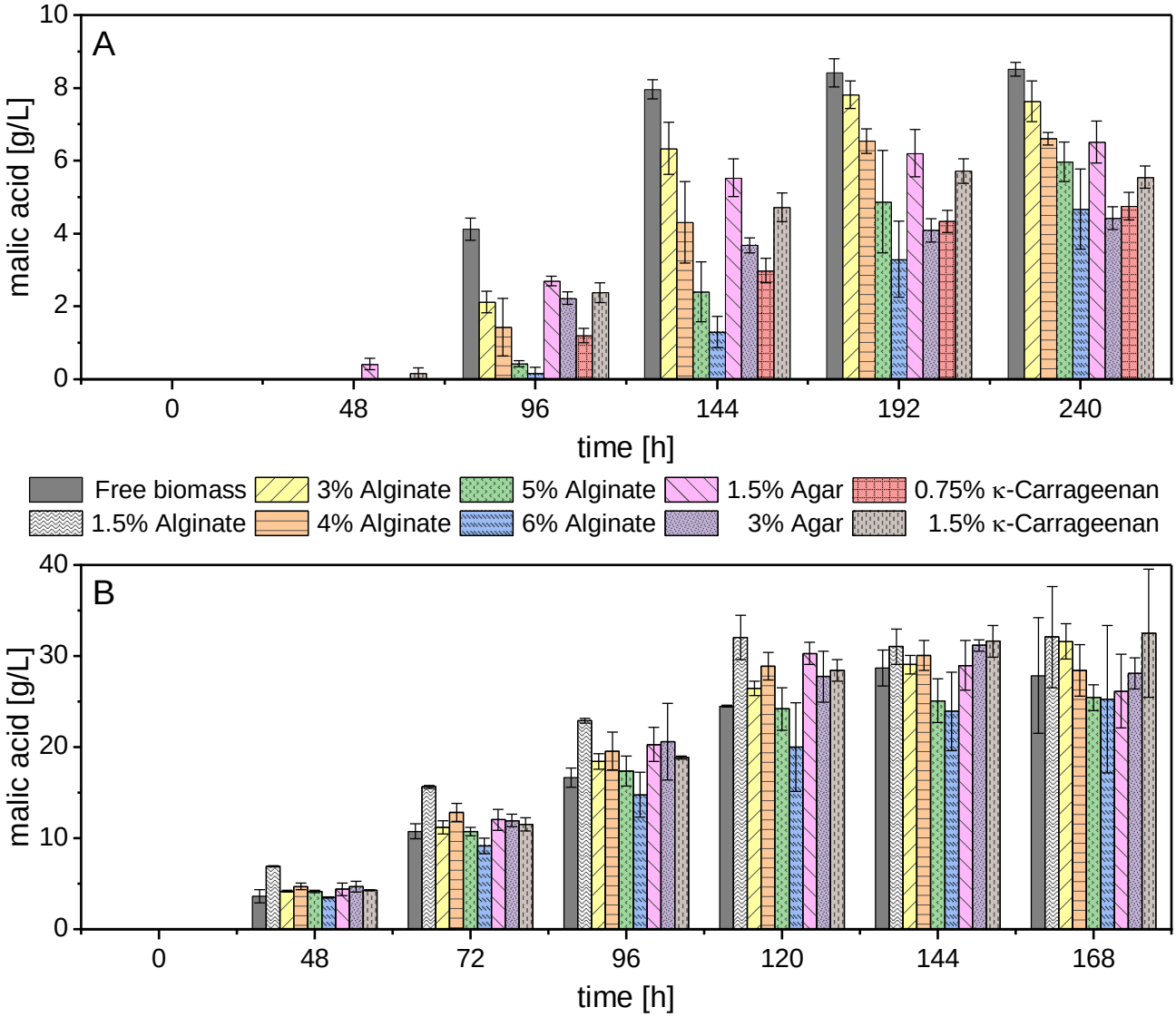

Figure 14. Malic acid production in shake flask cultivations with *A. oryzae* immobilized in different natural polymers using acetate (A) or glucose (B) as carbon source. Data are the means of triplicate experiments with respective standard deviations.

Table 11. Fermentation results for shake flask cultivations with free and immobilized *A. oryzae* after 144 h. Data are the means of triplicate experiments with respective standard deviations.

Sub-strate	Immobiliza-tion matrix	Consumed substrate [g/L]	Malic acid [g/L]	$Y_{P/S,\ malic\ acid}$ [g/g]	Productivity [g/L/h]	Total acids [g/L][a]
HAc	Free biomass	38.90 ± 1.09	7.96 ± 0.26	0.20 ± 0.01	0.055 ± 0.002	15.69 ± 0.67
	3% Alginate	32.15 ± 3.27	6.34 ± 0.72	0.19 ± 0.00	0.044 ± 0.005	12.03 ± 1.12
	4% Alginate	26.91 ± 5.35	4.31 ± 1.13	0.16 ± 0.02	0.030 ± 0.008	8.88 ± 2.12
	5% Alginate	20.30 ± 3.44	2.40 ± 0.82	0.12 ± 0.02	0.017 ± 0.006	5.26 ± 1.59
	6% Alginate	16.56 ± 1.73	1.30 ± 0.43	0.08 ± 0.02	0.009 ± 0.003	3.71 ± 0.61
	1.5% Agar	30.71 ± 1.28	5.53 ± 0.52	0.18 ± 0.01	0.038 ± 0.004	11.40 ± 1.00
	3% Agar	25.72 ± 0.66	3.68 ± 0.21	0.14 ± 0.00	0.026 ± 0.001	7.62 ± 0.74
	0.75% κ-Carra-geenan	23.71 ± 2.50	2.98 ± 0.34	0.13 ± 0.02	0.021 ± 0.002	6.80 ± 0.48
	1.5% κ-Carra-geenan	28.92 ± 0.99	4.72 ± 0.39	0.16 ± 0.02	0.033 ± 0.003	9.64 ± 0.84
Glc	Free biomass	48.99 ± 0.97	28.69 ± 1.98	0.59 ± 0.05	0.199 ± 0.014	39.08 ± 1.26
	1.5% Alginate	69.80 ± 1.06	31.04 ± 1.95	0.45 ± 0.03	0.216 ± 0.014	44.63 ± 3.10
	3% Alginate	64.96 ± 2.20	29.05 ± 1.03	0.45 ± 0.03	0.202 ± 0.007	40.18 ± 0.36
	4% Alginate	66.81 ± 1.53	30.08 ± 1.66	0.45 ± 0.03	0.209 ± 0.012	41.31 ± 1.85
	5% Alginate	64.83 ± 2.98	25.08 ± 2.40	0.39 ± 0.04	0.174 ± 0.017	35.68 ± 2.74
	6% Alginate	59.29 ± 6.96	23.92 ± 4.30	0.40 ± 0.03	0.166 ± 0.030	33.34 ± 6.81
	1.5% Agar	65.96 ± 2.43	28.96 ± 2.74	0.44 ± 0.05	0.201 ± 0.019	40.75 ± 2.76
	3% Agar	64.96 ± 0.64	31.16 ± 0.63	0.48 ± 0.01	0.216 ± 0.004	42.21 ± 0.37
	1.5% κ-Carra-geenan	61.62 ± 1.63	31.63 ± 1.74	0.51 ± 0.02	0.220 ± 0.012	41.81 ± 2.41

HAc = acetic acid, Glc = glucose, $Y_{P/S,\ malic\ acid}$ = substrate specific malic acid yield calculated as g(malic acid)/g(consumed substrate).

[a] Refers to all organic acids quantified (see Appendix D, Table D1).

For successful application of immobilized microorganisms, not only the production performance but also the matrix stability has to be considered. Ideally, the matrix is insoluble and chemically robust under the reaction conditions throughout the duration of the process. Differences in stability of the immobilized structures were observed between glucose and acetate-containing medium. As the cultivation progressed, the initially "fluffy" surface of the beads incubated in glucose medium and the free biomass pellets became smoother while all matrices remained intact with $CaCO_3$ adhering to the biomass layer (Figure 15).

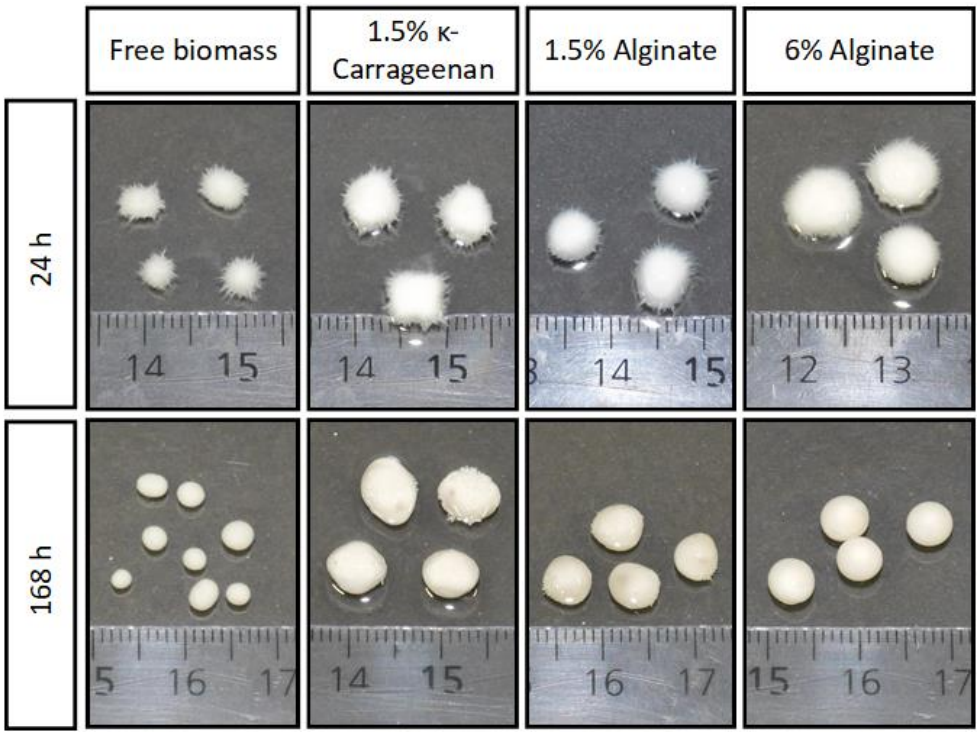

Figure 15. Appearance of free biomass pellets and immobilized *A. oryzae* during malic acid production in glucose medium.

In acetate medium, not all the immobilized structures were stable until the end of the fermentation. Between 96-144 h, the beads prepared with 3% and 4% alginate showed first signs of disintegration and were completely dissolved after 240 h. The alginate beads prepared with 5% and 6% of the polymer remained intact but gradually showed a softer consistency. In general, with a higher concentration of alginate the amount of outgrowth from the beads decreased, reducing the amount of biomass surrounding the beads (Figure 16). On the other hand, no difference in growth behavior was noticed for the two agar and κ-carrageenan concentrations tested. While the agar and κ-carrageenan matrices were stable and did not show any signs of degradation, an abrasion of the biomass was observed starting between 96 h and 144 h. Exemplarily, the development of cuboids prepared with 3% agar is shown in Figure 17. In the preculture, a dense biomass layer developed in the outer region and on the surface of the particles while the conidia located near the particle's cores did not germinate or remained in the early stages of growth (Figure 17A). In the main culture the cuboid structures were initially well covered with biomass which captured the $CaCO_3$ present in the main culture medium (Figure 17B). Towards the end of the fermentation, the biomass was largely stripped off and only a thin biomass layer remained on the surface (Figure 17C,D). This behavior was comparable for both agar and κ-carrageenan concentrations tested using acetate medium.

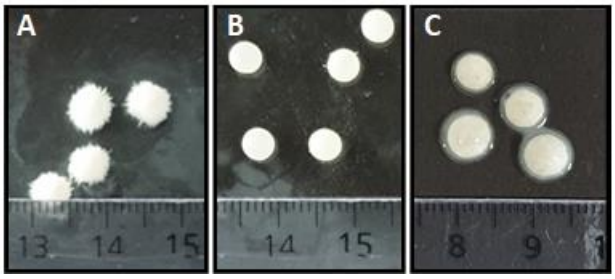

Figure 16. *A. oryzae* immobilized in 3% alginate after 48 h (A), 6% alginate after 48 h (B), and 6% alginate after 240 h (C) of incubation in main culture medium containing acetate.

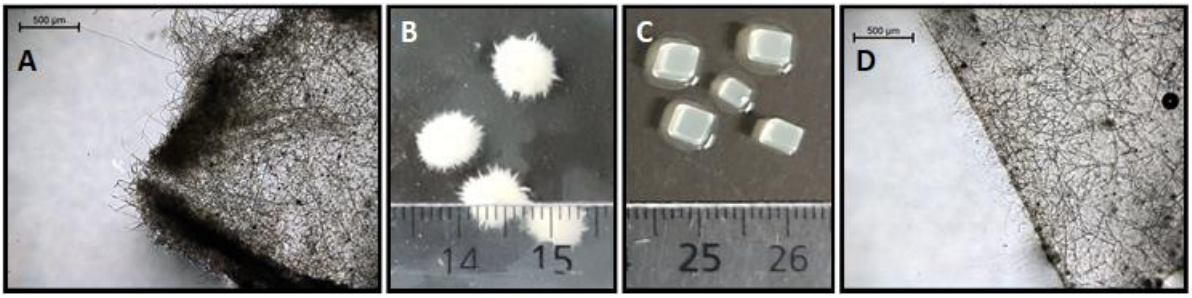

Figure 17. *A. oryzae* immobilized in 3% agar in the beginning (A), after 48 h (B), and after 240 h (C, D) of incubation in main culture medium containing acetate.

4.3.2. Malic acid production in bioreactor cultivations

2.5-L bioreactor cultivations were carried out with *A. oryzae* immobilized in alginate and κ-carrageenan using glucose or acetate as the main carbon source. *A. oryzae* entrapped in agar and κ-carrageenan performed similarly regarding acid production and growth behavior in shake flasks and therefore only the latter was tested in bioreactor fermentations. For a better comparison to the shake flask cultivations, the values summarized in Table 12 are calculated for a cultivation time of 144 h.

For the fermentations with glucose, *A. oryzae* immobilized in alginate (1.5% and 3%) and 1.5% κ-carrageenan was used. With the two alginate concentrations the progression of the fermentation was similar to the one with free biomass (Figure 18). Slightly lower malic acid concentrations were quantified compared to the free biomass fermentation, which yielded 29.77 ± 2.70 g/L of the product within 144 h (Table 12). Compared to that of shake flask cultivations, the malic acid concentrations obtained with free biomass and *A. oryzae* immobilized in alginate was similar. The yield, however, was increased compared to that of shake flask cultivations, which was associated with a decreased byproduct formation. While the malic acid percentage in shake flasks was around 70%, it increased to about 80% in the bioreactor cultivations with free biomass and *A. oryzae* immobilized in alginate (Appendix D, Table D2). At the same time, especially the citric acid production decreased to values below 1%. With *A. oryzae* immobilized in 1.5% κ-carrageenan, malic acid production was lowest (23.61 ± 4.68 g/L) and the yield was reduced to a value of 0.56 ± 0.12 g/g. Using glucose as carbon source, the malic acid concentration usually peaks around 168 h hours of fermentation, followed by a

decrease. With κ-carrageenan, this peak apparently occurred earlier than in the other fermentations, leading to a lower maximum concentration. As in shake flask experiments, ammonium was depleted within 48 h for all conditions and the pH decreased to values of 6.7-7.0.

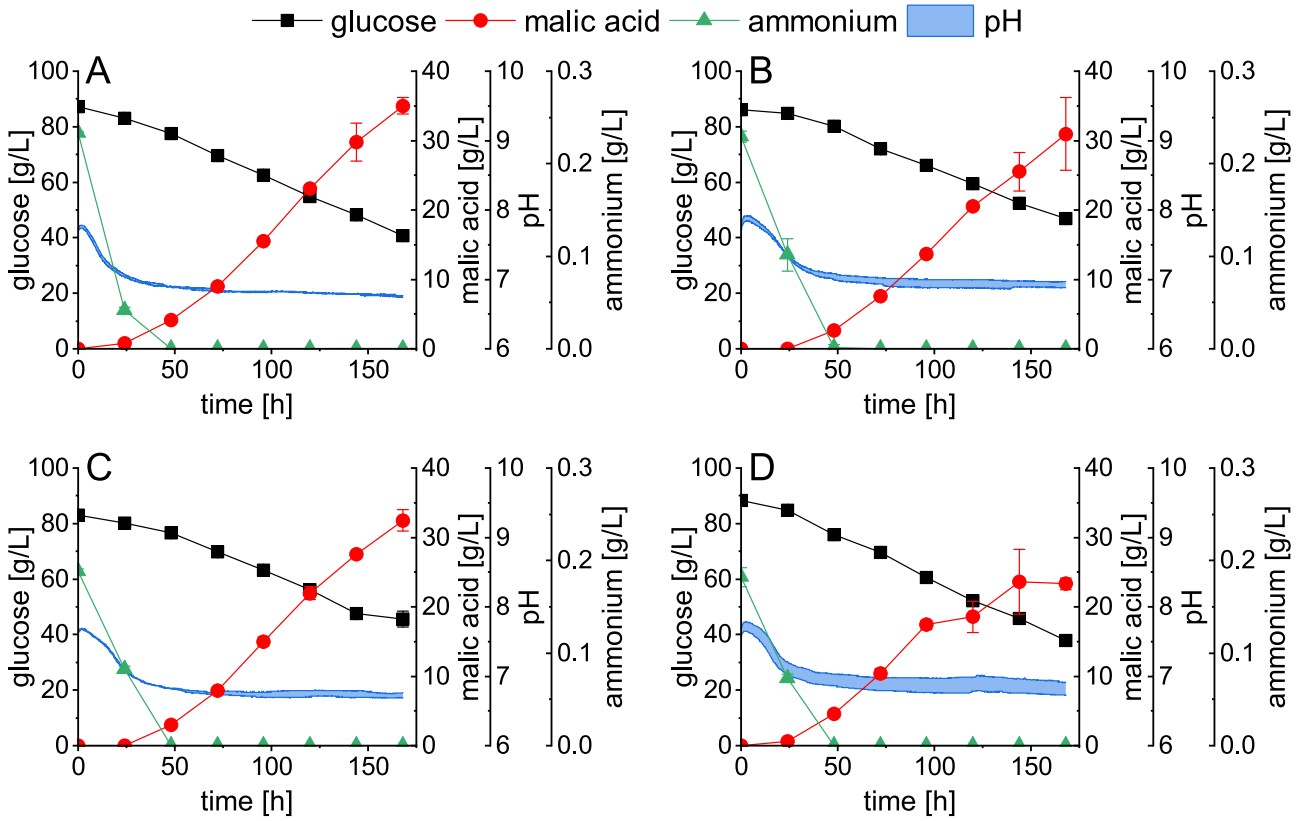

Figure 18. Malic acid production using glucose as carbon source in 2.5-L bioreactors with free biomass (A) and *A. oryzae* immobilized in 1.5% alginate (B), 3% alginate (C), and 1.5% κ-carrageenan (D). Data are the means of duplicate experiments with respective standard deviations.

Table 12. Fermentation results for 2.5-L bioreactor cultivations with free and immobilized *A. oryzae* after 144 h. Data are the means of duplicate experiments with respective standard deviations.

Main sub-strate	Immobilization matrix	Consumed substrate [g/L]	Malic acid [g/L]	$Y_{P/S,\ malic\ acid}$ [g/g]	Productivity [g/L/h]	Total acids [g/L][a]
HAc	Free biomass	HAc: 6.68 ± 4.91 Glc: 4.91 ± 0.54	0.73 ± 0.27	0.06 ± 0.00	0.005 ± 0.002	3.13 ± 0.64
	3% Alginate	HAc: 7.38 ± 0.29 Glc: 4.90 ± 0.03	2.60 ± 0.14	0.21 ± 0.02	0.018 ± 0.001	5.39 ± 0.07
	6% Alginate	HAc: 7.91 ± 0.76 Glc: 5.04 ± 0.02	1.70 ± 0.05	0.13 ± 0.01	0.012 ± 0.000	3.91 ± 0.06
	1.5% κ-Carrageenan	HAc: 6.82 ± 0.55 Glc: 4.67 ± 0.05	1.60 ± 0.09	0.14 ± 0.00	0.011 ± 0.001	4.23 ± 0.19
Glc	Free biomass	39.09 ± 1.65	29.77 ± 2.70	0.76 ± 0.10	0.207 ± 0.019	36.91 ± 3.81
	1.5% Alginate	35.26 ± 1.36	25.23 ± 3.13	0.72 ± 0.12	0.175 ± 0.022	32.12 ± 1.42
	3% Alginate	35.35 ± 2.90	27.62 ± 0.05	0.78 ± 0.06	0.192 ± 0.000	33.66 ± 0.30
	1.5% κ-Carrageenan	42.40 ± 0.99	23.61 ± 4.68	0.56 ± 0.12	0.164 ± 0.033	31.42 ± 5.45

HAc = acetic acid, Glc = glucose, $Y_{P/S,\ malic\ acid}$ = substrate specific malic acid yield calculated as g(malic acid)/g(total consumed substrate(s)).

[a] Refers to all organic acids quantified (see Appendix D, Table D2).

In bioreactor cultivations with acetate as main carbon source, malic acid production with *A. oryzae* entrapped in 3% and 6% alginate as well as 1.5% κ-carrageenan was studied. For all cultivations, including the one with free biomass, the same cultivation conditions were applied as for fermentations with glucose. However, different than with glucose, the malic acid concentration obtained with free biomass in the bioreactor cultivation was much lower compared to that measured in shake flasks (Figure 19). Within 144 h, cultures with free mycelium produced only 0.73 ± 0.27 g/L, which is about 10% of the concentration obtained in shake flasks during the same period (Table 12). Even though substrate was present at concentrations above 30 g/L until the end of the fermentation, the malic acid concentration stagnated after 192 h and did not surpass 2 g/L. All fermentations with immobilized *A. oryzae* performed better. The highest concentration of 2.60 ± 0.14 g/L malic acid was obtained with 3% alginate. Towards the end of the fermentation, this value further increased to a concentration of more than 4 g/L. However, this is still considerably lower than the titer determined in shake flask cultivations. In the bioreactor fermentations with acetate, glucose was added at a concentration of 5 g/L to shorten the lag phase. The additional carbon source was depleted within 48 h (free biomass) or 72 h (cultures with immobilized *A. oryzae*). The consumption of the main carbon source acetate within 144 h was below 10 g/L for all fermentations. Ammonium was depleted after 96 h in the cultivations with free biomass and after 120 h for the fermentations with immobilized *A. oryzae*. In terms of matrix stability, similar observations were made as for the shake flask cultivations, as disintegration of the alginate beads and abrasion of the

biomass from the κ-carrageenan particles were observed. However, in the bioreactors, also the 6% alginate beads dissolved starting between 144 h and 168 h, which could explain the slightly higher malic acid concentration compared to the shake flask cultures at the 144-h sampling point. Compared to shake flask experiments, the percentage of pyruvic acid was increased, especially for the cultivation with free biomass for which 37.0 ± 10.5% of the 3.13 ± 0.64 g/L total acids was attributed to this product (Appendix D, Table D2).

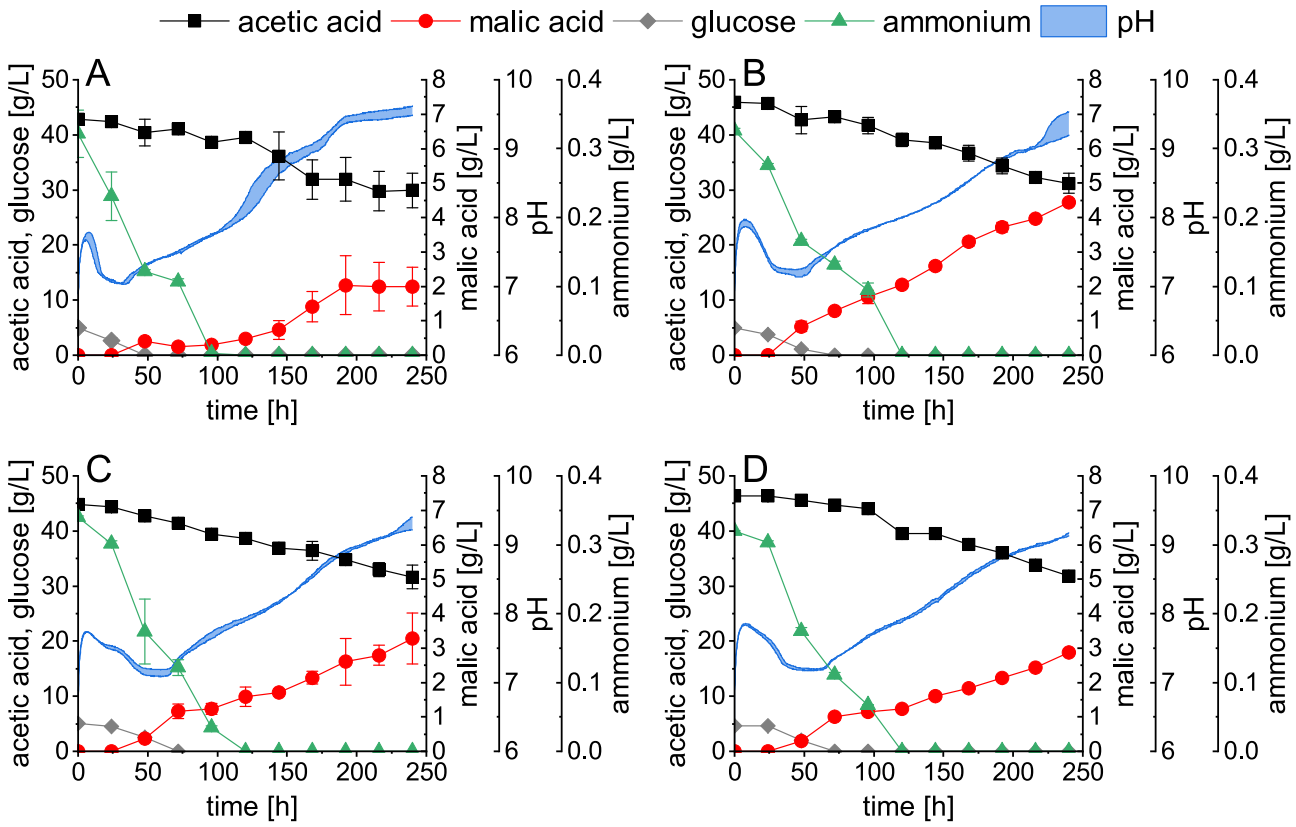

Figure 19. Malic acid production using acetate as carbon source in 2.5-L bioreactors with free biomass (A) and *A. oryzae* immobilized in 3% alginate (B), 6% alginate (C), and 1.5% κ-carrageenan (D). Data are the means of duplicate experiments with respective standard deviations.

4.3.3. Shake flask cultivations without carbon source in the preculture medium

Due to the challenges described for the cultivations in acetate medium, possibilities to improve the growth characteristics were evaluated. Albeit the biomass was mainly located on the surface of the beads or cuboids in the cultivations described in the previous chapters before biomass detachment occurred, growth of free mycelium was also observed. To confine the growth of *A. oryzae* to the immobilization matrix and reduce extensive outgrowth, the carbon source was omitted in the growth medium. The growth behavior was evaluated with *A. oryzae* immobilized in 3% and 6% alginate, 1.5% κ-carrageenan, and 1.5% agar, and several differences were observed. Regarding the alginate beads, *A. oryzae* grew throughout the matrix when the carbon source was absent instead of growing mainly on the bead's surface while growth for the other two polymers was still mainly located near or on the surface of the cuboids (Figure 20).

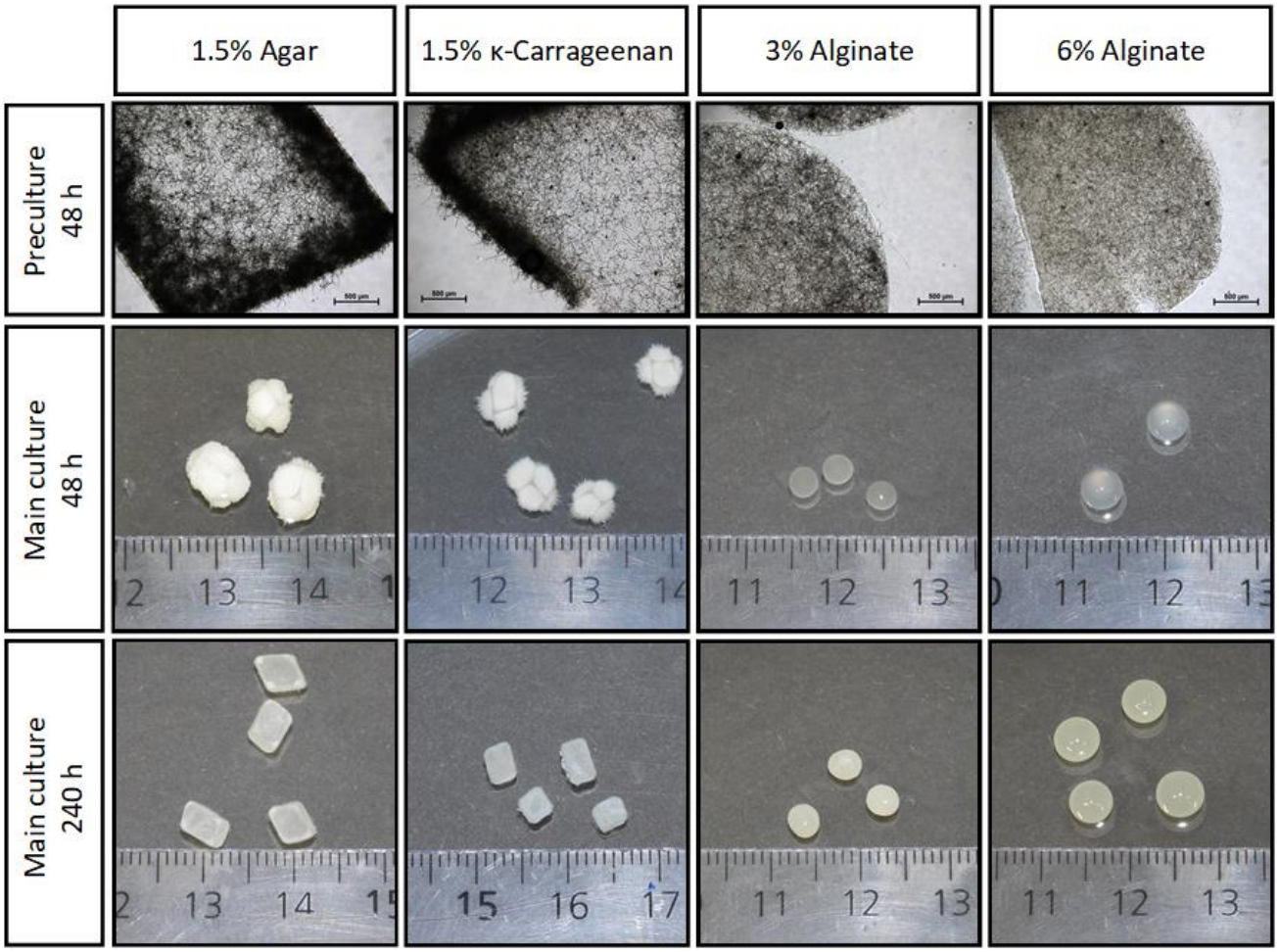

Figure 20. *A. oryzae* immobilized in different matrices in the pre- and main culture. The preculture was grown without carbon source in the medium while the main culture medium contained acetate.

Subsequently, malic acid production was evaluated with these matrices in shake flasks using main culture medium containing acetate as sole carbon source. With *A. oryzae* immobilized in alginate, malic acid production was delayed compared to that of the cultures grown in a preculture medium containing acetate, and much lower concentrations were obtained (Figure 21). For the 6% alginate beads no malic acid was detected after 144 h of cultivation (Table 13). Still, 1.06 ± 0.13 g/L total acids were obtained of which 99% was oxalic acid (Appendix D, Table D3). For cultures with agar and κ-carrageenan matrices, slightly higher concentrations were measured than after a preculture with acetate. As for the 1.5% agar cuboids, 6.14 ± 0.57 g/L malic acid compared to 5.53 ± 0.52 g/L were observed and with κ-carrageenan 5.59 ± 0.72 g/L compared to 4.72 ± 0.39 g/L, while the yields remained similar. Ammonium was depleted after 96 h in the cultures with agar and κ-carrageenan and was depleted or very close to depletion after 144 h for the alginate beads (Appendix D, Figure D3). In the first 48 h of the acid production phase, the agar and κ-carrageenan particles developed a biomass layer on the surface of the beads, which was comparable to that of the particles grown with a carbon source in the preculture, whereas the alginate beads did not (Figure 20). Between 48 and 96 h, the alginate beads prepared with 3% of the polymer formed a thin, filamentous

biomass layer around the particle's surface. The formation of a biomass layer on the surface of the immobilization material seems necessary for an effective malic acid production. While showing a markedly reduced malic acid productivity, the infiltration of the alginate beads by *A. oryzae* resulted in their stabilization in acetate medium. The 3% alginate beads did not dissolve during the fermentation since they were probably stabilized by the fungal filaments. As observed for the cultivations with matrices grown in the presence of acetate, the biomass layer formed in the main culture was detached in later stages of the fermentation (Figure 20).

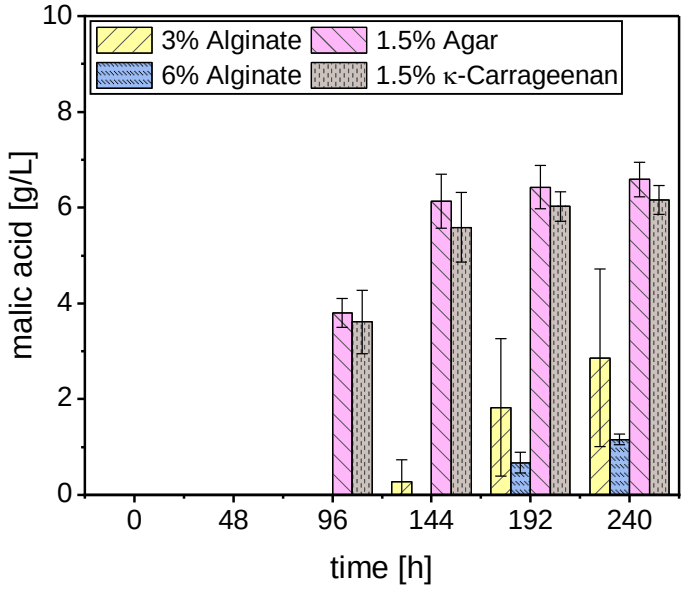

Figure 21. Malic acid production with immobilized *A. oryzae* grown in a preculture medium without carbon source. Acetate was used as carbon source for acid production in the main culture. Data are the means of triplicate experiments with respective standard deviations.

Table 13. Fermentation results for shake flask cultivations with immobilized *A. oryzae* after 144 h using acetate as carbon source in the main culture, while the preculture was grown without carbon source in the medium. Data are the means of triplicate experiments with respective standard deviations.

Immobilization matrix	**Consumed substrate [g/L]**	**Malic acid [g/L]**	**$Y_{P/S,\ malic\ acid}$ [g/g]**	**Productivity [g/L/h]**	**Total acids [g/L][a]**
3% Alginate	15.46 ± 1.25	0.27 ± 0.47	0.02 ± 0.03	0.002 ± 0.003	1.33 ± 0.59
6% Alginate	15.37 ± 0.88	0.00 ± 0.00	0.00 ± 0.00	0.000 ± 0.000	1.06 ± 0.13
1.5% Agar	34.28 ± 1.67	6.14 ± 0.57	0.18 ± 0.01	0.043 ± 0.004	10.85 ± 0.94
1.5% κ-Carra-geenan	32.04 ± 1.04	5.59 ± 0.72	0.17 ± 0.02	0.039 ± 0.005	10.51 ± 0.92

$Y_{P/S,\ malic\ acid}$ = substrate specific malic acid yield calculated as g(malic acid)/g(consumed acetic acid).

[a] Refers to all organic acids quantified (see Appendix D, Table D3).

4.4. Discussion

In this chapter of the dissertation, malic acid production was evaluated with *A. oryzae* entrapped in different concentrations of the natural polymers alginate, agar, and κ-carrageenan in shake flask and bioreactor cultivations.

With alginate in shake flask cultivations, the utilization of higher polymer concentrations resulted in decreased productivities with both acetate and glucose as shown in Figure 14 and Table 11 due to enhanced diffusion limitations. Decreased activity with increasing alginate concentration was also observed by other authors (Idris and Suzana, 2006; Jin et al., 2016; Li et al., 2015). These diffusion limitations affected the biomass growth and the subsequent production of malic acid especially for cultures with acetate. Growth on the surface of the alginate beads was reduced with higher concentrations of the immobilization matrix especially in acetate medium (Figure 16), resulting in a lower amount of active biomass which was associated with a reduced production of malic acid. Furthermore, the bead diameter was influenced by the alginate content, featuring slightly smaller diameters with lower polymer concentrations. This was particularly observed for the beads prepared with 1.5% alginate, whose diameter at the beginning of fermentation was about 1 mm smaller than that of the other alginate concentrations. In immobilized structures, the mass transfer towards the center of the particles is restricted which creates a region that can be considered biologically inactive (Galaction et al., 2012). The smaller bead diameter and lower diffusion limitations were probably the reasons for which cultures with 1.5% alginate beads featured the highest malic acid production in shake flask experiments (Figure 14, Table 11). However, the utilization of a low alginate concentration was associated with lower mechanical stability. Even though the 1.5% alginate beads were stable until the end of the fermentation in shake flasks, they were soft and easily deformable in a bioreactor environment. Therefore, 3% alginate beads were better suited for cultivation in the stirred tank reactor with glucose medium. Adjusting the alginate concentration offers the possibility of optimizing the characteristics of the beads, depending on the requirements of the microorganism and the process. Often, alginate concentrations of 2-3% seem to be a good balance between production performance and mechanical strength (Chen et al., 2012; Jin et al., 2016; Li et al., 2015), which is consistent with the results presented here.

Whereas in glucose medium all alginate concentrations were stable, this was not the case for the cultivations using acetate. Beads with lower alginate concentrations seemed to be more favorable for malic acid production in shake flasks but the stability of the beads was poor. Only the beads prepared with 5% and 6% alginate remained intact in shake flask cultivations, although showing a softer consistency. Alginate is a linear copolymer consisting of D-mannuronate and L-guluronate which forms hydrogels by cross-linking the chains with divalent cations such as Ca^{2+} (Lee and Mooney, 2012). These divalent ions can be replaced by monovalent ions which gradually weakens the hydrogel by reducing the cross-linking, leading to swelling and disintegration (Bajpai and Sharma, 2004). As the acetate medium was rich in sodium ions due to the adjustment of the pH with sodium hydroxide, this explains the dissolution of the alginate beads while they were stable in glucose

medium. Similar observations were reported with medium containing potassium acetate (Eikmeier et al., 1984). In 2.5-L bioreactor cultivations even the beads prepared with 6% alginate disintegrated at around 168 h of fermentation due to increased mechanical stress. The stability of alginate gels is affected by the polymer concentration, the molarity of the curing solution, the type of cross-linking ion and the curing time and temperature. Further stabilization of the matrix can be achieved by treatment with the cross-linker glutaraldehyde, which was reported to reduce the cell leakage of alginate-immobilized *E. coli* (Zhang et al., 2010). By preparing a mixed polymer gel composed of alginate and polyvinyl alcohol the mechanical stability of alginate beads could be increased considerably (Wei et al., 2018). However, in both reported applications the increased stability was accompanied by a decrease in relative activity. A denser matrix created by a higher alginate concentration, as presented in this work, was also associated with lower productivity. Coating of the beads might improve their stability without altering the properties of the alginate core. By coating alginate beads with a chitosan layer, the surface properties of the beads could be improved while maintaining similar catalytic activity with optimized coating conditions, resulting in an increased number of reuse cycles (Chen et al., 2012). Chitosan coating furthermore resulted in reduced swelling of the alginate beads by improving the surface characteristics (Pasparakis and Bouropoulos, 2006). Other authors, however, did not find an improved stability of alginate core beads with a chitosan layer and disintegration was still observed (Voo et al., 2011).

Regarding the immobilization in agar and κ-carrageenan, no disintegration of the matrices was found as these polysaccharides feature different gelling characteristics. Agar is composed of agaropectin and agarose, a linear polysaccharide consisting of D-galactose and 3,6-anhydro-L-galactose, which is mainly responsible for the gelling properties by forming a 3D structure of aggregated helixes upon cooling (Nordqvist and Vilgis, 2011). κ-Carrageenan is structurally similar to agarose consisting of D-galactose and 3,6-anhydro-D-galactose units, with the difference of the D-galactose residue being sulfated and the temperature-dependent gelation is assumed to be the same as for agarose, transitioning from a random coil structure at high temperatures to an ordered helical structure upon cooling in the presence of cations such as potassium, sodium, and calcium (Rhein-Knudsen et al., 2015). The cations shield the sulfate groups and reduce repulsion forces. The gelation temperature of κ-carrageenan solutions is therefore dependent on the cation concentration (Şen and Erboz, 2010). For this reason, the 0.75% κ-carrageenan solution was in a sol state at room temperature and beads were prepared by dripping the suspension in a KCl solution. The 1.5% κ-carrageenan solution, however, was in a gel state at ambient temperature and a film was prepared which was cut into cuboids after cooling. Due to the additional ions, beads prepared with 0.75% κ-carrageenan probably featured a higher gel strength than the 1.5% κ-carrageenan cuboids which were not further solidified by ion treatment. This likely resulted in the reduced malic acid production due to increased diffusion limitations even though the polymer concentration was lower (Figure 14, Table 11).

The diffusivity in the matrices was probably further influenced by the charged groups of the polymers. In acetate medium, the utilization of *A. oryzae* immobilized in 3% agar resulted in considerably reduced malic acid titers compared to the 1.5% agar cuboids (Figure 14, Table 11). With glucose, by contrast, no difference in malic acid production between *A. oryzae* immobilized in agar and κ-carrageenan was noticed, and titers were similar to the control with free biomass. Potentially, the diffusion of glucose, an uncharged monosaccharide, in the polymer matrices is easier than the diffusion of the negatively charged acetate anion. κ-Carrageenan and agar, particularly the agaropectin portion, feature sulfate groups and the alginate chains carboxyl groups. Especially in alginate beads, the diffusivity could be further hindered by the high pH values reached during cultivation in acetate, enforcing deprotonation. This possibly contributed to the larger difference in malic acid production between the alginate concentrations compared to fermentations with glucose (Figure 14, Table 11). Furthermore, it could explain the increased proportion of oxalic acid accompanied by a higher polymer concentration or enhanced growth within the matrix as observed for the alginate beads grown without carbon source (Appendix D, Table D1). The increased mass transfer limitations probably resulted in an undersupply of nutrients which likely caused a change in the metabolism. In Chapter 2, an increased oxalic acid production was associated with a low initial acetate concentration in the medium. Oxalic acid can potentially be produced from oxaloacetate by oxaloacetate hydrolase as demonstrated for *A. niger* (Han et al., 2007; Kubicek et al., 1988). Another difference in the side product spectrum between the carbon sources was the increased succinic acid and decreased malic acid percentage observed with acetate (Appendix D, Tables D1 and D2). This is likely due to the glyoxylate cycle being the main pathway for malic acid production with acetate as opposed to the reductive TCA cycle with glucose as discussed in Chapter 2.

While the agar and κ-carrageenan matrices were stable in acetate medium, biomass detachment was observed in later stages of fermentation (Figure 17, Figure 20). A possible explanation for this observation is fragmentation of the hyphae due to mechanical stress (Casas López et al., 2005; Nielsen and Krabben, 1995). As biomass loss was observed later in the fermentation, it was possibly associated with physiological changes caused by aging or nutrient limitation such as increased vacuolation which is a characteristic feature of the basal, older region of the hyphae (Ohneda et al., 2002; Shoji et al., 2006). Potentially, increased vacuolation weakened the older or nutrient-deprived hyphae, making them susceptible to fragmentation which contributed to the observed biomass abrasion (Papagianni, Mattey, Kristiansen, 1999a; Paul et al., 1994). However, the involvement of vacuolation needs to be confirmed in further experiments. With glucose, complete biomass abrasion was not observed but the particles transitioned from a fluffy to a dense and smooth biomass layer, which was maintained until the end of the cultivation as pictured in Figure 15. Fragmentation of the long filaments also happened but a major part of the biomass was located around the surface of the particles. Probably, the osmotic stress *A. oryzae* experiences in acetate medium contributed to these differences. Overall, malic acid production with immobilized *A. oryzae* was more successful in glucose media, as the immobilization matrices were stable and malic acid production was similar to

the control except for the cultures with high alginate concentrations. This suggests their suitability for prolonged use in repeated-batch or continuous processes which needs to be verified in further experiments.

Although challenges regarding the stability of the immobilization matrices were observed in acetate media, all bioreactor fermentations with immobilized *A. oryzae* showed improved malic acid production compared to the fermentation with free biomass (Figure 19, Table 12). Compared to that of shake flask cultivations, the bioreactor fermentation with free biomass resulted in considerably reduced malic acid titers. For the fermentations with acetate the same cultivation conditions were chosen as for cultivations with glucose. While the scale-up worked well for the saccharide (Figure 18), the conditions were not suited for the process using acetate. Interestingly, the pyruvate proportion was increased in all bioreactor fermentations with acetate and particularly high with free mycelium (Appendix D, Table D2). It is unlikely that this is related to the addition of glucose as an additional substrate, since no increased pyruvic acid production was observed in shake flask cultures with 45 g/L acetate and 5 g/L glucose (see Chapter 2). The accumulation of pyruvic acid might be associated with gas transfer limitations. *A. oryzae* presumably experiences oxygen limitation, reducing its ability to replenish redox equivalents required for the TCA cycle. Therefore, the process needs to be optimized, probably with regard to the stirrer velocity and aeration rate. During the fermentation, attachment of the filamentous biomass to the bioreactor installations such as baffles and the Rushton turbines was observed. The improved performance of fermentations with entrapped *A. oryzae* can be explained by the fact that the immobilization in a defined matrix ensured a proper dispersion of the biomass in the bioreactor as extensive attachment was not observed. However, increased shear forces in the bioreactor setting caused an earlier disintegration of the alginate matrices and biomass detachment of the κ-carrageenan cuboids. Testing the κ-carrageenan and agar cuboids for the possibility of catalyst recycling and regrowth of the biomass layer could still be interesting. This would enable a repeated-batch or continuous process management, which is challenging with dispersed filaments due to difficulties in biomass retention. Possibly, performing surface immobilization on a synthetic material instead of polymer entrapment might be better suited for the fermentation in acetate media. Materials described for surface immobilization of *Aspergillus* species include macro porous polymeric sponge (Arikan et al., 2019), polyurethane foam (Kautola et al., 1991), nylon mesh (Papagianni and Mattey, 2004), or celite beads (Papagianni et al., 2002).

The possibility of limiting biomass formation to the beads was investigated by performing experiments without carbon source in the preculture medium, and growth was observed with all matrices (Figure 20). This could be explained by either the consumption of the polymer or the glycerol in which the spores were suspended. Each 20 mL of the immobilization matrix, i.e., the amount for one triplicate, was prepared with 4 mL spore suspension consisting of 50% glycerol. The cultivation without an external carbon source was efficient in limiting the amount of free mycelium to a minimum. Interestingly, growth throughout the entire matrix was only observed with alginate. This might be explained by an enhanced diffusivity of nutrients and gas in this material. Growth

throughout the matrix was also reported for *A. niger* immobilized in alginate upon nitrogen limitation to a value of 0.05 g/L NH_4NO_3, whereas with a slightly higher concentration of 0.2 g/L NH_4NO_3, the formation of a biomass layer near the surface of the beads was observed (Eikmeier et al., 1984). The citric acid concentration obtained with *A. niger* grown with 0.05 g/L NH_4NO_3, however, was considerably lower than with 0.2 g/L NH_4NO_3 (Eikmeier and Rehm, 1984). This is in accordance with the results presented in this work as the infiltration of the alginate beads was associated with considerably reduced malic acid productivities (Figure 21, Table 13). Determining the optimum nutrient concentrations especially regarding the nitrogen content in both pre- and main culture can maximize acid production and limit the growth of free mycelium (Bayraktar and Mehmetoglu, 2000; Lee et al., 1989). The occurrence of free biomass during the cultivation of entrapped filamentous fungi probably cannot be completely prevented and some outgrowth appears to be necessary for efficient organic acid production.

4.5. Conclusions

This chapter investigated the production of L-malic acid with *A. oryzae* immobilized by entrapment in different natural polymers. Large differences were observed regarding the performance of the immobilization matrices with the two tested carbon sources acetate and glucose. Cultivation of immobilized *A. oryzae* in acetate medium was accompanied by several challenges, due to the low stability of the alginate beads and the abrasion of the biomass from the surface of the agar and the κ-carrageenan matrices. Optimization regarding the medium composition and growth conditions can potentially improve the growth characteristics and malic acid production. However, immobilization was able to improve malic acid production with acetate in bioreactor cultivations by ensuring a thorough distribution of the biomass. Generally, further research is required regarding the optimization of malic acid production with acetate in a bioreactor setting. In contrast, all matrices were stable in glucose medium, and productivities of cultures with entrapped *A. oryzae* were similar to that of the control with free biomass, except for high alginate concentrations. In further experiments, the reusability and long-term production potential in repeated-batch or continuous processes should be evaluated.

5. Optimization of L-malic acid production from acetate using a pH-coupled feeding strategy

This chapter is based on the publication:

Optimization of L-malic acid production from acetate with *Aspergillus oryzae* DSM 1863 using a pH-coupled feeding strategy
Aline Kövilein, Vera Aschmann, Lena Zadravec, Katrin Ochsenreither (2022)
Microbial Cell Factories 21, 242
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Author contributions

Aline Kövilein: conceptualization, investigation, data analysis, writing of first draft, manuscript revision.

Vera Aschmann: investigation, data analysis.

Lena Zadravec: investigation, data analysis.

Katrin Ochsenreither: conceptual advice, project supervision, manuscript revision.

5.1. Introduction

As described in Chapter 3, a fed-batch process with repeated feeds of acetic acid was suitable to increase the maximum malic acid titer and prolong the production period in shake flask cultivations. However, the production rate declined over time. One factor which contributed to this observation was likely the low medium pH of 5.5-6.0 after addition of the acid. Therefore, this chapter evaluates several strategies for malic acid production with a pH-coupled feed of acetic acid in 2.5-L bioreactor fermentations. In this way, a constant pH can be maintained while substrate is supplied to prevent depletion of the carbon source. Furthermore, this chapter investigates the influence of initial product concentrations of up to 50 g/L malate and succinate to evaluate a potential product inhibition.

5.2. Material and methods

5.2.1. Microorganism and media

Aspergillus oryzae DSM 1863 was obtained from DSMZ strain collection (German Collection of Microorganisms and Cell Cultures GmbH) and spore propagation was performed as described in Chapter 2.2.2.

Organic acid production was carried out as a two-stage process consisting of a preculture and a main culture. The preculture medium consisted of 45 g/L acetic acid, 4 g/L $(NH_4)_2SO_4$, 0.75 g/L KH_2PO_4, 0.98 g/L K_2HPO_4, 0.1 g/L $MgSO_4 \cdot 7H_2O$, 0.1 g/L $CaCl_2 \cdot 2H_2O$, 5 mg/L NaCl, 5 mg/L $FeSO_4 \cdot 7H_2O$ and 2 mL/L 1000x Hutner's Trace Element solution. 1000x Hutner's trace element solution consists of 5 g/L $FeSO_4 \cdot 7H_2O$, 50 g/L EDTA-Na_2, 22 g/L $ZnSO_4 \cdot 7H_2O$, 11 g/L H_3BO_3, 5 g/L $MnCl_2 \cdot 4H_2O$, 1.6 g/L $CoCl_2 \cdot 6H_2O$, 1.6 g/L $CuSO_4 \cdot 5H_2O$, and 1.1 g/L $(NH_4)_6Mo_7O_{24} \cdot 4H_2O$ with a pH of 6.5 (Hill and Kafer, 2001). The pH of the preculture medium was set to 6.5 with NaOH.

The main culture medium for organic acid production in 2.5-L bioreactors was composed of 45 g/L acetic acid, 1.2 g/L $(NH_4)_2SO_4$, 0.1 g/L KH_2PO_4, 0.17 g/L K_2HPO_4, 0.1 g/L $MgSO_4 \cdot 7H_2O$, 0.1 g/L $CaCl_2 \cdot 2H_2O$, 5 mg/L NaCl, 60 mg/L $FeSO_4 \cdot 7H_2O$, and 50 or 90 g/L $CaCO_3$. Furthermore, 5.0 g/L glucose were added to the production medium in the bioreactor fermentations in form of a stock solution (c = 400 g/L) sterilely after autoclaving to shorten the lag phase. The main culture media for evaluating the influence of different product concentrations on acid production in shake flask cultivations had the same composition. The only differences were that no glucose was added to these media, that 90 g/L $CaCO_3$ was used for all tested conditions, and that malic and succinic acid in concentrations of 1-50 g/L were added as indicated in the results section. The pH of the main culture media was adjusted to a value of 5.5 with NaOH. All media were sterilized by autoclaving for 20 min at 121 °C.

5.2.2. Preculture conditions

In 500-mL baffled shake flasks, 100 mL of the preculture medium were inoculated with 3 x 10^7 conidia and incubated at 30 °C and 100 rpm. After 48 h, the biomass was separated from the medium by filtration through Miracloth (Merck KGaA, Darmstadt, Germany), washed thoroughly with distilled water and resuspended in main culture medium. For the bioreactor cultivations, the biomass of four precultures was resuspended in 150 mL of main culture medium which was used for the inoculation of one bioreactor. For the shake flask cultivations, the biomass of two precultures was resuspended in 200 mL of the respective main culture medium and 10 mL were used for the inoculation of one shake flask.

5.2.3. Organic acid production in bioreactors

Organic acid production was performed in 2.5-L stirred tank reactors (Minifors, Infors HT, Bottmingen, Switzerland) with a working volume of 1.4 L. 0.3 mL of the antifoaming agent Contraspum A 4050 HAc (Zschimmer und Schwarz GmbH und Co KG, Lahnstein, Germany) was added before inoculation and when necessary during the fermentation. The agitator consisted of two 6-bladed Rushton turbine impellers with a diameter of 4.6 cm and a blade width of 1.1 cm. The distance between the two impellers was 8.0 cm. The cultivations were carried out at 32 °C, 600 rpm and an aeration rate of 0.5 vvm. In Table 14 an overview is given of the different cultivation conditions. Fermentations were run either without pH adjustment (fermentations I and II) or with an automatic pH control using 10 M acetic acid (fermentations III-VII). The pH control was activated when the pH value reached its minimum after an initial peak, which was approximately after 24 h of cultivation. The target pH was set to this minimum value measured online which was between pH 7.1-7.2. For the pH-adjustment to pH 3.0 and 4.5 of the 10 M acetic acid used in fermentations IV and V solid NaOH was used. In fermentation VII, besides the pH-coupled feed of 10 M acetic acid, manual feeds of 5-10 mL of 5 M NaOH were performed after each sampling between 48 h and 216 h to increase the amount of acetic acid required for pH control. The volume of the NaOH feed for each bioreactor was determined after HPLC measurement of the acetic acid concentration in the fermentation broth. The 5 M NaOH was added over a period of several minutes to prevent the pH from increasing to potentially inhibiting values. Additionally, 42 g of $CaCO_3$ (one third of the initial amount) was added after the sampling at 168 h during fermentation VII to prevent carbonate depletion. Cultivations were run in duplicate except for fermentation VII for which a triplicate was performed. The pH value was measured online. Samples were taken at the indicated time points for offline analysis of ammonium, glucose and organic acid concentration.

Table 14. Summary of cultivation conditions evaluated in 2.5-L bioreactors with a working volume of 1.4 L. Stirrer velocity was 600 rpm, aeration 0.5 vvm and temperature 32 °C for all fermentations.

Fermentation No.	$CaCO_3$ [g/L]	pH control
I	90	-
II	50	-
III	50	10 M acetic acid
IV	50	10 M acetic acid (pH 3.0)
V	50	10 M acetic acid (pH 4.5)
VI	90	10 M acetic acid
VII	90 + 42 g after 168 h	10 M acetic acid, 5 M NaOH

5.2.4. Organic acid production in shake flasks

In 500-mL baffled shake flasks, 100 mL of the main culture medium were inoculated with 10 mL resuspended preculture and incubated at 32 °C and 120 rpm for 240 h. Samples were taken at the indicated time points for the determination of pH as well as organic acid and ammonium concentration. All experiments were performed as triplicates.

5.2.5. Analytics

Organic acids and glucose were quantified by HPLC (Agilent 1100 Series) using a Rezex ROA organic acid H+ (8%) column (300 x 7.8 mm, 8 µm, Phenomenex) with a UV detector at 220 nm for organic acid and an RI detector for glucose determination. Sample preparation and conditions for analysis were the same as described in Chapter 2.2.5.

Ammonium was measured photometrically (Spectroquant Test Kit, 114752, Merck KGaA). The reaction volume of the assay was scaled down to 200 µL and samples were measured in duplicate in microtiter plates according to the instructions of the manufacturer.

5.3. Results

5.3.1. L-Malic acid production in 2.5-L bioreactor batch cultivations

At first, malic acid production with *A. oryzae* was evaluated in 2.5-L bioreactor batch fermentations. The detailed conditions for each fermentation are listed in Table 14 in the methods section.

In Chapter 4 of this dissertation a low malic acid production was reported with acetate as carbon source in 2.5-L bioreactor fermentations when using the same cultivation conditions as for glucose. While the malic acid titer with the saccharide in bioreactor fermentations was comparable to shake flask experiments, this was not the case using acetate. In these fermentations, a stirrer velocity of 300 rpm was used and only 0.73 ± 0.27 g/L malic acid was obtained within 144 h, representing less than 10% of the concentration obtained in shake flasks (Chapter 4.3.2). Increasing the stirrer velocity

to 600 rpm (Figure 22, I) considerably improved the fermentation outcome. Within 144 h, *A. oryzae* produced 5.62 ± 0.30 g/L malic acid, corresponding to a yield and productivity of 0.16 ± 0.01 g/g and 0.039 ± 0.002 g/L/h, respectively (Table 15). A distinct difference observed between the two stirrer velocities was the morphology of *A. oryzae*. During the bioreactor cultivation at 300 rpm, the filamentous-lumpy morphology obtained during growth in the seed culture was maintained and the biomass tended to attach to the bioreactor installations, limiting gas and nutrient transfer. By increasing the agitation intensity to 600 rpm, spherical to elongated biomass pellets of about 1-2 mm in diameter were formed, enabling a better distribution of the biomass. The stirrer velocity is thus an important parameter for managing the morphology in acetate media and for ensuring a successful scale-up.

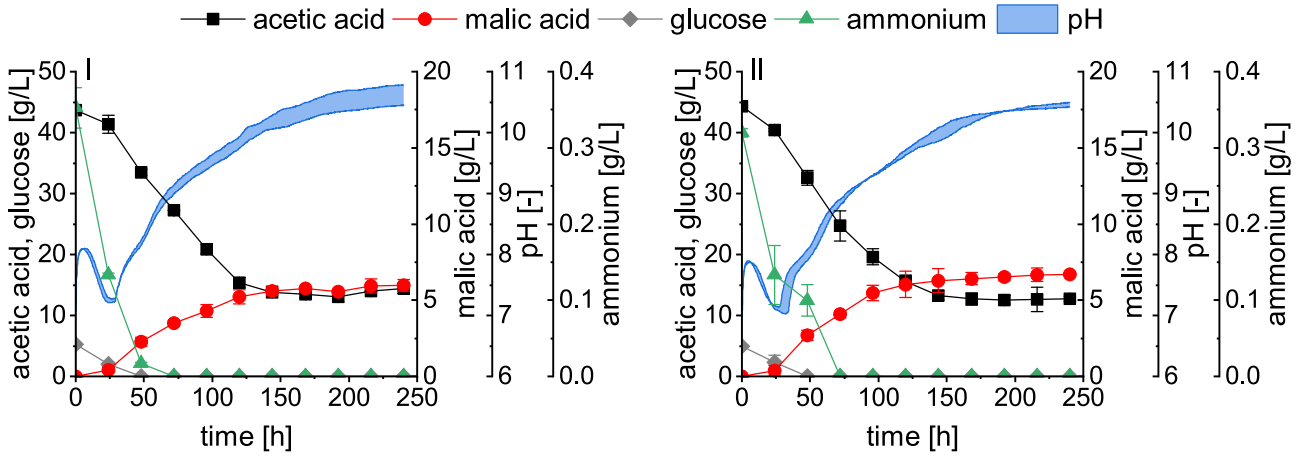

Figure 22. Bioreactor batch cultivations I (90 g/L $CaCO_3$) and II (50 g/L $CaCO_3$) without pH control. Data are the means of duplicate experiments with respective standard deviations.

Between 120 and 144 h the substrate consumption and malic acid production in fermentation I slowed down and ceased after 144 h. At this time point, the pH had exceeded a value of 10. This high pH seems to completely inhibit the metabolic activity of *A. oryzae*. During organic acid production with *A. oryzae* on glucose, $CaCO_3$ is added as buffering agent which maintains the pH in a stable range between 6.5-7.0 without the further addition of an acid or base (see Chapter 4.3.2). With glucose, the pH is mainly influenced by the consumption of ammonium and the production of organic acids, both leading to a decrease in pH. Using acetate, the consumption of the carbon source adds a further influencing factor. After a drop in pH during the consumption of ammonium and glucose at the beginning of the fermentation, as shown in Figure 22, the pH remains stable for a short time. This is followed by a phase of pH increase, which is accompanied by the consumption of acetate. When the metabolism is then inhibited due to a high pH, the pH slowly continues to increase, probably due to the loss of CO_2 from the remaining $CaCO_3$ through aeration. Compared to the experiments in shake flasks for which values close to pH 10 were only obtained after 240 h (see for example Appendix E, Figure E1), the pH showed a faster increase in the bioreactor environment, leading to an inhibition. Therefore, a complete substrate consumption was not observed. With about

a third of the substrate remaining unused, lower maximum malic acid concentrations were obtained than in shake flask cultivations.

The presence of $CaCO_3$ was shown to be beneficial for efficient malic acid production in shake flask cultivations as presented in Chapter 2. However, the addition of $CaCO_3$ also increases the pH value. For this reason, a lower $CaCO_3$ concentration of 50 g/L was evaluated (Figure 22, II) as opposed to the 90 g/L used in fermentation I. Even though the pH was slightly lower in the first part of the fermentation, inhibiting values were also reached between 120 and 144 h, resulting in the production of 6.29 ± 0.78 g/L malic acid and a yield of 0.17 ± 0.02 g/g within 144 h (Table 15). Hence, no considerable difference was observed between the two carbonate concentrations, even though malic acid production was correlated with the concentration of $CaCO_3$ in shake flasks. Therefore, the lower concentration of 50 g/L was used for fermentations III-V. In these experiments, a pH-coupled feeding strategy was evaluated.

Table 15. Fermentation results for 2.5-L bioreactor cultivations with *A. oryzae* for L-malic acid production. Data are the means of duplicate (I-VI) or triplicate (VII) experiments with respective standard deviations.

Fermentation No.	Consumed substrate [g][a]	Malic acid [g/L]	$Y_{P/S,\ malic\ acid}$ [g/g][b]	Malic acid productivity [g/L/h]	Total acid [g/L][c]	$Y_{P/S,\ total\ acid}$ [g/g]	Total acid productivity [g/L/h]	Sampling point for calculation [h]
I	50.12 ± 1.29	5.62 ± 0.30	0.16 ± 0.01	0.039 ± 0.002	11.99 ± 1.08	0.33 ± 0.04	0.083 ± 0.007	144
II	51.68 ± 0.74	6.29 ± 0.78	0.17 ± 0.02	0.044 ± 0.005	13.25 ± 1.47	0.35 ± 0.04	0.092 ± 0.010	144
III	104.47 ± 2.50	18.53 ± 0.90	0.26 ± 0.01	0.097 ± 0.005	35.82 ± 1.16	0.50 ± 0.00	0.187 ± 0.006	192
IV	96.62 ± 3.72	14.75 ± 1.20	0.23 ± 0.01	0.077 ± 0.006	28.90 ± 2.50	0.45 ± 0.01	0.151 ± 0.013	192
V	94.52 ± 2.78	16.22 ± 0.71	0.26 ± 0.00	0.084 ± 0.004	31.43 ± 1.02	0.50 ± 0.00	0.164 ± 0.005	192
VI	153.18 ± 6.12	29.53 ± 1.82	0.29 ± 0.01	0.112 ± 0.007	54.20 ± 3.23	0.53 ± 0.01	0.205 ± 0.012	264
VII	139.44 ± 6.60	18.66 ± 0.50	0.20 ± 0.01	0.071 ± 0.002	37.22 ± 2.49	0.40 ± 0.01	0.141 ± 0.009	264

[a] Refers to the sum of substrate metabolized from acetate and glucose. Initially, 45 g/L acetic acid and 5 g/L of glucose were present in the medium.

[b] $Y_{P/S,\ malic\ acid}$ = substrate specific malic acid yield calculated as g(malic acid)/g(consumed substrates).

[c] Refers to all organic acids produced during the cultivation (see Table 16).

Table 16. Organic acid distribution for bioreactor cultivations based on the sampling points summarized in Table 15. Data are the means of duplicate (I-VI) or triplicate (VII) experiments with respective standard deviations.

Fermentation No.	Organic acid distribution [%]						
	Malate	Succinate	Fumarate	Pyruvate	α-Keto-glutarate	Oxalate	Citrate
I	47.0 ± 1.7	39.7 ± 2.4	5.3 ± 0.3	3.4 ± 0.7	0.3 ± 0.0	3.3 ± 0.1	1.1 ± 0.2
II	47.4 ± 0.6	40.7 ± 0.1	5.4 ± 0.1	2.3 ± 0.5	0.3 ± 0.0	3.0 ± 0.1	0.9 ± 0.0
III	51.7 ± 0.8	42.1 ± 0.5	3.8 ± 0.1	1.2 ± 0.2	0.2 ± 0.0	0.1 ± 0.0	1.0 ± 0.0
IV	51.0 ± 0.3	42.5 ± 0.4	4.2 ± 0.4	1.3 ± 0.0	0.1 ± 0.0	0.6 ± 0.2	0.3 ± 0.2
V	51.6 ± 0.6	42.0 ± 0.5	4.3 ± 0.0	1.5 ± 0.1	0.0 ± 0.0	0.6 ± 0.1	0.0 ± 0.0
VI	54.5 ± 0.1	40.2 ± 0.2	3.3 ± 0.2	0.6 ± 0.1	0.2 ± 0.0	0.9 ± 0.2	0.5 ± 0.1
VII	50.2 ± 2.7	43.1 ± 2.8	2.9 ± 0.2	1.6 ± 0.1	0.2 ± 0.0	0.8 ± 0.6	1.1 ± 0.1

5.3.2. L-Malic acid production in 2.5-L bioreactors using a pH-coupled feeding strategy

To prevent the pH from reaching an inhibiting range, acetic acid was used as pH controlling agent which simultaneously provided additional substrate during the fermentation. As displayed for fermentations III-V in Figure 23, the pH control and feeding of acetic acid was started when the pH value reached its minimum, which was approximately after 24 h. During these first 24 h mainly glucose was consumed for growth and only a minor malic acid production was quantified. Glucose was added at a low concentration of 5 g/L to all bioreactor cultivations to reduce the lag phase and was depleted within 48 h in all fermentations.

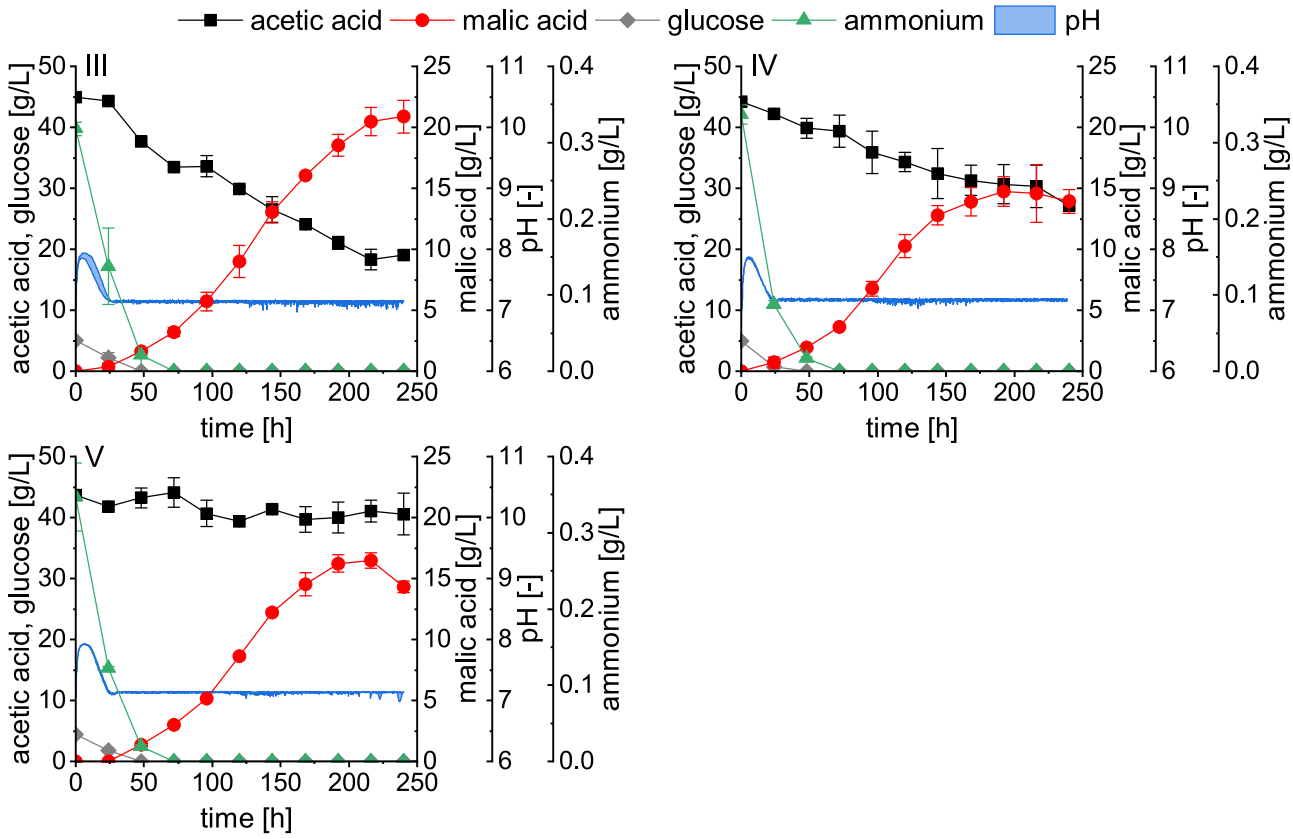

Figure 23. Bioreactor cultivations III-V with a pH-coupled feed and 50 g/L $CaCO_3$. The pH was controlled with 10 M acetic acid (III), 10 M acetic acid pH 3.0 (IV), or 10 M acetic acid pH 4.5 (V). Data are the means of duplicate experiments with respective standard deviations.

In fermentation III (Figure 23), the pH value was controlled with 10 M acetic acid. Compared to the batch fermentations displayed in Figure 22, the period of malic acid production was extended to approximately 216 h. Within this time, 20.47 ± 1.17 g/L malic acid was produced which is more than three times as much as obtained in a batch process without pH control. After 216 h, malic acid production ceased. At this time point, the acetic acid concentration had decreased to a value of 18.34 ± 1.71 g/L. Hence, it was not possible to maintain the substrate concentration stable with this feeding strategy. Chapter 2 demonstrated that low initial acetate concentrations resulted in poor malic acid production and negatively affected the organic acid spectrum produced by *A. oryzae*. The

substrate concentration which is ideal to be maintained over an extended period, however, has not been determined yet. Therefore, it was evaluated whether a constant substrate concentration close to the initial value of 45 g/L acetic acid would result in an increased maximum malic acid concentration and prolong the production time. One possibility to increase the amount of acetate added through the pH-coupled feed is to increase the pH value of the feeding solution. If the titrant is adjusted to a higher pH, a larger volume is required for pH correction, so more acetate is added to the fermentation broth. Therefore, fermentations with 10 M acetic acid set to a pH of 3.0 (Figure 23, IV) or 4.5 (Figure 23, V) as pH regulator were performed. When 10 M acetic acid with a pH of 3.0 was used, the substrate concentration also decreased during fermentation but to a lesser extent than with the unadjusted acid. After 216 h, 30.30 ± 3.50 g/L acetic acid were still available in the medium. In fermentation V, using acetic acid set to a pH of 4.5, the substrate concentration remained almost stable with a concentration of 41.07 ± 1.76 g/L determined after 216 h of fermentation. However, in both fermentations IV and V malic acid production ceased after about 192 h, thus earlier than in fermentation III when unadjusted acetic acid was used. Towards the end of these two fermentations, the concentration of malic acid decreased, indicating a reassimilation of the product. For comparison reasons, the calculations for fermentations III-V summarized in Table 15 are therefore based on the 192-h measurement point. Overall, fermentation III using the unadjusted 10 M acetic acid performed best resulting in a malic acid concentration of 18.53 ± 0.90 g/L, a yield of 0.26 ± 0.01 g/g and a productivity of 0.097 ± 0.005 g/L/h. With acetic acid of pH 3.0, 14.75 ± 1.20 g/L malic acid was quantified and 16.22 ± 0.71 g/L using acetic acid with a pH of 4.5. Hence, maintaining the substrate concentration at a high level did neither increase the maximum malic acid concentration nor the timespan of acid production.

In the later stages of fermentations III and IV a gradual dissolution of the $CaCO_3$ was observed visually until depletion at around 216 h of cultivation. This might have contributed to the cessation of malic acid production despite carbon availability. Therefore, a higher initial concentration of 90 g/L $CaCO_3$ was used again in fermentations VI and VII (Figure 24). In fermentation VI, the pH and substrate concentration were regulated with 10 M acetic acid, whose pH was not adjusted. In fermentation VII, in addition to the 10 M acetic acid used for the pH-coupled feed, 5 M NaOH was manually added after each sampling between 48 h and 216 h. Due to the addition of the base, more acetic acid was required for pH correction, resulting in higher substrate concentrations.

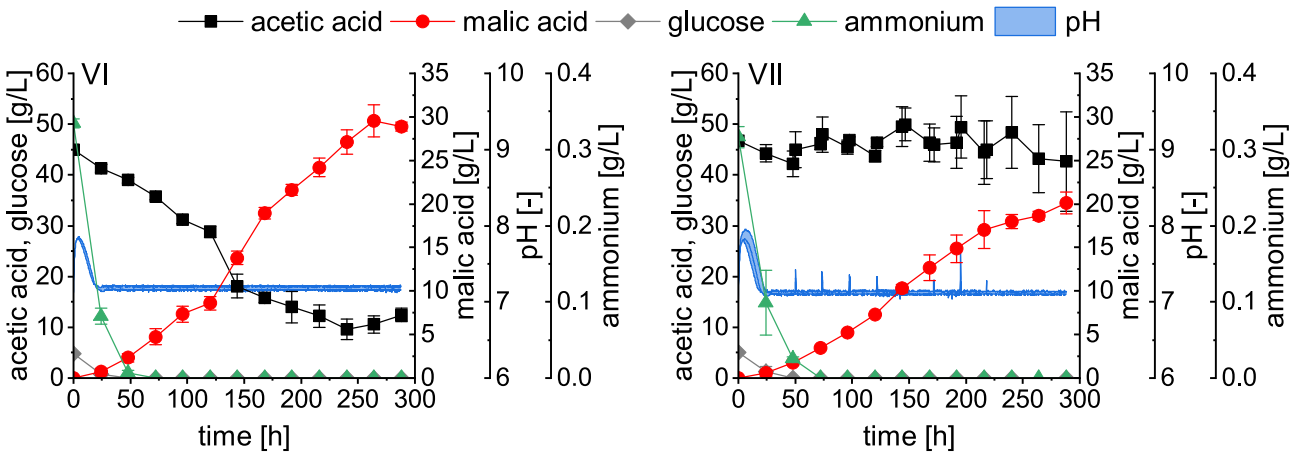

Figure 24. Bioreactor cultivations VI and VII with a pH-coupled feed and 90 g/L $CaCO_3$. The pH was controlled with 10 M acetic acid (VI), or 10 M acetic acid and 5 M NaOH (VII). Data are the means of duplicate (VI) or triplicate (VII) experiments with respective standard deviations.

By increasing the $CaCO_3$ concentration to 90 g/L and employing a pH-coupled feed consisting of 10 M acetic acid (fermentation VI) the highest malic acid concentration of 29.53 ± 1.82 g/L was obtained after 264 h (Table 15). This corresponds to a 5.3-fold increase compared to the batch process without pH control (fermentation I). The malate yield of 0.29 ± 0.01 g/g and productivity of 0.112 ± 0.007 g/L/h were the highest of all processes. Malic acid productivity was maintained until about 264 h which was longer than in the processes presented before. This suggests that a higher carbonate concentration has a positive effect on malic acid production with *A. oryzae* by extending the production time. During the 264 h, the acetic acid concentration decreased to 10.58 ± 1.69 g/L. Therefore, additional feeds of 5 M NaOH were used in fermentation VII. With these feeds, it was possible to maintain the acetic acid concentration above 40 g/L throughout the fermentation. To prevent $CaCO_3$ depletion during this process, a further 42 g $CaCO_3$ was added after the sampling at 168 h. Despite high substrate concentrations and additional supply of $CaCO_3$, malic acid yield and productivity were lower than in fermentation VI. This was comparable to the observations reported for fermentations IV and V. After 264 h, 18.66 ± 0.50 g/L malic acid were quantified, corresponding to a yield of 0.20 ± 0.01 g/g and a productivity of 0.071 ± 0.002 g/L/h.

Although malic acid was the main product of the cultivations presented in this work, *A. oryzae* secreted several other organic acids. With about 40% of the total acid production, succinic acid was the most significant side product, followed by fumarate with about 3-5% (Table 16). The pH-regulated feeding of acetic acid slightly increased the malate percentage and decreased the share of oxalate to below 1% compared to the batch fermentations. In the best performing fermentation VI, a total acid concentration of 54.20 ± 3.23 g/L, corresponding to a yield of 0.53 ± 0.01 g/g, was determined (Table 15). In this fermentation, malate production ceased after 264 h (Figure 24). It is possible that product concentrations in this range inhibit further organic acid production of *A. oryzae*. The effect of initially added malate and succinate on the organic acid production was therefore evaluated in the following chapter.

5.3.3. Organic acid production in the presence of malate and succinate

High product concentrations can inhibit further organic acid production. Evaluating the influence of varying product concentrations can thus be interesting regarding the development of process strategies such as a repeated-batch fermentation with partial medium replacement. Therefore, organic acid production of *A. oryzae* was evaluated with medium containing up to 50 g/L malic acid. With about 40%, succinate accounts for a large proportion of the totally produced organic acids for which reason combinations of malate and succinate were also tested. Figure 25 displays the production of malic, succinic and fumaric acid as well as the consumption of acetate.

All tested conditions first showed a decrease in malic acid concentration before an increase was observed. The only exceptions were the control and the lowest tested combination of malic (M) and succinic acid (S), i.e., 1+1 g/L M+S. For the other conditions, the consumption of the initially available malate was between 10-20% before production of the acid started. This is also evident in Figure 26, which shows the malic acid production rates calculated between the individual measurement points. The beginning of malic acid production after passing a minimum concentration was generally observed later with increasing product concentration. With 35 g/L and 50 g/L initially added malic acid, considerable acid production was only detected after 144 h, illustrated by the calculated productivity which was only clearly in the positive range from this measurement point onwards (Figure 26). The highest malic acid production rate was therefore detected later in these cultures. With 50 g/L malic acid, for example, the highest production rate of 0.082 ± 0.008 g/L/h was calculated between 144 and 192 h. The control and the cultures with a lower initial product concentration, on the other side, showed their maximum between 72 and 96 h which was in the range of 0.110-0.112 g/L/h. After 240 h a net production was determined for all tested conditions as all cultures had surpassed the initially added malic acid concentration. However, this net production was lower with a higher initial product concentration. The difference between the final malic acid concentration after 240 h and the concentration at the beginning, as well as the difference between the final and the minimal concentration, is displayed in Table 17. Regarding the difference between the final and the initial malate concentration, a net production of only 2.79 ± 0.75 g/L was determined with 50 g/L malic acid while 7.99 ± 0.42 g/L were measured for the control. When comparing the final minus the minimal concentration, however, a malic acid production of 8.02 ± 0.51 g/L was calculated for the cultures with 50 g/L malic acid which is comparable to the control. This shows that malic acid production is possible despite high product concentrations.

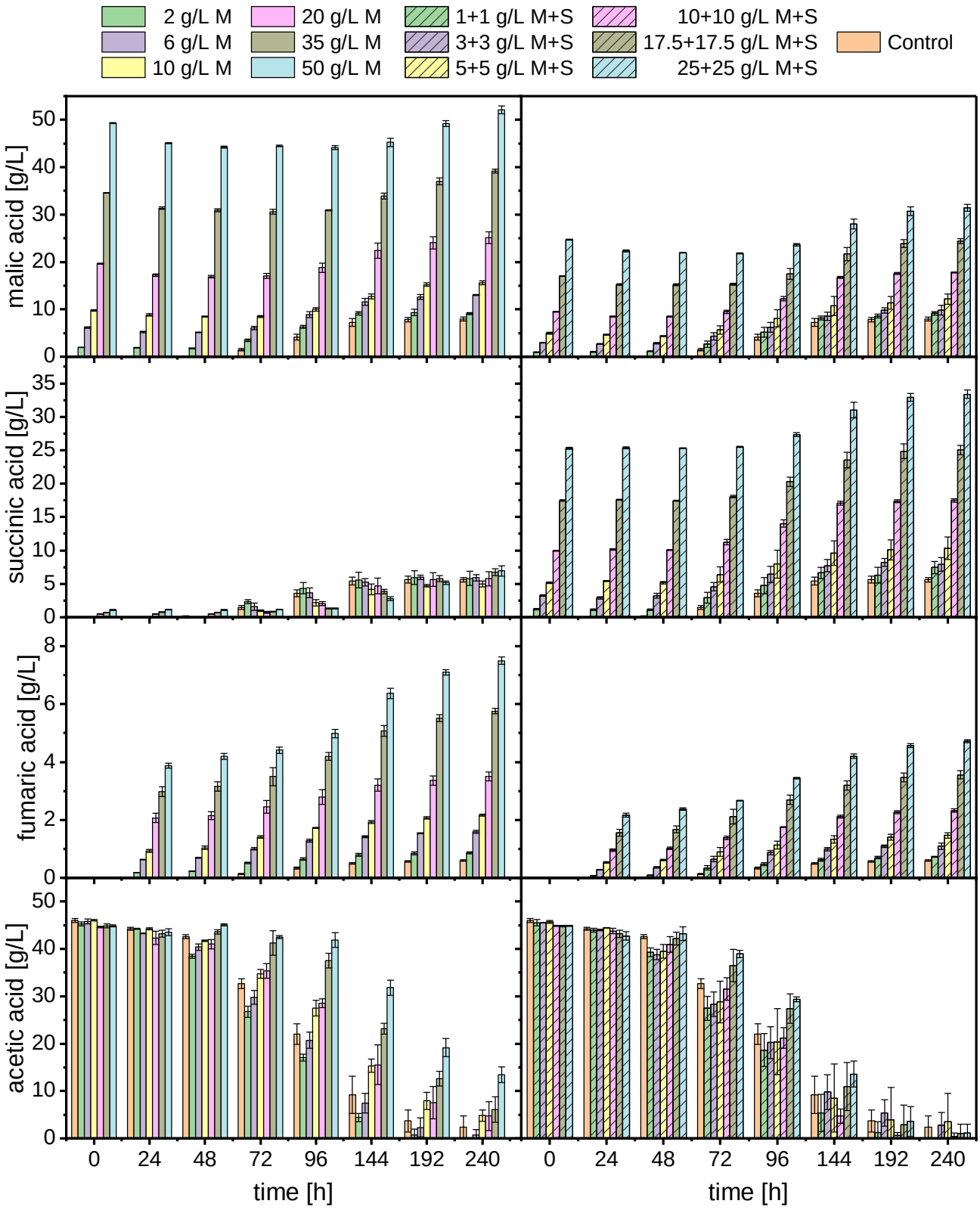

Figure 25. Organic acid production in shake flasks with different initial malic and succinic acid concentrations. Data are the means of triplicate experiments with respective standard deviations. M = malic acid, S = succinic acid.

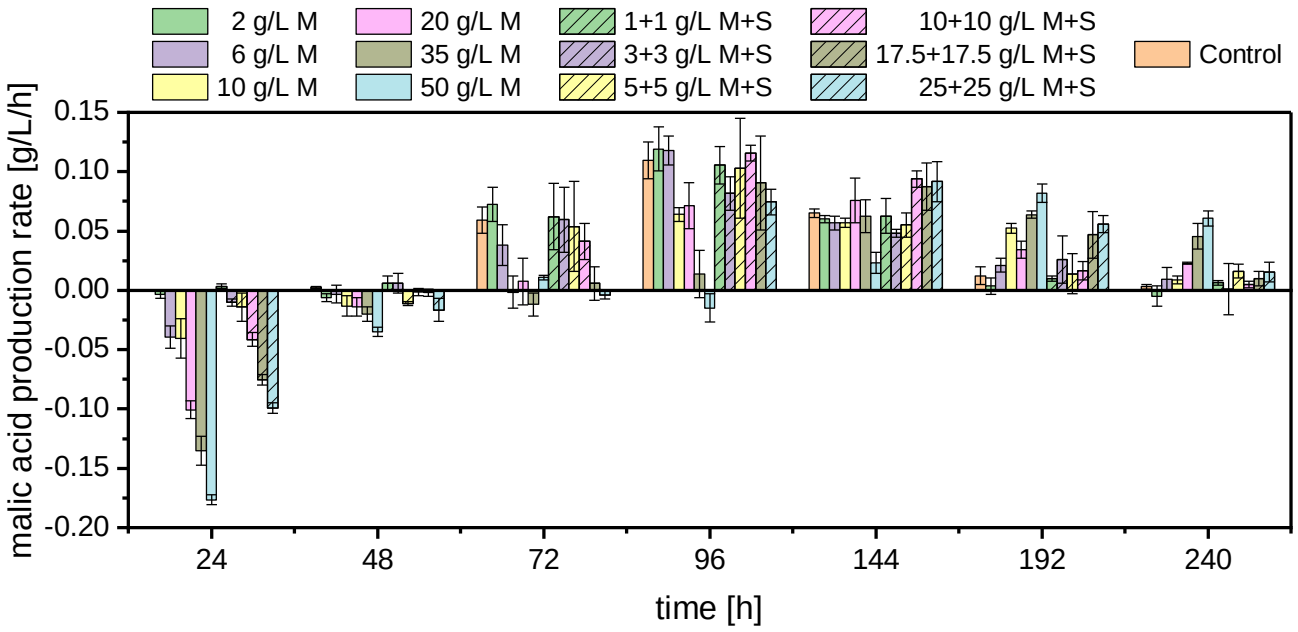

Figure 26. L-Malic acid production rates for cultures with different initial malic and succinic acid concentrations calculated between the sampling points. Negative values represent a consumption. Data are the means of triplicate experiments with respective standard deviations. M = malic acid, S = succinic acid.

The addition of succinate seems to have a lower impact on the organic acid production capacity of *A. oryzae* than malate. In the cultures with 20-50 g/L malic acid, low succinate concentrations were detected from the beginning of the cultivation which originated from impurities in the malic acid added to the medium. Different than malic acid, succinic acid was not consumed as no considerable decrease was observed even with the highest concentration of 25+25 g/L M+S. Most of the tested conditions with M+S showed a net malic acid production (final-initial) similar to the control (Table 17). Regarding the difference between the final and the minimal malate concentration, the cultures containing 10-25 g/L M+S even surpassed the control, with 25+25 g/L M+S producing 9.60 ± 0.58 g/L malate. This is an interesting observation as the acetate consumption was similar to the control. The net production of succinic acid over 240 h of cultivation was mostly between 5-6 g/L for all conditions and comparable to the control (5.63 ± 0.34 g/L). Only the cultures with 10-25 g/L M+S showed a higher production of up to 8.10 ± 0.64 g/L succinic acid.

Table 17. Fermentation results for shake flask cultivations of *A. oryzae* with different initial malic and succinic acid concentrations. Data are the means of triplicate experiments with respective standard deviations.

Condition[a]	Acetic acid, initial-final[b] [g/L]	Malic acid, final-initial [g/L]	Malic acid, final-minimal[c] [g/L]	$Y_{P/S,\ malic\ acid,\ final\text{-}initial}$ [g/g]	Productivity malic acid, final-initial [g/L/h]	Succinic acid, final-initial [g/L]	Fumaric acid, final [g/L]	Total acid, final-initial [g/L][d]	$Y_{P/S,\ total\ acid,\ final\text{-}initial}$ [g/g]	$Productivity_{total\ acid,\ final\text{-}initial}$ [g/L/h]
2 g/L M	44.33 ± 0.38	7.18 ± 0.25	7.41 ± 0.12	0.16 ± 0.01	0.030 ± 0.001	5.75 ± 0.73	0.87 ± 0.03	14.34 ± 0.88	0.32 ± 0.02	0.060 ± 0.004
6 g/L M	44.18 ± 1.63	6.92 ± 0.11	7.98 ± 0.15	0.16 ± 0.00	0.029 ± 0.000	5.69 ± 0.43	1.59 ± 0.05	14.82 ± 0.37	0.34 ± 0.02	0.062 ± 0.002
10 g/L M	40.45 ± 1.28	5.88 ± 0.28	7.31 ± 0.26	0.15 ± 0.00	0.024 ± 0.001	4.92 ± 0.44	2.16 ± 0.03	13.90 ± 0.65	0.34 ± 0.01	0.058 ± 0.003
20 g/L M	39.87 ± 3.19	5.51 ± 1.19	8.37 ± 0.99	0.14 ± 0.02	0.023 ± 0.005	5.28 ± 1.06	3.50 ± 0.15	15.19 ± 1.86	0.38 ± 0.02	0.063 ± 0.008
35 g/L M	38.77 ± 3.10	4.56 ± 0.35	8.57 ± 0.37	0.12 ± 0.01	0.019 ± 0.001	5.98 ± 0.51	5.76 ± 0.09	16.90 ± 0.60	0.44 ± 0.02	0.070 ± 0.002
50 g/L M	31.43 ± 1.54	2.79 ± 0.75	8.02 ± 0.51	0.09 ± 0.02	0.012 ± 0.003	5.83 ± 0.82	7.49 ± 0.12	16.56 ± 1.58	0.53 ± 0.03	0.069 ± 0.007
1+1 g/L M+S	44.95 ± 0.09	8.23 ± 0.38	8.23 ± 0.38	0.18 ± 0.01	0.034 ± 0.002	6.36 ± 0.97	0.73 ± 0.01	15.78 ± 1.22	0.35 ± 0.03	0.066 ± 0.005
3+3 g/L M+S	41.87 ± 2.62	6.91 ± 1.00	7.18 ± 1.04	0.16 ± 0.01	0.029 ± 0.004	4.59 ± 0.94	1.09 ± 0.11	13.29 ± 1.82	0.32 ± 0.02	0.055 ± 0.008
5+5 g/L M+S	41.35 ± 6.19	7.24 ± 0.93	7.85 ± 1.17	0.18 ± 0.01	0.030 ± 0.004	5.14 ± 1.77	1.47 ± 0.10	14.49 ± 2.51	0.35 ± 0.01	0.060 ± 0.010
10+10 g/L M+S	44.40 ± 0.69	8.28 ± 0.11	9.32 ± 0.14	0.19 ± 0.00	0.035 ± 0.000	7.52 ± 0.25	2.32 ± 0.06	18.83 ± 0.38	0.42 ± 0.00	0.078 ± 0.002
17.5+17.5 g/L M+S	43.74 ± 2.00	7.36 ± 0.54	9.28 ± 0.42	0.17 ± 0.01	0.031 ± 0.002	7.59 ± 0.80	3.56 ± 0.15	19.48 ± 1.04	0.45 ± 0.01	0.081 ± 0.004
25+25 g/L M+S	43.77 ± 1.97	6.73 ± 0.69	9.60 ± 0.58	0.15 ± 0.01	0.028 ± 0.003	8.10 ± 0.64	4.71 ± 0.05	20.43 ± 1.15	0.47 ± 0.02	0.085 ± 0.005
Control	43.68 ± 2.80	7.99 ± 0.42	7.99 ± 0.42	0.18 ± 0.00	0.033 ± 0.002	5.63 ± 0.34	0.60 ± 0.02	15.03 ± 0.69	0.34 ± 0.01	0.063 ± 0.003

[a] M = Malic acid; S = Succinic acid

[b] initial = acid concentration at 0 h; final = acid concentration at 240 h

[c] minimal = minimal acid concentration

[d] Refers to all organic acids quantified (see Table 18)

Most of the initial malate consumption was observed within the first 24 h of cultivation. During the same period considerable amounts of fumaric acid were detected (Figure 25) whereas fumaric acid was only detected after 72 h in the control cultures. The fumarate production was proportional to the initially added product concentration. Hence, the highest fumaric acid production of 3.88 ± 0.08 g/L after 24 h was determined for cultures with 50 g/L malic acid with a concurrent consumption of 4.24 ± 0.10 g/L malate. This corresponds to a molar production of 33.42 ± 0.73 mM fumaric acid and a consumption of 31.62 ± 0.75 mM malic acid. For the other tested conditions, the molar consumption and production were similar as well, and the proportion of malate converted to fumarate within the first 24 h of cultivation was between 9 and 13% (except for the control and 1+1 g/L M+S as no consumption was observed). This suggests that most of the initially consumed malate was converted to fumarate. In an abiotic control with medium incubated under the same conditions as the cultures with *A. oryzae*, no acid consumption or production was observed, clearly indicating that the fungal metabolism was involved in the production of fumaric acid (data not shown). After 240 h, the highest fumaric acid concentration of 7.49 ± 0.12 g/L was measured for cultures with 50 g/L malic acid compared to only 0.60 ± 0.02 g/L in the control cultures (Table 17). The addition of succinate had a lower impact on the production of fumaric acid since with the highest tested combination (25+25 g/L M+S) only about two thirds of the fumarate concentration obtained with 50 g/L malic acid, namely 4.71 ± 0.05 g/L, were quantified.

Besides the three main products malate, succinate and fumarate, only minor concentrations of other organic acids were detected. An overview of the organic acid spectrum for all tested conditions is listed in Table 18. The values in this table refer to the net acid production (final-initial). The total acid concentration ranged from 13.29 ± 1.82 g/L (3+3 g/L M+S) to 20.43 ± 1.15 g/L (25+25 g/L M+S) (Table 17). The highest share of malate was quantified in the control (53.1 ± 0.5%), followed by succinate (37.4 ± 0.6%) and fumarate (4.0 ± 0.1%). The addition of malic and succinic acid mainly impacted the malate and fumarate proportions, leading to a decrease or increase, respectively. With 50 g/L malic acid, the malate share was reduced to 16.6 ± 3.0% while the fumarate portion was increased to 45.5 ± 4.2%.

Regarding the acetate consumption, the substrate was depleted or close to depletion in most cultures after 240 h except for those containing higher malate concentrations. In the cultures with 50 g/L initial malic acid, 13.4 ± 1.7 g/L acetic acid were left at this measurement point. Considering the delayed consumption of acetate and the nitrogen source ammonium (Appendix E, Figure E1 which also displays the development of the pH), it is likely that high product concentrations inhibit the growth of *A. oryzae*. This inhibition, however, seems to originate from malic acid primarily as the effect of the combination of malate and succinate was less pronounced.

Table 18. Organic acid distribution for shake flask cultivations of *A. oryzae* with different initial malic and succinic acid concentrations. Data are the means of triplicate experiments with respective standard deviations.

Condition	Organic acid distribution [%][a]						
	Malate	**Succinate**	**Fumarate**	**Pyruvate**	**α-Keto-glutarate**	**Oxalate**	**Citrate**
2 g/L M	50.1 ± 1.3	40.0 ± 2.7	6.1 ± 0.5	0.9 ± 0.1	0.3 ± 0.0	1.7 ± 0.5	0.9 ± 0.4
6 g/L M	46.7 ± 0.9	38.4 ± 1.9	10.8 ± 0.4	0.8 ± 0.0	0.5 ± 0.1	1.9 ± 0.5	1.0 ± 0.5
10 g/L M	42.3 ± 1.6	35.3 ± 1.5	15.6 ± 0.5	0.9 ± 0.1	0.5 ± 0.1	3.9 ± 0.7	1.5 ± 0.8
20 g/L M	36.0 ± 3.4	34.6 ± 2.6	23.3 ± 3.6	1.0 ± 0.2	0.4 ± 0.1	3.7 ± 2.2	1.0 ± 0.3
35 g/L M	26.9 ± 1.6	35.3 ± 1.9	34.1 ± 1.7	1.0 ± 0.1	0.3 ± 0.0	2.0 ± 0.7	0.3 ± 0.3
50 g/L M	16.6 ± 3.0	35.1 ± 1.6	45.5 ± 4.2	0.9 ± 0.1	0.3 ± 0.0	1.6 ± 0.5	0.0 ± 0.0
1+1 g/L M+S	52.2 ± 1.6	40.2 ± 3.0	4.6 ± 0.3	0.7 ± 0.0	0.3 ± 0.1	1.5 ± 0.8	0.4 ± 0.2
3+3 g/L M+S	51.9 ± 0.6	34.3 ± 2.4	8.3 ± 0.5	0.8 ± 0.2	0.4 ± 0.1	3.3 ± 2.3	1.0 ± 0.1
5+5 g/L M+S	50.3 ± 2.6	34.7 ± 6.8	10.3 ± 1.2	0.9 ± 0.1	0.5 ± 0.1	2.8 ± 2.7	0.7 ± 0.4
10+10 g/L M+S	44.0 ± 0.5	40.0 ± 0.6	12.3 ± 0.1	0.8 ± 0.1	0.4 ± 0.0	1.5 ± 0.3	1.0 ± 0.1
17.5+17.5 g/L M+S	37.8 ± 0.8	38.9 ± 2.0	18.3 ± 0.7	0.8 ± 0.1	0.3 ± 0.0	3.2 ± 2.4	0.7 ± 0.1
25+25 g/L M+S	32.9 ± 1.6	39.6 ± 0.9	23.1 ± 1.5	0.8 ± 0.1	0.3 ± 0.0	2.7 ± 0.7	0.5 ± 0.1
Control	53.1 ± 0.5	37.4 ± 0.6	4.0 ± 0.1	0.7 ± 0.0	0.5 ± 0.0	2.8 ± 1.0	1.4 ± 0.2

M = Malic acid; S = Succinic acid

[a] Values refer to the organic acid distribution measured at 240 h minus the initial concentrations of malic and succinic acid (see Table 17).

5.4. Discussion

5.4.1. L-Malic acid production in 2.5-L bioreactor batch cultivations

This chapter aimed to improve malic acid production with *A. oryzae* using acetate as carbon source in 2.5-L bioreactor fermentations. At first, organic acid production was evaluated in batch processes (Figure 22). Compared to the results reported in the previous chapter, malate production was increased considerably by applying a stirrer velocity of 600 rpm instead of 300 rpm (see Figure 19). The increase in agitation velocity induced a change in morphology from filamentous to pelleted which likely was the main reason for the improved acid production. The morphology developed by filamentous microorganisms can take a variety of different forms from dispersed hyphae to compact pellets. Thereby, the ideal morphology seems to vary between target products and microorganisms (Veiter et al., 2018). The pellet formation is influenced by various factors such as the inoculum concentration, osmolality, pH, aeration rate, stirrer velocity, and the presence of additives including surfactants and particles (Böl et al., 2020; Veiter et al., 2018). In the cultivations presented in this work, biomass pellets were formed only during the fermentation in the bioreactor, not in the seed culture in which a filamentous-lumpy morphology was obtained. Thus, the different cultivation conditions in the bioreactor, likely the higher shear rate in particular, caused this transition. A high stirrer velocity usually results in the formation of smaller and more compact biomass pellets due to the breaking of pellets or the erosion of the pellet surface (Casas López et al., 2005; Cui et al., 1997; Rodríguez Porcel et al., 2005). However, comparing low agitation intensities, it seems that the pellet diameter first increases before high shear forces lead to a decrease. For *Trichoderma reesei*, a transition towards larger and more defined pellets was observed when increasing the stirrer velocity from 100 rpm to 400 rpm in 7.5-L bioreactor cultivations (Yu et al., 2012). Similar observations were reported for the cultivation of *A. oryzae* FCD15 in 7.5-L bioreactors for malate production. When the stirrer velocity was increased from 200 rpm to 600 rpm, the morphology changed from a mycelial to a pelleted form and the malate concentration after 144 h increased from approximately 40 g/L to 83.3 g/L (Chen et al., 2019). Further experiments should evaluate the influence of the stirrer velocity on morphology and organic acid production with *A. oryzae* from acetate in more detail to find the optimum balance between malate production and energy demands.

5.4.2. L-Malic acid production in 2.5-L bioreactors using a pH-coupled feeding strategy

In the bioreactor batch processes, malic acid production ceased after 144 h despite carbon availability which was most likely a result of the high and inhibiting pH values. Using a pH-coupled feed of acetic acid, the pH was maintained at physiological values of about 7.1-7.2 and additional substrate was provided at the same time. With a feed of 10 M acetic acid and a $CaCO_3$ concentration of 90 g/L (fermentation VI, Figure 24), the maximum malic acid titer was increased about 5.3-fold compared to the batch process without pH control, yielding 29.53 ± 1.82 g/L (Table 15). This is the highest malic acid concentration obtained with acetate as the main carbon source so far. Regarding

the overall production rate, a value of 0.112 ± 0.007 g/L/h was determined for a fermentation time of 264 h. This is comparable to the maximum production rate calculated for batch cultivations performed in shake flasks for which a value of 0.122 g/L/h at 85 h was determined by fitting the data and calculating the maximum of the first derivative (see Chapter 2.3.2). Hence, a high production rate could be maintained over an extended period by feeding of acetic acid. The amount of substrate provided through the pH-coupled feed, however, was not sufficient to maintain a stable acetate concentration. With an acetic acid feed adjusted to a pH of 4.5 (fermentation V, Figure 23), and by applying manual feeds of 5 M NaOH (fermentation VII, Figure 24), the acetate concentration was successfully maintained close to the initial concentration of 45 g/L acetic acid. These strategies, however, resulted in lower malic acid concentrations compared to the fermentations using unadjusted 10 M acetic acid only (Table 15). A reason for this observation might be a high ion concentration. To adjust the pH of the 10 M acetic acid with a pH of 4.5, sodium hydroxide was used. Due to the pH-dependent feeding of acetic acid with pH 4.5, or the manual feeds of 5 M NaOH, sodium ions accumulated during the fermentation. It is possible that these additional ions inhibited malic acid production in later stages of the cultivation. Inhibition of production caused by sodium chloride concentrations of 40 g/L (0.68 mol/L) and above was also observed during Ochratoxin A synthesis with *Aspergillus ochraceus* and *Penicillium nordicum* (Wang et al., 2020). Another factor which might have contributed to the decreased malic acid production in fermentations IV, V and VII is a higher concentration of acetic acid in the medium. It is assumed that the growth inhibiting effect of acetate is caused by the presence of its protonated form which can enter the cells by diffusion, causing an acidification of the cytoplasm upon deprotonation (Stratford et al., 2009). In the fermentations with pH control through 10 M unadjusted acetic acid (III and VI), the acetate concentration decreased more rapidly, leading to a lower concentration of the protonated form in the fermentation medium. When a higher acetate concentration was maintained, *A. oryzae* was subjected to elevated concentrations of the protonated form for a longer period. Even though the ratio of acetic acid to acetate at a pH close to neutrality is low (more than 99% is deprotonated at pH 7.0), a prolonged subjection may be inhibiting and contribute to a lower productivity. In further experiments, the feeding strategy should be optimized and the effect of maintaining lower substrate concentrations on malic acid production should be evaluated. Additionally, the optimum pH value for organic acid production with *A. oryzae* in acetate media should be determined. The utilization of acetic acid for substrate supply and pH control has also been described for other processes and different regulation strategies were employed. For lipid production with *Rhodotorula glutinis*, the pH was maintained using 30% (v/v) acetic acid. In the reported process, this could keep the acetate supply at a sufficiently high level with only little fluctuations around the initial concentration (Zhang et al., 2019). Qian et al. (2020) employed the same strategy for lipid production with the yeasts *Cryptococcus podzolicus* and *Trichosporon porosum* using 4 M acetic acid for pH control and substrate supply. For lipid production with *Yarrowia lipolytica*, it was reported that utilizing acetic acid for pH control alone was not sufficient to provide an adequate substrate supply. The authors therefore added a continuous sodium acetate feed to maintain a low acetate concentration throughout

the semicontinuous fermentation (Xu et al., 2017). Another strategy combines the pH-coupled feeding of acetic acid with feeds of an acetate solution depending on the dissolved oxygen concentration. This was employed for itaconic acid production with *Corynebacterium glutamicum*. When the DO-concentration surpassed an upper limit upon the depletion of the carbon source, acetate solution was fed which allowed for the production of 29.2 g/L itaconate within 46 h (Merkel et al., 2022). Maintaining a low acetate concentration with a continuous or DO-coupled acetate feed could also be evaluated for malic acid production with *A. oryzae*.

Interestingly, malic acid production ceased despite the availability of residual acetate in fermentations III-VI, followed by a reassimilation in fermentations IV and V. This was also observed in batch processes with *A. oryzae* using glucose as substrate, which reported malate reassimilation despite a residual glucose concentration of about 30 g/L (Schmitt et al., 2021). In this regard, optimizing the supply of medium components such as the nitrogen availability or salt concentration could be advantageous. While nitrogen limitation is important for an efficient organic acid synthesis in filamentous fungi, the C:N ratio can influence the organic acid composition (Ding et al., 2011; Ochsenreither et al., 2014). Thus, an adequate supply of nitrogen can positively influence malic acid production. Ji et al. (2021) showed that an optimized nitrogen supply strategy could result in an prolonged phase of rapid malate production with *A. oryzae*, leading to an increase in product concentration from 130.5 to 164.9 g/L. Regarding malate production with *Ustilago trichophora*, increasing the NH_4Cl concentration enhanced the maximum volumetric productivity and therefore enabled a higher malate titer in a shorter time (Zambanini et al., 2016). Schmitt et al. (2021) did not observe a positive effect of supplementing the glucose feed with nitrogen in fed-batch processes with *A. oryzae* but found an increased malate concentration when complete medium was added. Maybe, the C:N ratio in these experiments was not ideal. In a study with *A. oryzae* FMME218-37, the C:N ratio was found to have a significant impact on the maximum malate titer. At a fixed C:N ratio, however, also the concentrations of the carbon and nitrogen source were shown to influence malic acid production. With a C:N ratio of 50 and optimized glucose and ammonium sulfate concentrations of 146 g/L and 5.5 g/L, respectively, a high malate titer of 88.4 g/L was reported (Ding et al., 2018). However, an increased availability of nitrogen enhances biomass formation, which diverts the carbon from malate synthesis. The nitrogen availability must thus be balanced between productivity and yield. As demonstrated in Chapter 3 of this dissertation, especially the addition of yeast extract proved beneficial by accelerating malic acid production in the beginning of the cultivation. Therefore, optimizing the nitrogen availability and testing nitrogen sources besides $(NH_4)_2SO_4$ in bioreactor cultivations, possibly in combination with the adjustment of other medium components, could help to increase malate synthesis and prolong the production period, and should be evaluated in further studies.

Two concentrations of calcium carbonate were evaluated both in batch mode (fermentations I and II, Figure 22) and in cultivations with a pH-coupled feed (fermentations III and VI, Figure 23 and Figure 24). While malic acid production with the tested carbonate concentrations was similar in

batch fermentations, the utilization of 90 g/L $CaCO_3$ resulted in an increased malate titer in cultivations with a pH-coupled feed as carbonate depletion was prevented. Hence, an adequate supply of $CaCO_3$ throughout the fermentation seems necessary for efficient production of malic acid. $CaCO_3$ can influence the cultivation in several ways. It neutralizes the produced organic acids, provides insoluble solids, and is a source for calcium ions and CO_2. The availability of carbon dioxide potentially plays a role in malate production from glucose as pyruvate carboxylase fixes CO_2 in the reductive tricarboxylic acid cycle, which is assumed to be the main pathway towards malate production in *Aspergillus* using this substrate (Peleg et al., 1989). Furthermore, the provided Ca^{2+} can form poorly soluble calcium malate which precipitates and helps prevent product inhibition (Zambanini et al., 2016). $CaCO_3$ is therefore often used in processes for organic acid production. In cultivations with *A. oryzae* performed by Geyer et al. (2018), a high $CaCO_3$ concentration of 120 g/L was reported advantageous as an increased carbon percentage was attributed to malic acid. The concentration of $CaCO_3$ was also found to affect organic acid production by *Rhizopus delemar* as demonstrated by Ronoh et al. (2022). With 60 or 100 g/L $CaCO_3$ compared to 20 g/L, carbonate was available until the end of the fermentation, which prevented a drop in pH and resulted in a higher malic to fumaric acid ratio. The authors ruled out the presence of insoluble solids as influencing factor but found increased ion concentrations and different pH values to affect the production profile (Ronoh et al., 2022). Schmitt et al. (2021) also observed a positive effect of calcium carbonate on malic acid productivity and associated it with its ability to control the pH as buffering agent, although the authors pointed out that this may not be its only function. A high carbonate concentration is accompanied by a high ion concentration which remains in the medium once the carbonate is exhausted and might as well become inhibiting. With a concentration of 50 or 90 g/L $CaCO_3$, 20 or 36 g/L Ca^{2+} are provided, respectively. Ronoh et al. (2022) reported a decreased glucose consumption and fumaric acid production of *R. delemar* with 20 g/L Ca^{2+} in the medium, which suggests inhibition. However, they also observed an increased, albeit delayed, formation of malic acid which might be attributed to a stimulatory effect of Ca^{2+} on the enzyme fumarase (Ronoh et al., 2022). The results described in all these references were obtained for cultivations using glucose as carbon source. So far, the function of $CaCO_3$ in malic acid production from acetate could not be identified conclusively and should be the subject of future studies. Furthermore, a potential inhibition of *A. oryzae* by calcium ions needs to be evaluated. Considering a possible inhibition by both the calcium ions provided through the $CaCO_3$ and the sodium ions added through an acetate feed required for maintaining a stable substrate concentration, a fermentation strategy with partly removal of the fermentation medium will probably be necessary to maintain a high production rate.

Compared to the maximum malic acid productivities and yields reported with glucose, the values obtained with acetate presented in this study are considerably lower. High productivities and yields were for example determined by Ji et al. (1.14 g/L/h, 0.77 g/g), Chen et al. (1.08 g/L/h, 0.75 g/g), Liu et al. (1.38 g/L/h, 0.68 g/g) and Xu et al. (1.05 g/L/h, 1.22 g/g) for different species of *Aspergillus* (Chen et al., 2019; Ji et al., 2021; Liu, Xie et al., 2017; Xu et al., 2020). These strains,

however, were all genetically modified. Given the high production of side products, especially succinate, with acetate as carbon source (Table 16), a possibility for increasing the malate yield is the reduction of these organic acids by directing the carbon flux towards malic acid. Acetate is metabolized through the glyoxylate pathway. In this bypass of the citric acid cycle, isocitrate is first converted to glyoxylate and succinate by the enzyme isocitrate lyase, followed by a conversion of the glyoxylate and acetyl-CoA to malate by malate synthase. The use of this pathway for acetate metabolization in *Aspergillus* was demonstrated in cultivations with *A. nidulans*, where induction of the two enzymes was observed in the presence of acetate. Furthermore, mutants deficient in either enzyme were not able to grow on this carbon source (Armitt et al., 1970; Armitt et al., 1971). Experiments with ^{13}C-labeled acetate furthermore demonstrated the incorporation of the labeled carbon into citrate, malate and succinate in *A. fumigatus* (Ries et al., 2021). The production of high succinate concentrations is thus a result of the glyoxylate cycle as main pathway towards malic acid production. Liu et al. (2018) reported a decreased concentration of succinate in favor of an enhanced malate titer in *A. oryzae* by employing two strategies. As the authors found that succinic acid was mainly present in the cytosol, they overexpressed a dicarboxylate carrier from *Saccharomyces cerevisiae* which transports succinate from the cytosol to the mitochondria. Furthermore, they engineered the redox metabolism by overexpression of a water-forming NADH oxidase (Liu et al., 2018). As the succinic acid proportion obtained during malic acid production from acetate represents approximately 40% of the total acids produced, there is a large potential for improvement.

5.4.3. Organic acid production in the presence of malate and succinate

The observed cessation of malic acid production in later stages of the fermentation could also be caused by product inhibition. Therefore, the effect of malate and succinate addition on organic acid production from acetate was evaluated in shake flask cultivations. Interestingly, a high fumarate production was observed especially in the first 24 h of the cultivation with a concurrent consumption of malate (Figure 25). This fumarate production was proportional to the amount of malic acid added while the addition of succinate only had a minor effect. Fumarase, which was shown to be present both in the mitochondria and the cytosol in *Aspergillus* species, catalyzes the reversible hydration of fumaric to L-malic acid (Peleg et al., 1989). Experiments with purified porcine fumarase and permeabilized yeast cells showed that the maximal enzymatic activity to fumaric acid is considerably higher than to malic acid and that about 80% of fumarate are converted to malate under equilibrium conditions (Presečki et al., 2007). The high fumarate production observed at high product concentrations is likely a result of the dehydration of malate to fumarate. Interestingly, in fermentation VI, the fumarate proportion amounted to only 3.3 ± 0.2% total acid despite the presence of almost 30 g/L malic acid. In the bioreactor processes, however, high malate concentrations were obtained only in later stages of the fermentation when the cultivation conditions such as the pH and nutrient concentrations were different than in shake flasks. The pH value was shown to influence the direction of the fumarase-catalyzed reaction as the maximum activity towards fumarate hydration was pH 7, while it was about pH 8 for the dehydration using porcine fumarase (Presečki et al., 2007).

The different metabolic states of *A. oryzae* or the C:N ratio may also have played a role in fumarate production. The steepest increase in fumarate concentration in shake flasks was observed within the first 24 h. During this time, *A. oryzae* was still in the lag phase as only minor differences in the ammonium concentration were observed (Appendix E, Figure E1). In the bioreactor fermentations, however, nitrogen was depleted between 48 and 72 h and the growth phase was therefore completed when high malic acid titers were present (Figure 24). It is also conceivable that the transport of malic acid out of the cell during the production phase is rapid, preventing dehydration to fumaric acid. For a better understanding of the fumarate production observed at high product concentrations, fumarase activity in *A. oryzae* needs to be further evaluated. These experiments should ideally be performed in the controlled environment of a bioreactor with pH control.

Another interesting observation of the experiments with addition of malate and succinate is the total acid concentration in relation to the consumed substrate. In the control, the total acid production was 15.03 ± 0.69 g/L (Table 17). Especially the addition of succinate increased the total acid concentration, since the highest titer of 20.43 ± 1.15 g/L was measured with 25+25 g/L M+S. The substrate consumption in these cultures, however, was similar. The reassimilation of malate or succinate, or the conversion of these compounds into other intermediates or products such as fumarate, might be more favorable regarding the demand for energy or reduction equivalents. It is also possible that the biomass production was influenced by the availability of the two compounds which then affected the production capacity. The biomass concentration was not measured in this work but considering the ammonium measurements, fungal growth was delayed with increasing concentration of succinate and especially malate. This delay in ammonium consumption might as well originate from an inhibition caused by a higher sodium ion concentration as more sodium hydroxide was required for pH correction in these media and not necessarily from the products themselves. Further research is therefore required to clarify these observations.

The question whether product concentrations of up to 50 g/L inhibit malate production cannot be answered conclusively by the results presented here. Even though product formation was delayed, and lower maximum productivities were obtained with high initial product concentrations (Figure 26), malic acid production was still observed. Given that the differences between the final and the minimal malic acid concentrations were comparable or, in the case of 10-25 g/L M+S, even higher than in the control (Table 17), an inhibition regarding the organic acid production capacity in a range up to 50 g/L seems not to be very pronounced. Evaluating the effects of high malate and succinate concentrations for selected key enzymes involved in malate production from acetate individually could be a tool to elucidate a possible product inhibition in more detail. Furthermore, adding the products after the depletion of the nitrogen source rather than at the beginning of the cultivation should be considered.

5.5. Conclusion

This chapter evaluated several strategies to improve malic acid production using acetate as carbon source in 2.5-L bioreactor fermentations with *A. oryzae*. The utilization of a higher stirrer velocity induced the formation of biomass pellets which seemed crucial for a successful scale-up from shake flask to bioreactor. With a pH-coupled feeding strategy, the malic acid concentration was increased, resulting in a maximum value of 29.53 ± 1.82 g/L which is the highest concentration obtained with acetate as the main carbon source so far. It was found that the pH-coupled feed of unadjusted acetic acid was not sufficient to provide a constant substrate concentration. Maintaining the substrate supply within a constant range close to the initial concentration of 45 g/L acetic acid, however, did not prove beneficial. Shake flask experiments to evaluate the effects of high product titers showed a stronger influence of malate than of combinations of malate and succinate. Despite a delay in growth and acid secretion observed with high product concentrations, malate production was similar to the control after an initial reassimilation, illustrating that product synthesis with *A. oryzae* beyond 50 g/L is possible in acetate medium. In further experiments the feeding strategy should be optimized with the aim to prolong the period of maximum productivity. Aspects to consider could be a feed of medium components such as salts and nitrogen and maintaining a lower acetate concentration throughout the fermentation. Since succinic acid accounts for about 40% of the total acid production, strategies such as metabolic engineering aiming to decrease the concentration of this side product should be considered to maximize the malic acid yield.

6. Final conclusions and future perspectives

The utilization of inexpensive biomass-derived substrates can contribute to the development of microbial processes for malic acid production that are able to compete with chemical synthesis. Since acetic acid is commonly contained in these substrates, it is a promising carbon source in a bio-based economy. This study therefore evaluated malic acid production from acetate with *Aspergillus oryzae*, a filamentous fungus which is already widely used in the food industry and produces malic acid naturally.

The process was first evaluated in shake flasks to characterize growth and organic acid production and better understand the differences to cultivations employing glucose. Whereas with the carbohydrate, high initial carbon concentrations can be applied, this is not the case when acetate is used due to substrate inhibition. Thus, in a batch process, an initial acetate concentration of 45 g/L was found to be best regarding the production rate and the percentage of substrate conversion which resulted in about 8.4 g/L malic acid. A detailed evaluation of other organic acids secreted by *A. oryzae* besides the main product malate was provided in this study which has not been reported before. It was found that the carbon source type and concentration, as well as the cultivation temperature had a considerable effect on the product spectrum. Compared to cultivations with glucose, the malate percentage decreased, and the succinate share increased when acetate was used. The succinic to malic acid ratio was further enhanced with an increase in cultivation temperature until it became the main product at 38 °C. Hence, the cultivation temperature and the initial product concentration are important parameters to influence malic acid synthesis. The $CaCO_3$ concentration, however, did not affect the acid spectrum but the acid concentration. The highest malate titer and production rate was observed with the highest tested carbonate concentration. Further studies are required to investigate the function of calcium carbonate addition conclusively. In shake flask experiments, it was also shown that the supplementation of acetate medium with small amounts of glucose can accelerate malate production. Therefore, 5 g/L glucose were added to bioreactor fermentation media to reduce the lag phase. In these experiments, it was found that both acetate and glucose are consumed in the first 48 h of cultivation. This finding is especially interesting regarding the future utilization of complex biomass-derived substrates. The potential of co-feeding acetate as main carbon source with low concentrations of carbohydrates such as glucose, xylose or levoglucosan should be explored in more detail in further studies. This will allow to better assess the suitability of acetate-containing biomass-derived substrates for microbial metabolization. Since a concentration of about 45 g/L acetic acid is required for efficient malate synthesis in a batch process, suitable substrates from biomass could be condensates of pyrolysis or products of syngas fermentation which can feature acetate concentrations in this range.

Filamentous microorganisms can display a large variety of morphological forms ranging from dispersed filaments to compact biomass pellets. When glucose is used as carbon source, biomass pellets are naturally obtained with *A. oryzae*, but with acetate the situation proved to be more

complex. As demonstrated in this study, the initial acetate concentration had a considerable effect on the morphology, most likely due to an increased ion concentration. Whereas pellets were obtained up to a concentration of 30 g/L acetic acid, a filamentous-lumpy morphology was observed with an acetate concentration of 40 g/L and above. A pelleted morphology is advantageous from a process engineering point of view as it leads to a lower viscosity of the fermentation broth and facilitates separation of the biomass. Therefore, entrapment of *A. oryzae* was evaluated as a tool for morphological control. The natural polymers alginate, κ-carrageenan and agar were selected as entrapment matrices due to their non-toxic nature and good biocompatibility. Although the entrapment was successful in glucose medium and similar fermentation outcomes were obtained as with free biomass pellets, abrasion of biomass from the beads or their disintegration was observed during cultivation in acetate medium. Therefore, other options such as entrapment in synthetic polymers or the immobilization on porous carriers should be considered if this strategy is pursued further.

Another possibility to alter the morphology of filamentous microorganisms is the choice of fermentation parameters such as the stirrer velocity. By increasing the stirrer speed from 300 to 600 rpm during the cultivation in 2.5-L bioreactors, an agglomeration of the biomass was induced, and small pellets were obtained. This was a key for a successful transfer of the fermentation from shake flask to bioreactor scale. The control of the morphology in the production of malic acid from acetate therefore appears to be of critical importance and needs to be considered in a further scale-up. Since the morphology can considerably affect product synthesis, the development of the biomass pellet should be investigated in further studies with respect to the shear rate, the time point of pellet formation, or the effect of particle addition other than $CaCO_3$.

Different than in processes which are based on carbohydrates, the metabolization of acetate affects the pH value which shows an increase up to potentially inhibiting values when left uncontrolled. To avoid additional accumulation of ions in the fermentation medium by using acids that are commonly applied for pH control such as H_2SO_4 or H_3PO_4, a pH-coupled feed of acetic acid for pH control and additional substrate supply was tested. This resulted in the highest titer (29.53 ± 1.82 g/L), yield (0.29 ± 0.01 g/g) and overall production rate (0.112 ± 0.007 g/L/h) obtained from acetate so far. Maintaining both the substrate concentration and pH at a constant level was possible with this strategy but did not prove beneficial due to a lower maximum titer and earlier cessation of malate production. This might have been due to the accumulation of ions or the subjection of *A. oryzae* to a higher concentration of undissociated acetic acid, which is assumed to be the main cause of inhibition observed when using acetate as carbon source. However, also in this most successful fermentation malate production ceased despite a residual acetate concentration of about 10 g/L. Evaluation of the effect of malate and succinate addition up to a concentration of 50 g/L demonstrated that *A. oryzae* is capable of further organic acid production, and product inhibition in a range of 30 g/L malic acid seems unlikely. In order to maintain the maximum production rate over an extended period of time, it appears to be necessary to further optimize the culture conditions such as the carbon availability and C:N ratio. Shake flask experiments evaluating different nitrogen sources

for growth and organic acid synthesis demonstrated that especially the addition of yeast extract considerably increased the biomass concentration and accelerated germination as well as malic acid production compared to the utilization of $(NH_4)_2SO_4$. Therefore, it will be interesting to investigate the repeated addition of low concentrations of this nitrogen source to potentially regenerate biomass and improve production in further studies.

The production rates obtained from acetate as described in this study are currently not sufficient for industrial application. Although the productivity could be considerably enhanced with suitable process management strategies, the maximum rate of 0.112 ± 0.007 g/L/h calculated for a fermentation period of 264 h is only about 10% of the highest rates reported for glucose. However, the high volumetric productivities on glucose were obtained with genetically modified strains in optimized fed-batch processes. Reducing the side product concentrations, especially the one of succinic acid, is crucial for improving malate production with *A. oryzae* from acetate. Since the glyoxylate cycle is involved in malate synthesis when acetate is the carbon source, especially the share of succinic acid is increased to about 40% of the total acid concentration. Hence, improving the pathway from succinate to malate has the potential to almost double the malate concentration. Evaluating the metabolic pathway from acetate to malate in *A. oryzae* in more detail through ^{13}C NMR analysis, for example, would help to better understand the process and elucidate the potential for genetic modification. Thus, the application of genetic engineering tools in combination with a suitable feeding strategy has great potential to further improve malic acid synthesis from acetate.

Whereas the metabolization of glucose generates ATP, the activation of acetate to acetyl-CoA requires ATP, and the yield is reduced. To obtain value-added products from acetate in an economic process, the use of inexpensive side or waste streams therefore seems essential, especially since raw material costs represent a large part of the total production costs of microbial processes. Pyrolytic aqueous condensates could be a potential candidate for such substrates, since an energetic exploitation is often not or only partially possible due to their low heating value. For instance, in the case of the bioliq® process developed at the Karlsruhe Institute of Technology, these condensates contain acetic acid at a concentration of 30-50 g/L, which is a suitable range for the production of malic acid, as shown in this study. Instead of potentially having to dispose of these side streams, they could serve as a substrate for the synthesis of value-added products such as malic acid. However, they are complex mixtures and require a detoxification step to be efficiently used by micro-organisms, which increases production costs. Since *A. oryzae* accepts a broad substrate spectrum, biomass-derived substrates with low levels of inhibitors could potentially perform better than pure acetate due to the presence of carbohydrates. It is also conceivable to co-feed a mainly carbohydrate-containing substrate such as a lignocellulosic hydrolysate at a low concentration to boost product synthesis. Thus, there are various options which can be explored for the development of an economic acetate-based process and further studies are required to prove their validity.

Research concerning microbial malic acid production has intensified especially in the past ten years, but this study is the first to provide a detailed evaluation of malic acid production from acetate. The utilization of this carbon source presents several challenges such as substrate inhibition at rather low carbon concentrations, an increased proportion of side products, a limited suitable pH range for product synthesis, and an altered morphology of *A. oryzae* depending on the acetate concentration. However, due to the abundance of acetate in biomass-derived substrates, it has great potential as a carbon source in a bioeconomy. As demonstrated in this study, product synthesis can be considerably enhanced with suitable process management strategies and various options for further improvements were highlighted. The lessons learned will contribute to the further development of acetate-based processes and microbial organic acid production from biomass-derived substrates.

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List of figures

List of tables

List of abbreviations

ACK: Acetate kinase

ACN: Aconitase

ACS: Acetyl-CoA synthetase

CS: Citrate synthase

FRD: Fumarate reductase

FUM: Fumarase

Glc: Glucose

HAc: Acetic acid

HPLC: High performance liquid chromatography

ICL: Isocitrate lyase

IDH: Isocitrate dehydrogenase

KDH: α-Ketoglutarate dehydrogenase

M: Malic acid

MDH: Malate dehydrogenase

MES: Microbial electrosynthesis

MS: Malate synthetase

NMR: Nuclear magnetic resonance

PC: Pyruvate carboxylase

PDH: Pyruvate dehydrogenase

PEP: Phosphoenolpyruvate

PEPC: Phosphoenolpyruvate carboxylase

PHAs: Polyhydroxyalkanoates

PK: Pyruvate kinase

PTA: Phosphate acetyltransferase

RI: Refractive index

S: Succinic acid

SCS: Succinyl-CoA synthetase

SDH: Succinate dehydrogenase

TCA: Tricarboxylic acid cycle

rTCA: reductive Tricarboxylic acid cycle

Appendix A

Calculation of theoretical maximum molar yield of malic acid from acetate through the glyoxylate cycle

Acetate is first converted to acetyl-CoA through acetyl-CoA synthetase (ACS) or acetate kinase (ACK) and phosphate acetyltransferase (PTA) which requires energy in the form of ATP (A1). Catalyzed by citrate synthase, acetyl-CoA then enters the TCA cycle through condensation with oxaloacetate, generating citrate (A2) which is converted to isocitrate by aconitase (A3). Subsequently, isocitrate lyase catalyzes the transformation of isocitrate to succinate and glyoxylate (A4). While the succinate undergoes further oxidation to malate (A6, A7), glyoxylate and acetyl-CoA are condensed to malate by malate synthase (A5). One of the malate molecules reenters the cycle through oxaloacetate, eventually resulting in the formation of one mol of malate from two mol of acetate (A9).

$$2\ \text{Acetate} + 2\ \text{ATP} + 2\ \text{CoA} \rightarrow 2\ \text{Acetyl-CoA} + 2\ P_i^{1} + 2\ \text{ADP}^{2} \quad \text{(A1)}$$

$$\text{Acetyl-CoA} + \text{Oxaloacetate} + H_2O \rightarrow \text{Citrate} + \text{CoA} \quad \text{(A2)}$$

$$\text{Citrate} \rightarrow \text{Isocitrate} \quad \text{(A3)}$$

$$\text{Isocitrate} \rightarrow \text{Succinate} + \text{Glyoxylate} \quad \text{(A4)}$$

$$\text{Glyoxylate} + \text{Acetyl-CoA} + H_2O \rightarrow \text{Malate} + \text{CoA} \quad \text{(A5)}$$

$$\text{Succinate} + \text{FAD} \rightarrow \text{Fumarate} + FADH_2 \quad \text{(A6)}$$

$$\text{Fumarate} + H_2O \rightarrow \text{Malate} \quad \text{(A7)}$$

$$\text{Malate} + NAD^+ \rightarrow \text{Oxaloacetate} + \text{NADH} + H^+ \quad \text{(A8)}$$

$$2\ \text{Acetate} + 2\ \text{ATP} + 3\ H_2O + \text{FAD} + NAD^+ \rightarrow \text{Malate} + 2\ P_i^{1} + 2\ \text{ADP}^{2} + FADH_2 + \text{NADH} + H^+ \quad \text{(A9)}$$

[1] P_i in case of involvement of ACK/PTA, PP_i in case of ACS.
[2] ADP in case of involvement of ACK/PTA, AMP in case of ACS.

Considerations regarding the energy requirements

As described above, the activation of acetate to acetyl-CoA can follow either the route via ACS or ACK/PTA. If the more energy-intense pathway through ACS is considered, 4 ATP are required for the assimilation of 2 acetate due to the production of AMP instead of ADP. AMP can be regenerated by adenylate kinase according to the following equation (Hoang et al., 1991):

$$\text{AMP} + \text{ATP} \rightarrow 2\ \text{ADP} \quad \text{(A10)}$$

Taking the AMP regeneration into consideration, Equation (A1) becomes the following:

$$2\ \text{Acetate} + 4\ \text{ATP} + 2\ \text{CoA} \rightarrow 2\ \text{Acetyl-CoA} + 2\ PP_i + 4\ \text{ADP} \quad \text{(A11)}$$

And Equation (A9) then results in:

$$2\ \text{Acetate} + 4\ \text{ATP} + 3\ H_2O + \text{FAD} + NAD^+ \rightarrow \text{Malate} + 2\ PP_i^1 + 4\ \text{ADP} + FADH_2 + \text{NADH} + H^+ \quad \text{(A12)}$$

Thus, 2 to 4 ATP are required to produce one molecule of malate. In this process, one molecule of $FADH_2$ and NADH is generated, respectively. Under aerobic conditions, electrons from these reduction equivalents are transferred to oxygen via the electron transport chain (ETC) while protons are pumped over the inner mitochondrial membrane in eukaryotes or the plasma membrane in prokaryotes. These protons are then used by ATP synthase to regenerate ATP from ADP. The number of protons required for the regeneration of one ATP is generally assumed to be between 2 and 4, depending on the organism (Jiang et al., 2001; Petersen et al., 2012). The number of protons which can be obtained from one NADH or $FADH_2$ is often calculated to be 10 and 6, respectively, assuming a complete pathway of the electrons through complexes I-IV (NADH) or II-IV ($FADH_2$) of the mitochondrial ETC (Guerra et al., 2002). However, there are branches of the ETC such as the one introduced by alternative oxidase which directly transfers electrons from ubiquinol (QH_2) from complex II to O_2. Alternative oxidase seems to be associated with metabolic stress and has also been found in *Aspergillus* species (Magnani et al., 2007; Tian et al., 2020). This reaction limits the number of protons per NADH to the one pumped through complex I, which is generally assumed to be 4, resulting in a considerably reduced ATP regeneration.

Hence, there are various possibilities to calculate the theoretical yield of malate from acetate if the energy levels are considered. Assuming the more energy-intense pathway involving ACS for acetate assimilation, which appears to be the main pathway in eukaryotic microorganisms (Connerton et al., 1990; Ingram-Smith et al., 2006), 4 ATP are required for the production of one molecule of malate. If furthermore the electron transfer through the complete ETC, and an H^+/ATP ratio of 4 is assumed, this leads to the regeneration of 4 ATP (2.5 from NADH and 1.5 from $FADH_2$), which closes the energy balance. However, if a less efficient electron transport is assumed, such as through alternative oxidase, the ATP yield is considerably reduced and more acetate would be required to enter the oxidative TCA cycle for respiration, which lowers the malate yield.

In conclusion, a maximum theoretical yield of malate from acetate of 0.5 mol/mol seems plausible considering the carbon balance. Depending on the pathway for acetate assimilation, potential branches in the ETC which reduce the proton flow, and the assumptions made for the H^+/ATP ratio, the yield can be lower. A detailed analysis of these aspects for the respective production organism are therefore required to determine the yield conclusively.

Appendix B

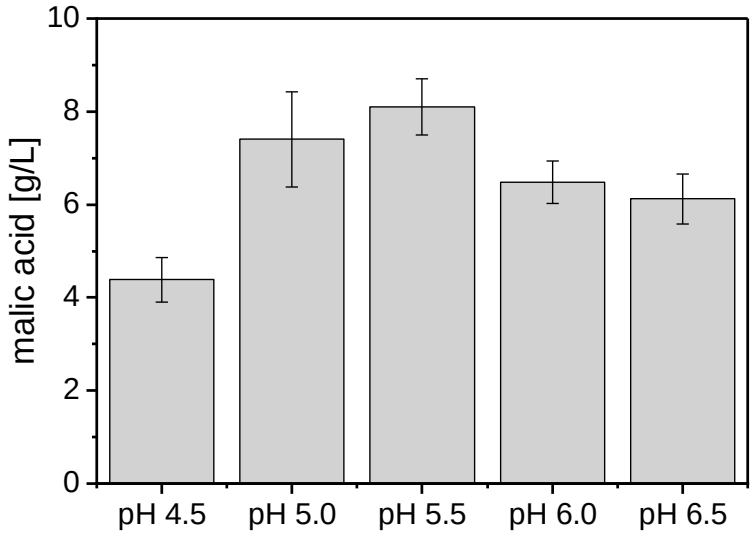

Figure B1. Evaluation of optimum initial pH for malic acid production with acetate. Initial medium pH-values of 4.5, 5.0, 5.5, 6.0 and 6.5 were tested and malic acid concentration after 192 h is displayed. Experiments were performed with 45 g/L acetic acid, 1.2 g/L $(NH_4)_2SO_4$ and 90 g/L $CaCO_3$. Data are the means of triplicate experiments with respective standard deviations.

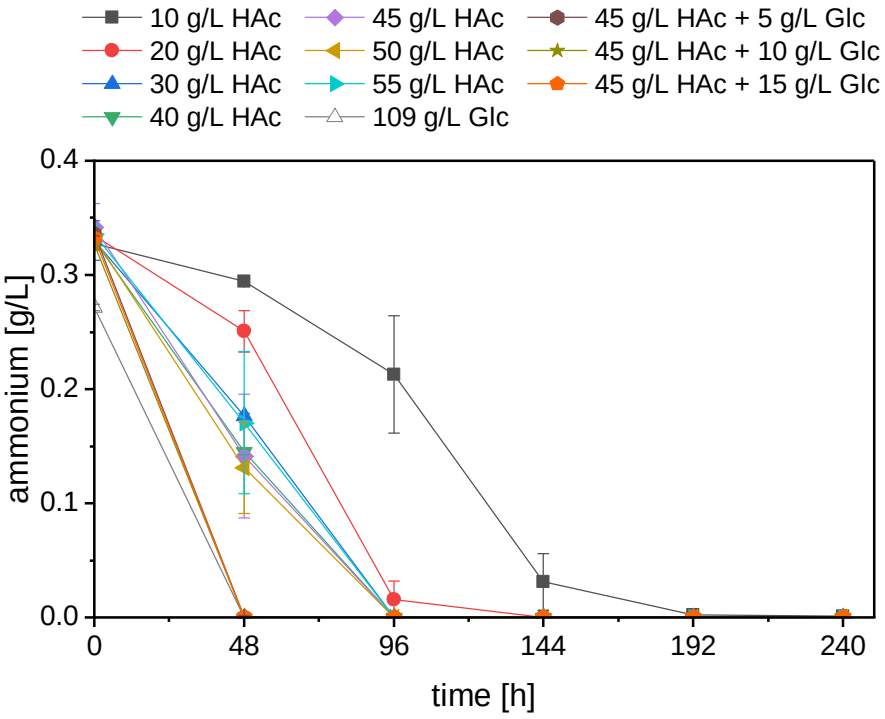

Figure B2. Determination of ammonium consumption with different substrate concentrations. Data are the means of triplicate experiments with respective standard deviations. HAc = acetic acid, Glc = glucose.

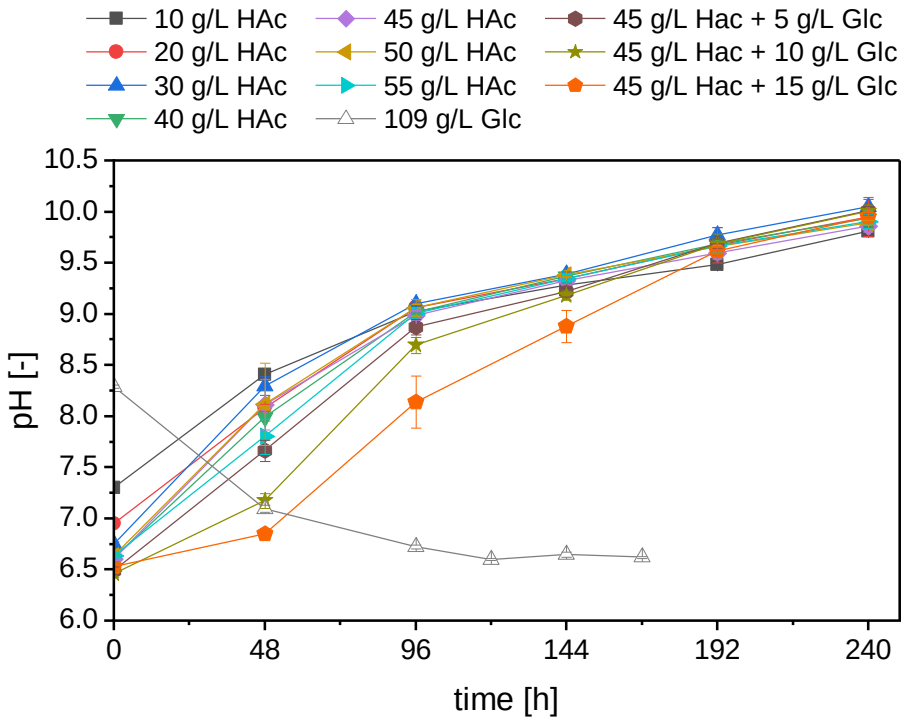

Figure B3. pH-values depending on substrate type and concentration. Data are the means of triplicate experiments with respective standard deviations. HAc = acetic acid, Glc = glucose.

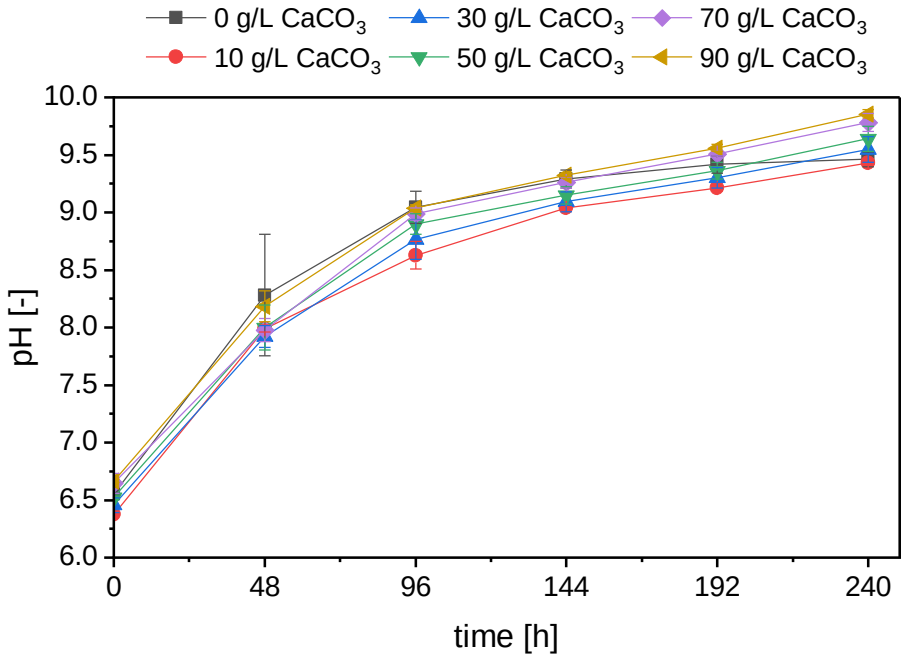

Figure B4. pH-values depending on the $CaCO_3$ concentration. Data are the means of triplicate experiments with respective standard deviations.

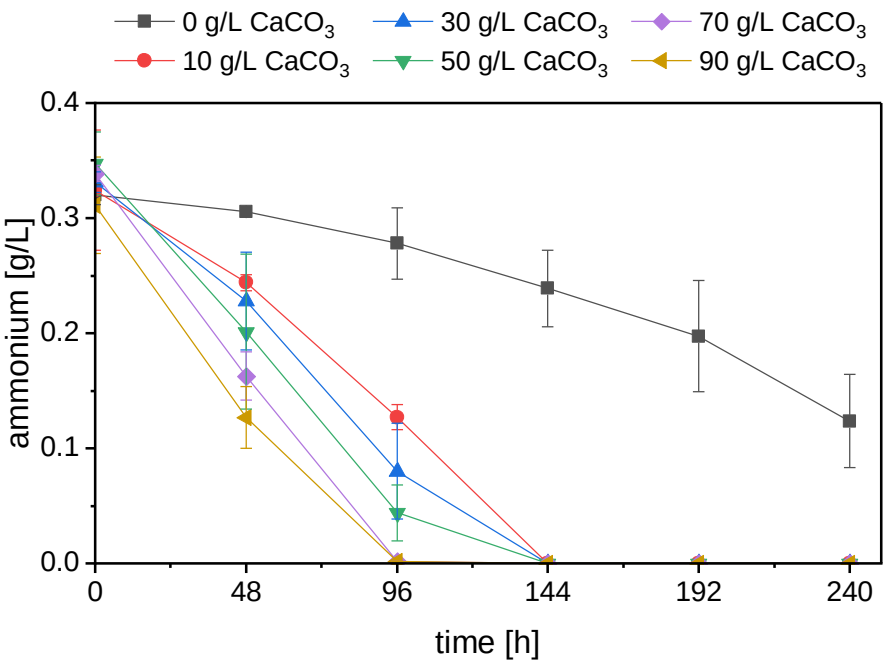

Figure B5. Determination of ammonium consumption depending on the $CaCO_3$ concentration. Data are the means of triplicate experiments with respective standard deviations.

Appendix C

Table C1. Organic acid distribution for cultivations of *A. oryzae* at different temperatures after 144 h. Data are the means of quadruplicate experiments with respective standard deviations.

Substrate	T [°C]	Organic acids [%]						
		Malate	Succinate	Fumarate	Pyruvate	α-Keto-glutarate	Oxalate	Citrate
Acetic acid	29	52.5 ± 2.4	34.8 ± 2.3	4.5 ± 0.4	0.6 ± 0.1	0.2 ± 0.1	6.2 ± 1.6	1.2 ± 0.6
	32	53.7 ± 1.6	38.7 ± 1.4	3.8 ± 0.4	0.7 ± 0.0	0.3 ± 0.1	2.2 ± 1.9	0.6 ± 0.2
	35	48.6 ± 0.9	43.9 ± 0.6	4.0 ± 0.1	1.0 ± 0.1	0.3 ± 0.1	1.2 ± 0.1	1.0 ± 0.4
	38	43.3 ± 1.1	51.5 ± 1.0	2.8 ± 0.1	1.1 ± 0.0	0.4 ± 0.1	0.3 ± 0.1	0.8 ± 0.2
Glucose	29	64.7 ± 2.4	16.5 ± 1.4	2.5 ± 0.0	0	0.2 ± 0.0	1.7 ± 0.1	14.4 ± 1.7
	32	73.8 ± 1.0	16.7 ± 0.2	1.5 ± 0.1	0.8 ± 0.2	0.4 ± 0.0	0.2 ± 0.1	6.6 ± 0.9
	35	68.8 ± 2.1	18.8 ± 1.3	1.4 ± 0.1	4.0 ± 0.4	0.9 ± 0.1	0.1 ± 0.0	6.1 ± 0.5
	38	63.4 ± 2.0	21.3 ± 1.4	1.5 ± 0.2	7.3 ± 0.5	1.7 ± 0.1	0.1 ± 0.0	4.7 ± 0.2

Table C2. Organic acid distribution for cultivations of *A. oryzae* with different nitrogen sources after 144 h. Data are the means of triplicate experiments with respective standard deviations.

Nitrogen source	Organic acids [%]						
	Malate	Succinate	Fumarate	Pyruvate	α-Keto-glutarate	Oxalate	Citrate
$(NH_4)_2SO_4$	54.8 ± 2.3	37.1 ± 2.2	3.7 ± 0.4	0.6 ± 0.0	0.4 ± 0.0	1.8 ± 0.4	1.6 ± 0.5
Urea	49.3 ± 1.5	38.1 ± 1.5	5.7 ± 0.5	1.0 ± 0.0	0.6 ± 0.1	4.5 ± 1.2	0.8 ± 0.8
Glutamate	53.9 ± 0.8	35.9 ± 1.9	4.1 ± 0.1	0.6 ± 0.0	0.7 ± 0.1	2.9 ± 1.1	1.9 ± 0.4
Yeast extract	52.5 ± 1.6	37.1 ± 1.0	4.1 ± 0.4	0.6 ± 0.0	0.5 ± 0.1	3.2 ± 1.3	1.9 ± 0.4
Casein hydrolysate	52.0 ± 1.3	36.1 ± 1.4	4.5 ± 0.6	1.0 ± 0.1	0.5 ± 0.0	3.8 ± 2.3	2.1 ± 0.1
Peptone	50.7 ± 0.7	38.7 ± 0.7	4.1 ± 0.1	0.7 ± 0.1	0.5 ± 0.1	3.9 ± 1.3	1.4 ± 0.3

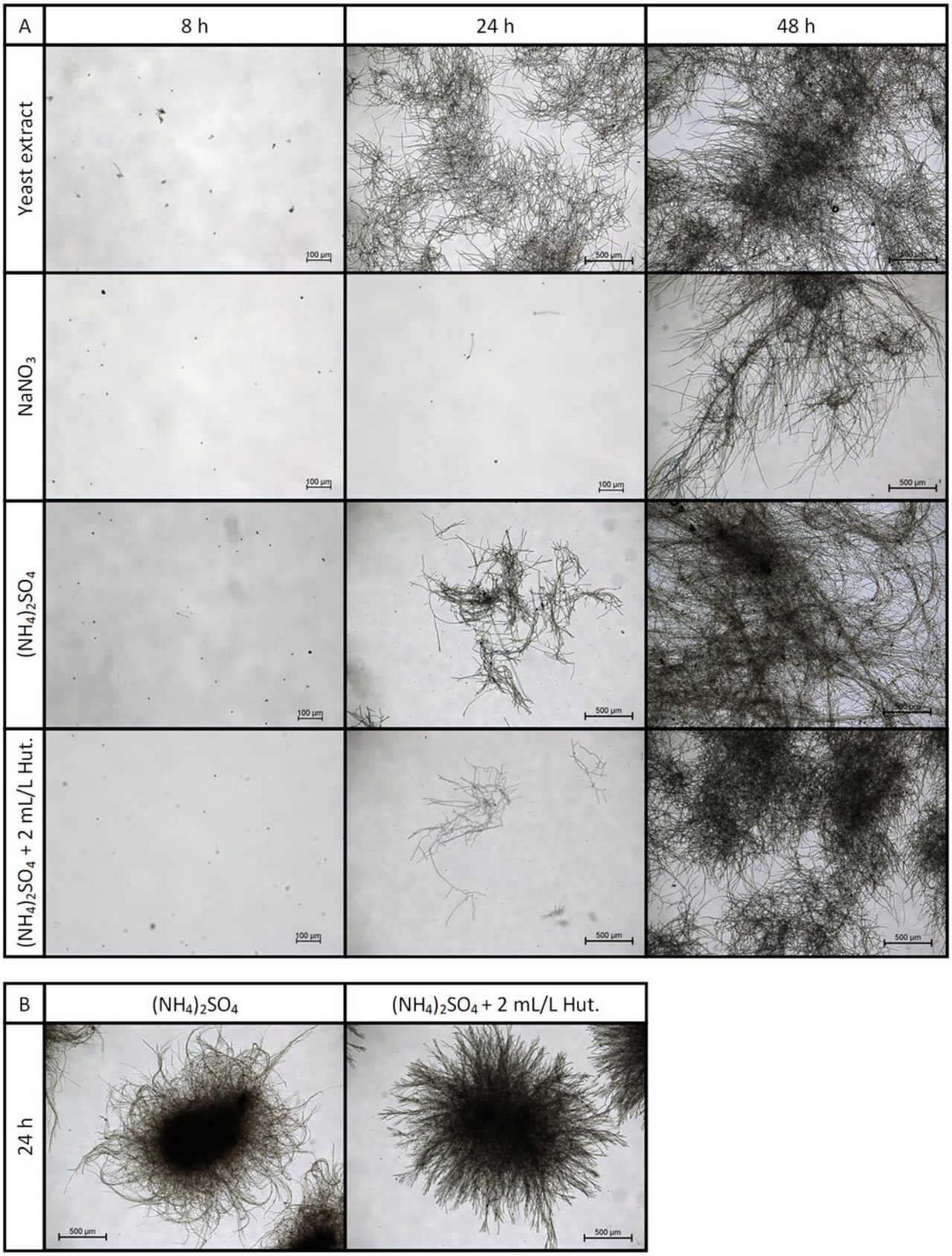

Figure C1. Morphology of *A. oryzae* after growth with different nitrogen sources and Hutner's trace element solution using acetate (A) or glucose (B) as carbon source.

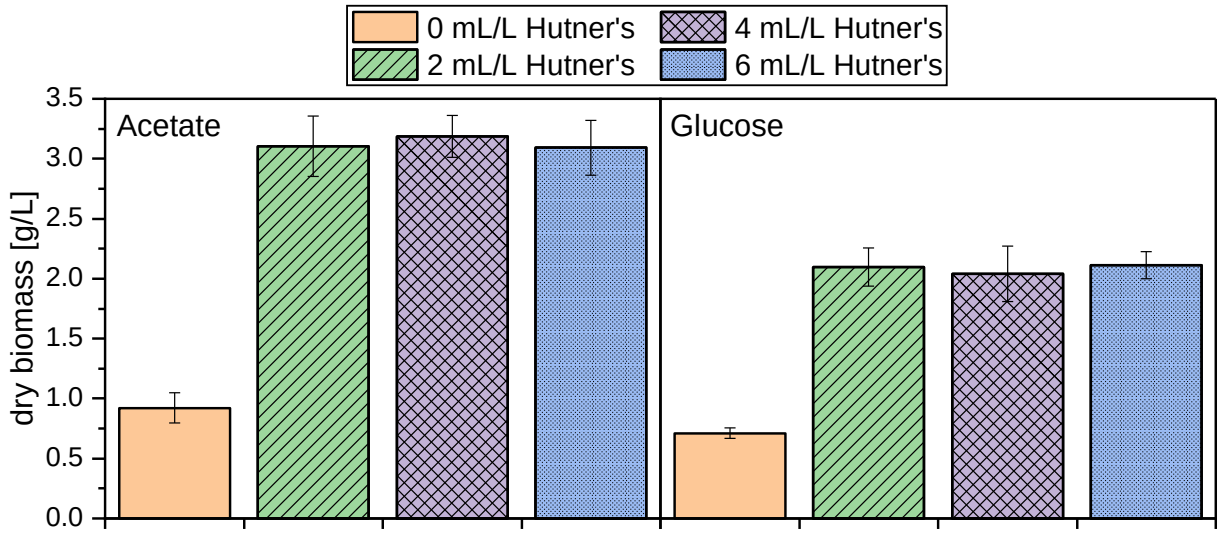

Figure C2. Biomass formation of *A. oryzae* after 48 h (acetate) or 24 h (glucose) of growth in preculture medium with different concentrations of 1000x Hutner's trace element solution. Data are the means of triplicate experiments with respective standard deviations.

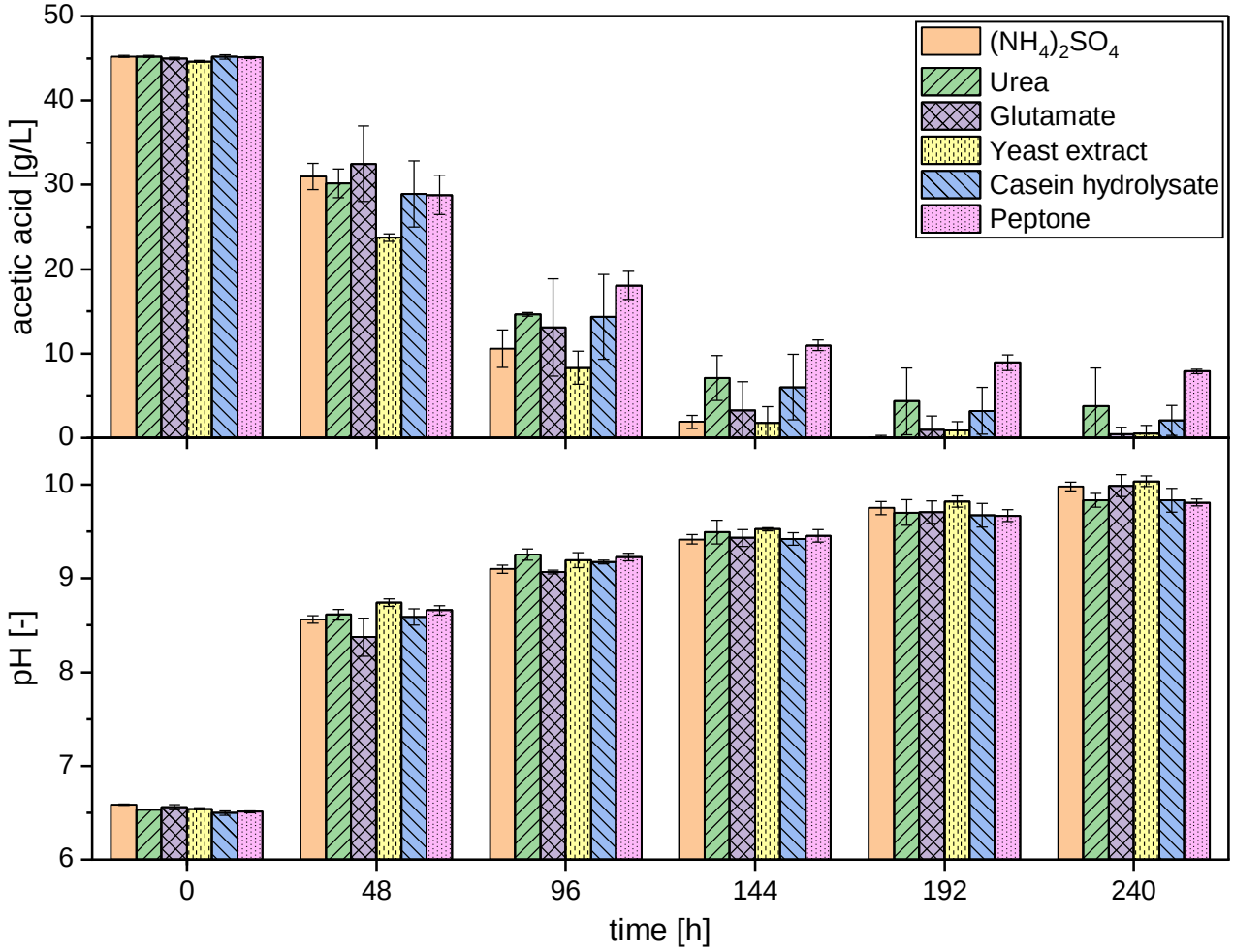

Figure C3. Time course of acetic acid concentration and pH during cultivations of *A. oryzae* with different nitrogen sources. Data are the means of triplicate experiments with respective standard deviations.

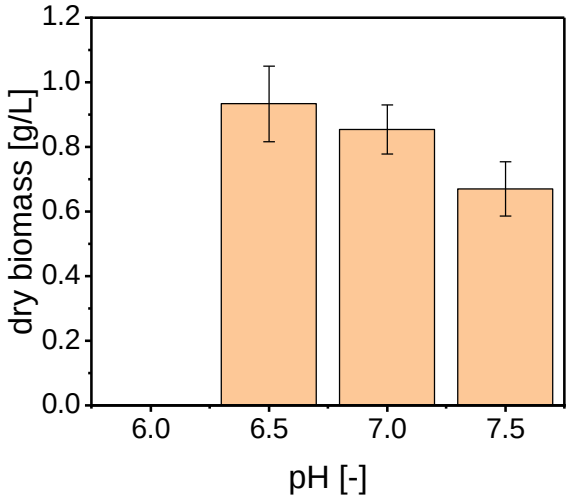

Figure C4. Dry biomass concentration of *A. oryzae* after 48 h of cultivation in medium with 45 g/L acetic acid adjusted to different pH values. Data are the means of triplicate experiments with respective standard deviations.

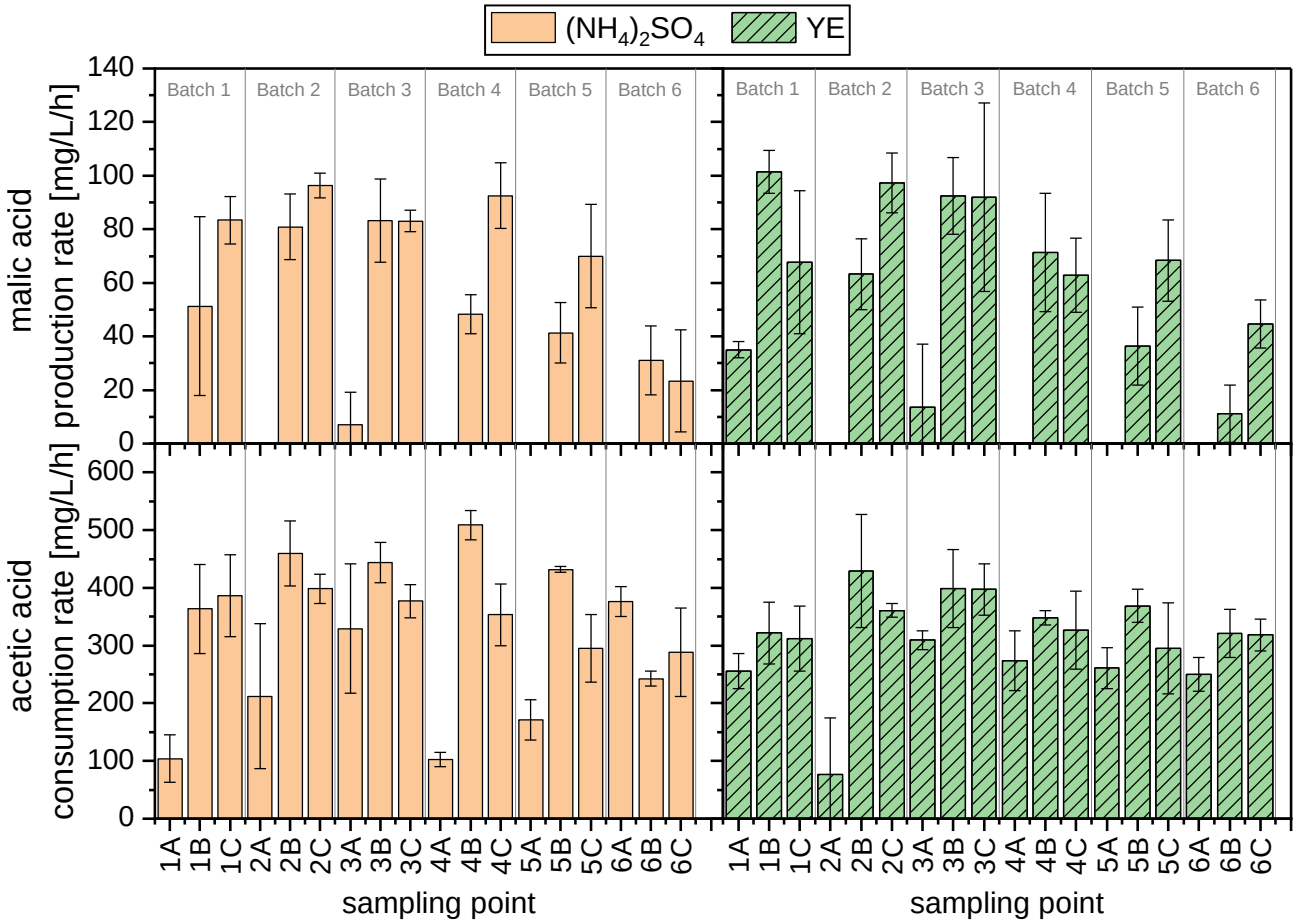

Figure C5. Malic acid production rate and acetic acid consumption rates calculated between each measurement point (see Figure 12) for the repeated-batch process. Hence, "1A" refers to the rates calculated for the first 48 h in the first batch cycle, "1B" are the rates calculated between 48 h and 72 h in the first batch, values for "2A" were determined for the first 24 h of the second batch and so on. Data are the means of triplicate experiments with respective standard deviations.

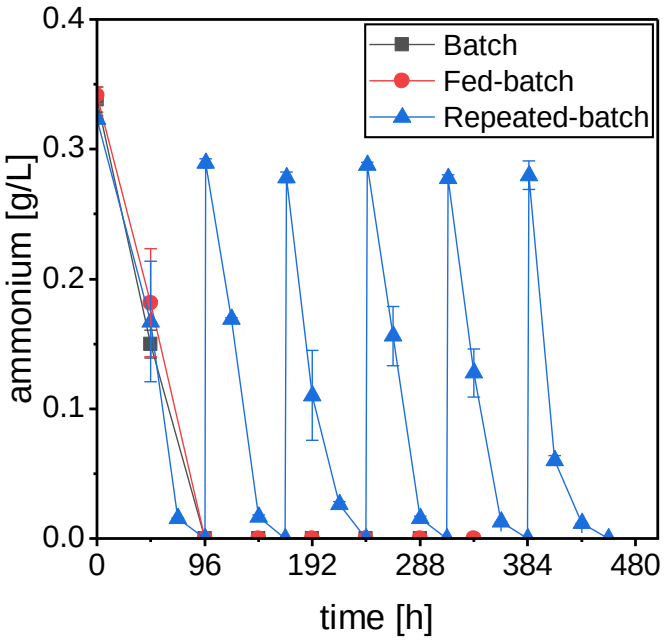

Figure C6. Ammonium concentration for batch, fed-batch and repeated-batch production of L-malic acid with $(NH_4)_2SO_4$ as nitrogen source. Data are the means of triplicate experiments with respective standard deviations.

Table C3. Organic acid composition for cultivations of *A. oryzae* using different process modes after 240 h. Data are the means of triplicate experiments with respective standard deviations.

Process mode	Nitrogen source	Organic acids [%]						
		Malate	**Succinate**	**Fumarate**	**Pyruvate**	**α-Keto-glutarate**	**Oxalate**	**Citrate**
Batch[a]	$(NH_4)_2SO_4$	54.6 ± 2.0 (52.3 ± 1.4)	34.9 ± 4.1 (37.5 ± 1.0)	3.9 ± 0.3 (3.9 ± 0.4)	0.6 ± 0.1 (0.6 ± 0.1)	0.4 ± 0.0 (0.3 ± 0.1)	3.7 ± 2.4 (3.5 ± 2.2)	1.9 ± 0.7 (1.9 ± 0.4)
	Yeast extract	56.7 ± 0.8 (55.1 ± 1.2)	35.2 ± 0.3 (37.0 ± 0.7)	3.4 ± 0.1 (3.4 ± 0.0)	0.6 ± 0.1 (0.6 ± 0.1)	0.4 ± 0.0 (0.4 ± 0.0)	1.9 ± 0.5 (1.8 ± 0.4)	1.7 ± 0.1 (1.8 ± 0.3)
Fed-batch	$(NH_4)_2SO_4$	54.2 ± 1.0	36.9 ± 1.4	3.1 ± 0.1	0.6 ± 0.1	0.1 ± 0.1	4.0 ± 1.7	1.1 ± 0.2
	Yeast extract	55.2 ± 0.7	39.6 ± 1.0	3.3 ± 0.2	0.6 ± 0.1	0.1 ± 0.1	0.7 ± 0.2	0.5 ± 0.7
Repeated-batch	$(NH_4)_2SO_4$	52.5 ± 2.9	36.3 ± 3.7	5.2 ± 0.7	1.0 ± 0.2	0.1 ± 0.1	4.6 ± 1.9	0.4 ± 0.7
	Yeast extract	54.9 ± 3.5	36.5 ± 1.6	4.2 ± 0.5	0.8 ± 0.4	0.3 ± 0.2	2.7 ± 1.6	0.6 ± 0.5

[a] Values in parentheses show the results after 144 h of cultivation, during which most of the product formation was detected in batch processes.

Appendix D

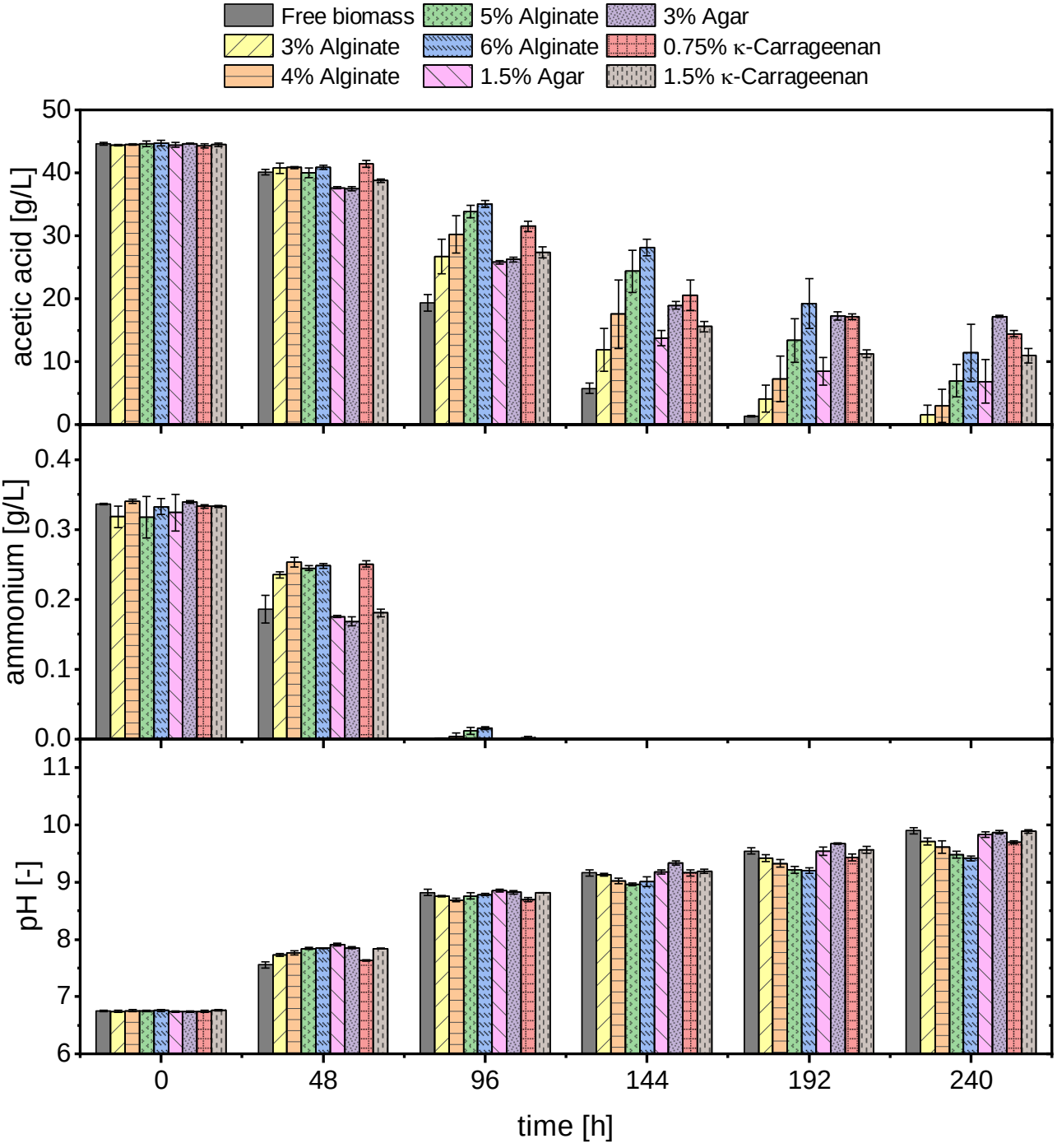

Figure D1. Acetic acid and ammonium concentration as well as progression of the pH value for shake flask experiments with free and immobilized *A. oryzae* using acetate as carbon source. Data are the means of triplicate experiments with respective standard deviations.

Table D1. Acid distribution for shake flask cultivations of free and immobilized *A. oryzae* after 144 h. Data are the means of triplicate experiments with respective standard deviations.

Substrate	Immobilization matrix	Organic acid distribution [%]						
		Malate	Succinate	Fumarate	Pyruvate	α-Keto-glutarate	Oxalate	Citrate
Acetic acid	Free biomass	50.8 ± 0.7	41.8 ± 0.7	4.2 ± 0.5	0.8 ± 0.1	0.3 ± 0.1	1.3 ± 0.3	0.7 ± 0.3
	3% Alginate	52.6 ± 1.2	38.9 ± 0.7	4.6 ± 0.2	1.0 ± 0.3	0.4 ± 0.1	2.1 ± 0.8	0.5 ± 0.4
	4% Alginate	48.4 ± 1.1	41.8 ± 1.0	5.1 ± 0.3	0.9 ± 0.3	0.5 ± 0.0	3.2 ± 1.9	0.1 ± 0.2
	5% Alginate	45.1 ± 2.5	36.3 ± 4.4	4.6 ± 1.0	0.6 ± 0.6	0.4 ± 0.4	12.5 ± 9.1	0.4 ± 0.7
	6% Alginate	34.4 ± 5.5	34.5 ± 1.8	3.1 ± 1.0	1.3 ± 0.2	0.3 ± 0.3	26.4 ± 8.1	0.0 ± 0.0
	1.5% Agar	48.5 ± 0.7	41.4 ± 1.4	5.1 ± 0.4	1.3 ± 0.1	0.4 ± 0.1	2.9 ± 1.2	0.4 ± 0.3
	3% Agar	48.5 ± 2.2	34.6 ± 5.7	5.6 ± 0.2	1.3 ± 0.2	0.3 ± 0.2	9.2 ± 2.6	0.6 ± 0.6
	0.75% κ-Carrageenan	43.8 ± 2.1	41.0 ± 2.8	4.8 ± 0.3	1.0 ± 0.2	0.6 ± 0.2	8.0 ± 1.1	0.8 ± 0.9
	1.5% κ-Carrageenan	49.0 ± 0.9	38.9 ± 1.0	5.4 ± 0.2	1.1 ± 0.1	0.4 ± 0.0	4.5 ± 0.6	0.7 ± 0.3
Glucose	Free biomass	73.3 ± 2.7	18.4 ± 1.4	1.7 ± 0.2	1.5 ± 0.6	0.4 ± 0.0	0.2 ± 0.0	4.6 ± 1.8
	1.5% Alginate	69.6 ± 1.6	19.1 ± 1.9	1.1 ± 0.1	2.5 ± 0.3	0.4 ± 0.0	0.2 ± 0.0	7.1 ± 0.5
	3% Alginate	72.3 ± 1.9	15.0 ± 1.8	1.7 ± 0.0	2.3 ± 0.4	0.3 ± 0.0	1.4 ± 0.5	7.0 ± 0.4
	4% Alginate	72.8 ± 0.8	15.0 ± 0.5	1.5 ± 0.2	2.8 ± 0.3	0.3 ± 0.0	0.8 ± 0.2	6.8 ± 0.4
	5% Alginate	70.2 ± 1.5	16.0 ± 0.7	1.9 ± 0.1	2.6 ± 1.0	0.3 ± 0.1	1.3 ± 0.7	7.7 ± 1.1
	6% Alginate	72.0 ± 1.8	14.7 ± 1.8	2.0 ± 0.2	2.2 ± 1.7	0.2 ± 0.1	1.8 ± 1.0	7.0 ± 0.7
	1.5% Agar	71.0 ± 2.1	16.3 ± 0.9	1.5 ± 0.2	3.0 ± 0.3	0.3 ± 0.0	0.8 ± 0.2	7.1 ± 1.6
	3% Agar	73.8 ± 0.9	15.6 ± 1.6	1.4 ± 0.0	2.7 ± 0.5	0.3 ± 0.0	0.6 ± 0.1	5.6 ± 0.4
	1.5% κ-Carrageenan	75.7 ± 0.5	13.8 ± 0.2	1.4 ± 0.1	2.9 ± 0.2	0.3 ± 0.1	0.5 ± 0.1	5.3 ± 0.4

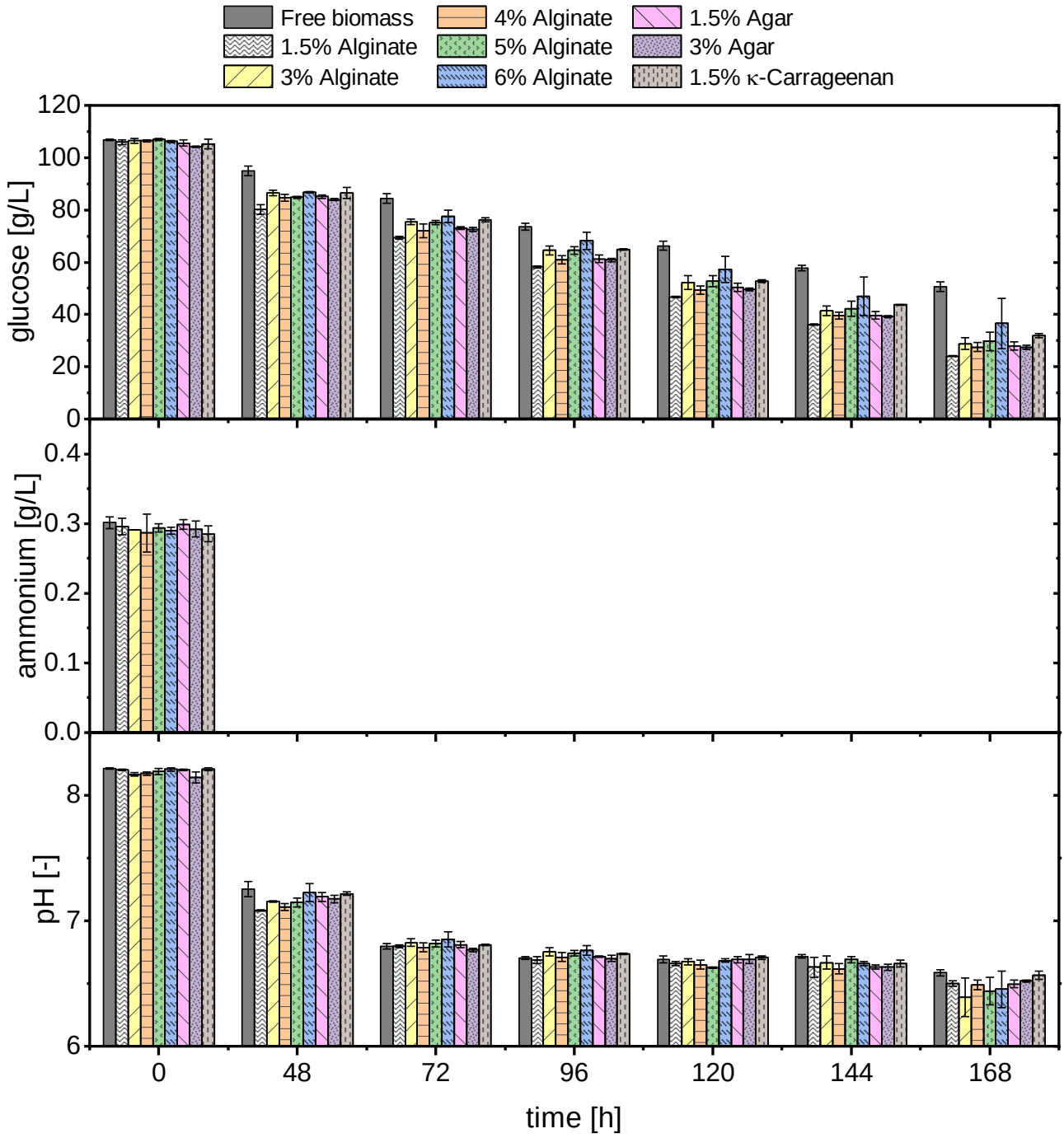

Figure D2. Glucose and ammonium concentration as well as progression of the pH value for shake flask experiments with free and immobilized *A. oryzae* using glucose as carbon source. Data are the means of triplicate experiments with respective standard deviations.

Table D2. Acid distribution for 2.5-L bioreactor cultivations of free and immobilized *A. oryzae* after 144 h. Data are the means of duplicate experiments with respective standard deviations.

Main substrate	Immobilization matrix	Organic acid distribution [%]						
		Malate	Succinate	Fumarate	Pyruvate	α-Keto-glutarate	Oxalate	Citrate
Acetic acid	Free biomass	23.1 ± 4.0	34.1 ± 3.9	1.4 ± 0.5	37.0 ± 10.5	2.1 ± 0.5	1.2 ± 0.9	1.1 ± 1.6
	3% Alginate	48.1 ± 2.0	35.0 ± 2.1	5.1 ± 0.6	10.6 ± 0.5	0.6 ± 0.0	0.6 ± 0.0	0.0 ± 0.0
	6% Alginate	43.6 ± 2.0	36.5 ± 1.4	5.9 ± 1.3	10.6 ± 0.3	1.0 ± 0.7	2.4 ± 0.3	0.0 ± 0.0
	1.5% κ-Carrageenan	37.9 ± 0.5	33.7 ± 0.0	5.1 ± 0.1	21.5 ± 0.7	1.1 ± 0.1	0.7 ± 0.1	0.0 ± 0.0
Glucose	Free biomass	80.7 ± 1.0	15.2 ± 1.5	1.6 ± 0.0	2.2 ± 0.0	0.1 ± 0.1	0.0 ± 0.0	0.3 ± 0.4
	1.5% Alginate	78.4 ± 6.3	17.7 ± 5.8	1.9 ± 0.1	1.2 ± 0.6	0.1 ± 0.1	0.2 ± 0.2	0.5 ± 0.8
	3% Alginate	82.0 ± 0.6	13.7 ± 1.1	2.0 ± 0.1	1.2 ± 0.0	0.2 ± 0.0	0.2 ± 0.1	0.7 ± 0.4
	1.5% κ-Carrageenan	75.0 ± 1.9	21.1 ± 1.6	1.9 ± 0.2	1.5 ± 0.5	0.1 ± 0.0	0.1 ± 0.0	0.3 ± 0.5

Table D3. Acid distribution for shake flask cultivations of immobilized *A. oryzae* after 144 h using acetate as carbon source in the main culture while the preculture was grown without carbon source. Data are the means of triplicate experiments with respective standard deviations.

Substrate	Immobilization matrix	Organic acid distribution [%]						
		Malate	Succinate	Fumarate	Pyruvate	α-Keto-glutarate	Oxalate	Citrate
Acetic acid	3% Alginate	13.5 ± 23.4	12.3 ± 21.3	2.9 ± 0.8	0.0 ± 0.0	0.0 ± 0.0	71.4 ± 44.7	0.0 ± 0.0
	6% Alginate	0.0 ± 0.0	0.0 ± 0.0	0.9 ± 0.1	0.0 ± 0.0	0.0 ± 0.0	99.1 ± 0.1	0.0 ± 0.0
	1.5% Agar	56.6 ± 1.3	34.0 ± 1.3	5.0 ± 0.0	1.2 ± 0.1	0.6 ± 0.2	1.4 ± 0.6	1.3 ± 0.4
	1.5% κ-Carrageenan	53.0 ± 2.4	35.8 ± 1.0	5.1 ± 0.2	1.2 ± 0.1	0.5 ± 0.2	2.1 ± 1.0	1.3 ± 0.5

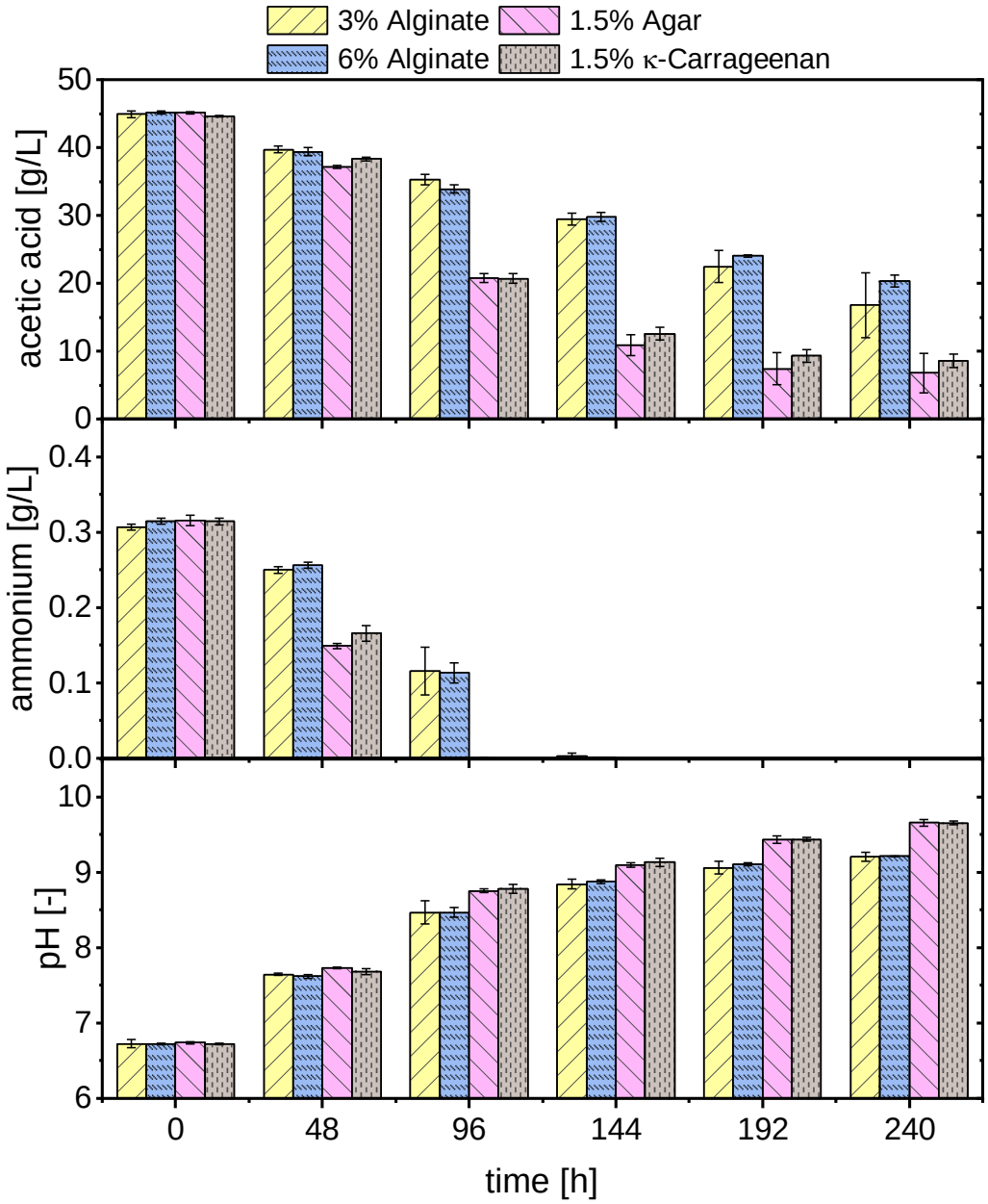

Figure D3. Acetic acid and ammonium concentration as well as progression of the pH value for main culture shake flask experiments using immobilized *A. oryzae* grown without external carbon source in the preculture. Data are the means of triplicate experiments with respective standard deviations.

Appendix E

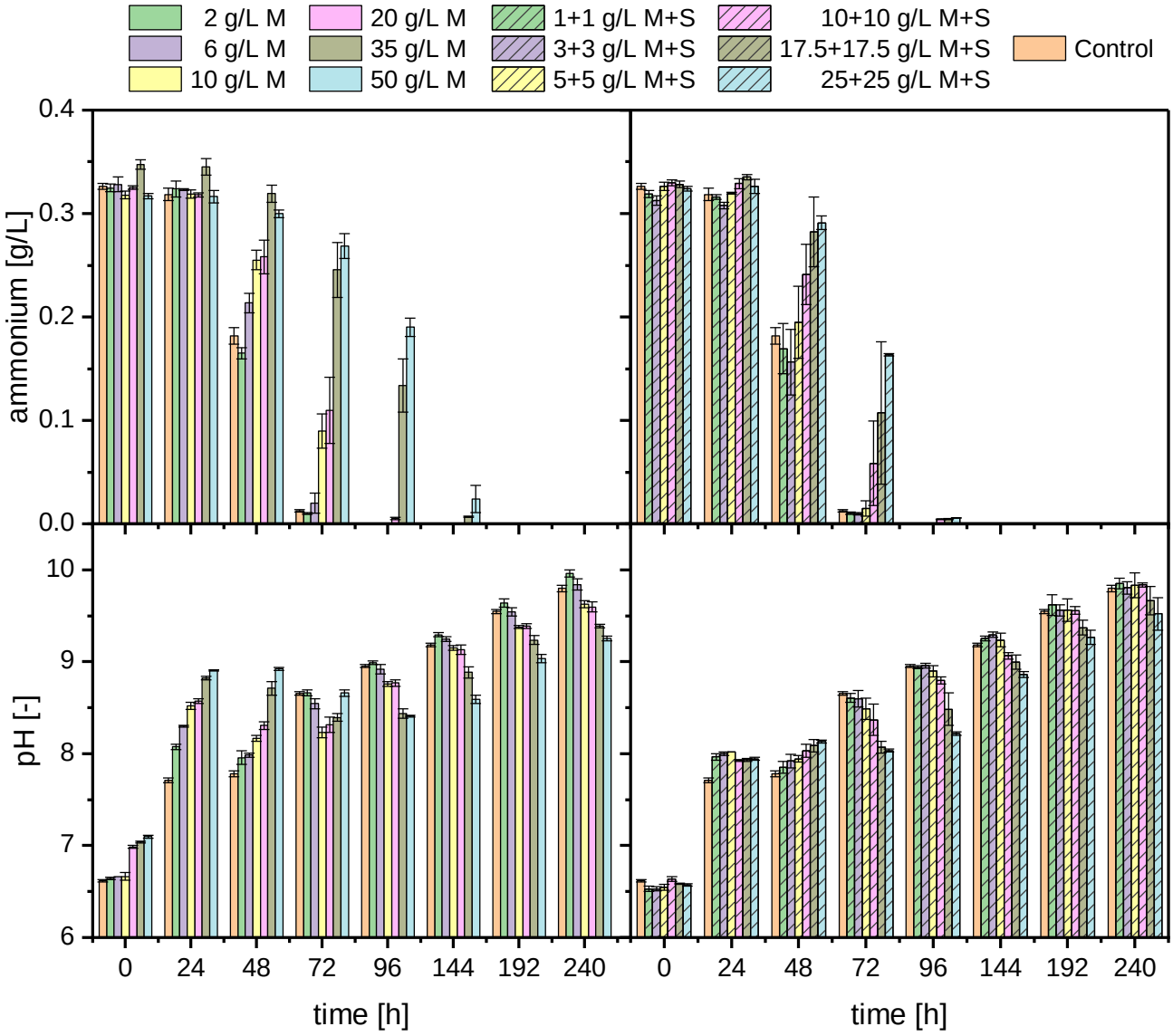

Figure E1. Ammonium consumption and pH development during shake flask cultivation of *A. oryzae* with different initial malic (M) and succinic acid (S) concentrations. Data are the means of triplicate experiments with respective standard deviations.