EVALUATION OF RENEWABLE RESOURCES AS CARBON SOURCES FOR ORGANIC ACID PRODUCTION WITH FILAMENTOUS FUNGI

zur Erlangung des akademischen Grades eines
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DISSERTATION

von
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Preamble

Parts of this thesis are based on peer reviewed research articles. All articles have been drafted during this work and describe the major results of the evaluation of renewable resources for malic and fumaric acid production with the filamentous fungi *A. oryzae* and *R. delemar*. Chapters based on previous published work are indicated as such at the beginning of the chapter. The text of these chapters is partly identical to the content of the publications. Layout, citation style, figures and formatting have been modified and adjusted to the style of this dissertation.

List of Publications

Peer reviewed original Publications:

Dörsam S., Fesseler J., Gorte O., Hahn T., Zibek S., Syldatk C. and Ochsenreither K. (2017). "Sustainable Carbon Sources for Microbial Organic Acid Production with Filamentous Fungi," *Biotechnology for Biofuels* 10 (1). BioMed Central: 242. doi:10.1186/s13068-017-0930-x.

Dörsam S., Kirchoff J., Bigalke M., Dahmen N., Syldatk C. and Ochsenreither, K. (2016). "Evaluation of Pyrolysis Oil as Carbon Source for Fungal Fermentation," *Frontiers in Microbiology* 7 (December): 1–11. doi:10.3389/fmicb.2016.02059

Oswald F.*, **Dörsam S.***, Veith N., Zwick M., Neumann A., Ochsenreither K. and Syldatk, C. (2016). "Sequential Mixed Cultures: From Syngas to Malic Acid." *Frontiers in Microbiology* 7 (June): 1–12. doi:10.3389/fmicb.2016.00891

***Co-first authorship**

Book Chapter

Neumann A., **Dörsam S.**, Oswald F., Ochsenreither K. (2016) Microbial Production of Value-Added Chemicals from Pyrolysis Oil and Syngas. In: Xian M. (eds) *Sustainable Production of Bulk Chemicals*. Springer, Dordrecht: 69–105. doi:10.1007/978-94-017-7475-8_4

Conference Poster

Dörsam, S., Syldatk, C. and Ochsenreither, K. (2017). "Evaluation of renewable resources for fungal organic acid production," 2. Bioökonomiekongress Baden-Württemberg

Dörsam S., Syldatk C. and Ochsenreither, K. (2016). "Toxic effects of different substances from pyrolysis oil on growth and production of malic acid by *Aspergillus oryzae*," VAAM Jahrestagung

Dörsam S., Syldatk C. and Ochsenreither K. (2015). "Malate production from renewable resources by *Aspergillus oryzae*," FEMS, 6th Congress of European Microbiologists

Dörsam S., Syldatk C. and Ochsenreither, K. (2014). "Evaluation of pyrolysis oil as platform for fungal fermentation," 1. Bioökonomiekongress Baden-Württemberg

Abstract

The transformation of the modern economy to a bio-economy, based on biological raw materials requires completely new approaches in research, development and production. Above all, the combination of biotechnological and chemical substance conversion plays an important role. While developing processes based on renewable resources, the "food or fuel" dilemma has to be strongly considered. Lignocellulose as feedstock for those kinds of processes could be a part of the solution for this problem. The lignocellulosic bio refinery supplies pretreated fractions of lignocellulose, accessible for chemistry and biotechnology that possibly can be metabolized and converted by microorganisms to more valuable chemicals and platform chemicals. Promising candidates for these demands are dicarboxylic acids. Because of the high diversity of convertible carbon sources, as well as the known robustness fungi are the optimal organic acid producer used for the evaluation of renewable resources as carbon sources for organic acid production. In addition to L-malate, fumarate is one of the most important, high-quality basic chemical that can be produced from renewable resources through microbial fermentation.

Two different pretreatment methods of lignocellulose for fermentation were evaluated in this work. The pyrolysis process leads to two fractions, an organic (pyrolysis oil) and an aqueous condensate. Further gasification of a mixture of both condensates leads to the formation of syngas. During the organosolv-process, lignocellulose is separated into its basic components, lignin, cellulose and hemicellulose. In secondary refining, saccharification of the cellulose into glucose and hemicellulose into xylose is carried out by enzymatic hydrolysis.

Pyrolysis oil, a complex mixture of several organic compounds, produced during flash pyrolysis of organic lignocellulosic material was evaluated for its suitability as alternative carbon source for fungal growth and fermentation processes (chapter 2). Therefore several fungi from all phyla were screened for their tolerance towards pyrolysis oil. Additionally *Aspergillus oryzae* and *Rhizopus delemar*, both established organic acid producers, were chosen as model organisms to investigate the suitability of pyrolysis oil as carbon source in fungal production processes. It was observed that *A. oryzae* tolerates pyrolysis oil concentrations between 1 - 2 % depending on growth phase or stationary production phase, respectively. To investigate possible reasons for the low tolerance level, eleven compounds from pyrolysis oil representing aldehydes, organic acids, small organic compounds and phenolic substances were selected to determine maximum concentrations still allowing growth and organic acid production. Furthermore, the effect

of various substances to malic acid production were analyzed and compounds were categorized regarding their properties. To validate the results, further tests were also performed with *R. delemar*. For the first time it could be shown that small amounts of phenolic substances are even beneficial for organic acid production and *A. oryzae* might be able to degrade isoeugenol. Regarding pyrolysis oil toxicity, 2-cyclopenten-1-on was identified as the most toxic compound for filamentous fungi. This compound has never been described for anti-fungal or any other toxic properties before and possibly is responsible for the low fungal tolerance levels towards pyrolysis oil.

The aqueous condensate produced during flash pyrolysis of organic lignocellulosic material, was analyzed as substrate in the third chapter of this work. The main components are acetic acid with 4.5%, hydroxyacetone with 3.5%, methanol with 1.7%, ethylene glycol with 0.5% and propionic acid with 0.4%. As the effect of methanol in main culture medium in this concentration range is minimal, hydroxyacetone is the only main component with an inhibitory concentration below the concentration in the aqueous condensate. The cultivation in aqueous condensate model mixtures showed the possible suitability of the aqueous condensate when diluted 1:1. For evaluating the aqueous condensate as carbon source, shake flask cultivations with diluted and undiluted fractions, as well as several detoxifying pretreatments done to reduce the amount of phenolic and solvent like compounds but did not result in product formation.

The fourth chapter of this thesis describes the evaluation of synthesis gas (Syngas) as a possible carbon source for fermentation with the filamentous fungus *A. orzae*. Because this fungus is not able to metabolize sygase directly, the idea of a coupled process in which one organism produces a product from syngas which can also be the carbon source for the fungus was implemented. Synthesis gas fermentation using acetogenic bacteria is an approach for production of bulk chemicals like acetate, ethanol, butanol or 2,3-butandiol avoiding the “food or fuel” dilemma by using carbon monoxide, carbon dioxide and hydrogen from gasification of biomass or industrial waste gases. In this study, it was shown that *Aspergillus oryzae* is able to produce malic acid using acetate as sole carbon source which is a main product of acetogenic syngas fermentation. During the syngas fermentation as part of the sequential mixed culture, *Clostridium ljungdahlii* was grown with artificial syngas modeling a composition of clean syngas from entrained bed gasification of straw (32.5 vol-% CO, 32.5 vol-% H_2, 16 vol-% CO_2 and 19 vol-% N_2). Syngas consumption was monitored via automated gas chromatographic measurement of the off-gas. For the sequential fungal fermentation part gas sparging was switched from syngas to 0.6 L/min of air. Ammonia content of medium for syngas fermentation was reduced to

0.33 g/L NH_4Cl to meet the requirements for fungal production of dicarboxylic acids. Malic acid production performance of *A. oryzae* in organic acid production medium and syngas medium with acetate as sole carbon source was verified and gave $Y_{P/S}$ values of 0.28 g/g and 0.37 g/g respectively. Growth and acetate formation of *C. ljungdahlii* during syngas fermentation were not affected by the reduced ammonia content and 66 % of the consumed syngas was converted to acetate. The overall conversion of CO and H_2 into malic acid was calculated to be 3.5 g malic acid per mol of consumed syngas or 0.22 g malic acid per gram of syngas.

The fifth chapter of this work describes the evaluation of the second pretreatment method for lignocellulose used in this study. The organic acid producer *A. oryzae* and *R. delemar* are able to convert several alternative carbon sources to malic and fumaric acid. Thus, carbohydrate hydrolysates from lignocellulose separation are likely suitable as substrate for organic acid production with these fungi. Before lignocellulose hydrolysate fractions were tested as substrates, experiments with several mono- and disaccharides, possibly present in pretreated biomass, were conducted for their suitability for malic acid production with *A. oryzae*. *A. oryzae* is able to convert every sugar investigated to malate, albeit with different yields. Based on the promising results from the pure sugar conversion experiments, fractions of the organosolv process from beechwood (*Fagus sylvatica*) and *Miscanthus giganteus* were further analyzed as carbon sources for cultivation and fermentation with *A. oryzae* for malic acid and *R. delemar* for fumaric acid production. The highest malic acid concentration of 37.9 ± 2.6 g/L could be reached using beechwood cellulose fraction as carbon source in bioreactor fermentation with *A. oryzae* and 16.2 ± 0.2 g/L fumaric acid with *R. delemar*. In this chapter it was shown, that the range of convertible sugars for *A. oryzae* is even higher than known before. The suitability of fiber/cellulose hydrolysate obtained from the organosolv process as carbon source for *A. oryzae* in shake flasks as well as in a small-scale bioreactor was approved. The more challenging hemicellulose fraction of *F. sylvatica* was also positively evaluated for malic acid production with *A. oryzae*.

Thus, the organosolv process was evaluated to be a promising pretreatment method for providing sustainable carbon sources for biotechnological application. In contrast, pyrolysis oil might be usable for biomass formation, but the further processed syngas is more suitable for microbial conversion.

Zusammenfassung

Die Umwandlung der modernen Ökonomie in eine Bioökonomie, basierend auf biologischen Rohstoffen, erfordert völlig neue Ansätze in Forschung, Entwicklung und Produktion. Vor allem spielt die Kombination von biotechnologischer und chemischer Stoffumwandlung eine wichtige Rolle. Bei der Entwicklung von Prozessen, die auf nachwachsenden Rohstoffen basieren, muss das „Tank oder Teller"-Dilemma in den Fokus gestellt werden. Lignocellulose als Ausgangsmaterial zu verwenden, könnte ein Teil der Lösung für dieses Problem sein. Die Lignocellulose-Bioraffinerie liefert vorbehandelte Fraktionen von Lignocellulose, die für Chemie und Biotechnologie zugänglich sind und von Mikroorganismen zu Fein- und Plattformchemikalien umgewandelt werden können. Vielversprechende Kandidaten für diese Anforderungen sind Dicarbonsäuren, da sie für die Synthese von Polymeren geeignet sind. Aufgrund ihres breiten Substratspektrums sowie ihrer bekannten Robustheit sind Pilze die optimalen organischen Säureproduzenten für die Evaluierung nachwachsender Rohstoffe als Kohlenstoffquellen. Fumarat ist neben L-Malat eine der wichtigsten Grundchemikalien die durch mikrobielle Fermentation aus nachwachsenden Rohstoffen gewonnen werden können.

In dieser Arbeit wurden zwei verschiedene Vorbehandlungsmethoden von Lignocellulose für die Fermentation untersucht. Der Pyrolyseprozess führt zu zwei Fraktionen, einem organischen (Pyrolyseöl) und einem wässrigen Kondensat. Die weitere Vergasung einer Mischung beider Kondensate führt zur Bildung von Syngas. Während des Organosolv-Prozesses wird Lignocellulose in ihre Grundkomponenten Lignin, Cellulose und Hemicellulose aufgetrennt. Beim sekundären Raffinierprozess wird die Verzuckerung der Cellulose zu Glucose und Hemicellulose zu Xylose durch enzymatische Hydrolyse erreicht.

Pyrolyseöl, eine komplexe Mischung vieler organischer Verbindungen die während der Flash-Pyrolyse von organischen Materialien entsteht, wurde auf seine Eignung als alternative Kohlenstoffquelle für Pilzwachstums- und Fermentationsprozessen untersucht. Dazu wurden ausgewählte Pilze aus allen drei Phylla auf ihre Toleranz gegenüber Pyrolyseöl hin untersucht. Im Detail wurden *Aspergillus oryzae* und *Rhizopus delemar*, beide etablierte Produzenten von organischen Säuren, als Modellorganismen ausgewählt, um die Eignung von Pyrolyseöl als Kohlenstoffquelle zu untersuchen. Es wurde beobachtet, dass *A. oryzae* Pyrolyseölkonzentrationen zwischen 1 - 2% in Abhängigkeit von der Wachstumsphase bzw. der stationären Produktionsphase toleriert. Um mögliche Ursachen für das niedrige Toleranzniveau zu untersuchen,

wurden elf Substanzen aus Pyrolyseöl einschließlich Aldehyden, organischen Säuren, kleinen organischen Verbindungen und phenolischen Substanzen ausgewählt und die maximalen Konzentrationen, die noch Wachstum und organische Säureproduktion erlauben, bestimmt. Darüber hinaus wurden die Auswirkungen von Substanzen auf die Apfelsäureproduktion analysiert und die Verbindungen bezüglich ihrer Eigenschaften in drei Toxizitätsgruppen eingeteilt. Um die Ergebnisse zu validieren, wurden weitere Tests mit *R. delemar* durchgeführt. Zum ersten Mal konnte gezeigt werden, dass geringe Mengen phenolischer Substanzen für die Produktion organischer Säuren nützlich sind. Außerdem konnten Anzeichen dass *A. oryzae* in der Lage ist Isoeugenol abzubauen beobachtet werden. Hinsichtlich der Toxizität von Pyrolyseölen wurde 2-Cyclopenten-1-on als die toxischste Verbindung für filamentöse Pilze identifiziert. Dabei handelt es sich um eine Substanz, die bisher noch nicht für antimykotische oder andere toxische Eigenschaften beschrieben wurde, und möglicherweise für die geringe Toleranz der Pilze gegenüber Pyrolyseöl verantwortlich ist.

Das wässrige Kondensat, das während der Flash-Pyrolyse von organischen Materialien entsteht wurde im dritten Kapitel dieser Arbeit auf Eignung als Substrat analysiert. Die Hauptkomponenten sind Essigsäure mit 4,5%, Hydroxyaceton mit 3,5%, Methanol mit 1,7%, Ethylenglycol mit 0,5% und Propionsäure mit 0,4%. Da die Wirkung von Methanol im Hauptkulturmedium in diesem Konzentrationsbereich minimal ist, ist Hydroxyaceton die einzige Hauptkomponente mit einer Hemmkonzentration unterhalb der Konzentration im wässrigen Kondensat. Die Kultivierung in Modellmischungen zeigte die mögliche Eignung des wässrigen Kondensats bei Verdünnung 1: 1. Zur Bewertung des wässrigen Kondensats als Kohlenstoffquelle wurden Schüttelkolbenkultivierungen mit verdünnter und unverdünnter Fraktionen durchgeführt. Außerdem wurden verschiedene Vorbehandlungen durchgeführt, um die Menge an phenolischen und lösungsmittelartigen Verbindungen zu reduzieren. Allerdings führte keiner der Ansätze zu Produktbildung.

Das vierte Kapitel dieser Arbeit beschreibt die Bewertung von Synthesegas (Syngas) als mögliche Kohlenstoffquelle für die Fermentation mit *A. orzae*. Da dieser Pilz Syngas nicht direkt verstoffwechseln kann, wurde die Idee eines gekoppelten Prozesses implementiert, bei dem ein Organismus ein Produkt aus Sygnas herstellt, das als Kohlenstoffquelle für den Pilz geeignet ist. Synthesegasfermentation unter Verwendung von acetogenen Bakterien ist ein Ansatz zur Herstellung von Massenchemikalien wie Acetat, Ethanol, Butanol oder 2,3-Butandiol, wobei das „Tank oder Teller" Dilemma vermieden wird, indem Kohlenmonoxid, Kohlendioxid und

Wasserstoff aus der Vergasung von Biomasse oder Industrieabfällen verwendet werden. In diesem Kapitel wurde gezeigt, dass *A. oryzae* in der Lage ist, Malat unter Verwendung von Acetat als einziger Kohlenstoffquelle herzustellen, die ein Hauptprodukt der acetogenen Syngas-Fermentation ist. *Clostridium ljungdahlii* wurde dafür zunächst mit Modellsynthesegas als Substrat kultiviert. Dabei wurde die Zusammensetzung von sauberem Synthesegas aus der Vergasung von Stroh (32,5 Vol .-% CO, 32,5 Vol .-% H_2, 16 Vol .-% CO_2 und 19 Vol .-% N_2) imitiert. Der Syngasverbrauch wurde über eine automatisierte gaschromatographische Messung des Abgases überwacht. Für die Anschließende Pilzfermentation wurde der Gaseintrag von Syngas auf 0,6 L/min Luft umgeschaltet. Der Ammoniakgehalt des Mediums für die Synthesegas-Fermentation wurde auf 0,33 g/l NH_4Cl reduziert, um die Produktion von Malat zu unterstützen. Die Säureproduktionsleistung von *A. oryzae* in organischem Säureproduktionsmedium und Syngasmedium mit Acetat als einziger Kohlenstoffquelle wurde verifiziert und ergab eine Ausbeute von 0,28 g/g bzw. 0,37 g/g. Wachstum und Acetatbildung von *C. ljungdahlii* während der Synthesegas-Fermentation wurden durch den reduzierten Ammoniakgehalt nicht beeinflusst und 66% des verbrauchten Syngases wurden in Acetat umgewandelt. Die Gesamtumwandlung von CO und H_2 in Malat war 3,5 g Malat pro Mol verbrauchtem Syngas oder 0,22 g Malat pro Gramm Syngas.

Das fünfte Kapitel dieser Arbeit beschreibt die Bewertung der zweiten Vorbehandlungsmethode für Lignocellulose, die in dieser Arbeit verwendet wurde. Die Säureproduzenten *A. oryzae* und *R. delemar* sind in der Lage, mehrere alternative Kohlenstoffquellen in Malat und Fumarat umzuwandeln. Somit sind kohlenhydrathaltige Hydrolysate aus der Lignocellulose vielversprechend als Substrat für die organische Säureproduktion mit diesen Pilzen. Bevor Lignocellulosehydrolysat-Fraktionen als Substrate getestet wurden, wurden Versuche mit mehreren Mono- und Disacchariden, die möglicherweise in vorbehandelter Biomasse vorhanden sind, auf ihre Eignung zur Apfelsäure-Produktion mit *A. oryzae* untersucht. Dies schließt Levoglucosan, Glucose, Galactose, Mannose, Arabinose, Xylose, Ribose und Cellobiose ebenso wie preiswerte und leicht verfügbare Zucker, wie z.B. Fructose und Maltose ein. *A. oryzae* ist in der Lage, jeden untersuchten Zucker in Malat umzuwandeln, wenn auch mit unterschiedlichen Ausbeuten. Basierend auf den vielversprechenden Ergebnissen der reinen Zuckerexperimente wurden Fraktionen des Organosolv-Prozesses aus Buchenholz (*Fagus sylvatica*) und Riesen-Chinaschilf (*Miscanthus giganteus*) als Kohlenstoffquelle für Kultivierung und Fermentation mit *A. oryzae* für Malat und *R. delemar* für Fumarat Produktion weiter untersucht. Die höchste Malatkonzentration von 37,9 ± 2,6 g/L konnte unter Verwendung der Buchenholzcellulosefraktion als Kohlenstoffquelle in der Bioreaktorfermentation mit *A. oryzae* und 16,2 ± 0,2 g/L

Fumarat mit *R. delemar* erreicht werden. In diesem Kapitel wurde gezeigt, dass die Bandbreite der verwertbaren Zucker für *A. oryzae* sogar noch höher ist als bisher bekannt. Die Eignung des aus dem Organosolv-Verfahren erhaltenen Faser/Zellulosehydrolysats als Kohlenstoffquelle für *A. oryzae* und *R. delemar* im Schüttelkolben sowie im Bioreaktor wurde bestätigt. Die anspruchsvollere Hemicellulosefraktion aus Buchenholz wurde ebenfalls positiv auf Apfelsäureproduktion mit *A. oryzae* bewertet.

Daher hat sich der Organosolv-Prozess als vielversprechendes Vorbehandlungsverfahren zur Bereitstellung nachhaltiger Kohlenstoffquellen für die biotechnologische Anwendung herausgestellt. Im Gegensatz dazu könnte Pyrolyseöl für die Biomassebildung verwendbar sein, während sich das weiterverarbeitete Synthesegas besser für mikrobielle Umsetzung eignet.

Table of content

1 Theoretical background & research proposal

Parts of this chapter are based on the author's contribution to the bookchapter:

Microbial Production of Value-Added Chemicals from Pyrolysis Oil and Syngas

Sustainable Production of Bulk Chemicals

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The subchapter “Pyrolysis” is based on the author´s contribution to the Publication.

To date, the production of most chemicals is still based on fossil resources like coal, gas and crude oil. It is consensus that, due to dwindling resources and climate change, it is necessary to develop sustainable methods for the production of industrially relevant chemicals. The transformation of the modern economy to a bio-economy, based on biological raw materials requires completely new approaches in research, development and production. Bio refineries combine the necessary technologies between biological raw materials and industrial intermediates and value-added products. Above all, the combination of biotechnological and chemical substance conversion plays an important role. While developing processes based on renewable resources, the "food or fuel" dilemma has to be strongly considered. Lignocellulose as feedstock for those kinds of processes could be a part of the solution for this problem. The lignocellulosic bio refinery supplies pretreated fractions of lignocellulose, accessible for chemistry and biotechnology that possibly can be metabolized and converted by microorganisms to more valuable fine chemicals and platform chemicals. Promising candidates for these demands are various dicarboxylic acids because of their suitability to be used for the synthesis of various polymers, as was summarized by Lee *et al.* (2011). In 2004, the US Department of Energy selected the C_4 dicarboxylic acids malic acid, fumaric acid and succinic acid to be one of the 12 most important platform chemicals produced from biomass (Werpy and Petersen 2004). Because of the high diversity of convertible carbon sources, fungi are the optimal organic acid producer used for the evaluation of renewable resources as carbon sources for organic acid production.

1.1 Organic acids

Organic acids or carboxylic acids are aliphatic or aromatic compounds, containing at least one carboxylic group. Carboxylic and dicarboxylic acids are an important part of metabolic pathways, especially in the energy metabolism in every living system, and are abundantly used in households and in daily life. Prominent examples are acetic acid from vinegar and citric acid from citric fruits used e.g. as cleaning agents. Organic acid are also used in pharmaceuticals like isobutylphenylpropionic acid (Ibuprofen) and salicylic acid (as acetylsalicylic acid) which acts as pain killers and against inflammation. Important dicarboxylic acids are malic and fumaric acid, and the respective salts malate and fumarate.

1.1.1 Malic acid

Malic acid, also called 2-hydroxysuccinic acid, belongs to the group of C4-dicarboxylic acids and to the group of hydroxycarboxylic acids and occurs as an intermediate in the citrate cycle. Due to the chiral carbon atom connected to the alcohol group, two isomeric forms of malic acid exist: D (-) - malic acid and L- (+) - malic acid (**Figure 1-1**)

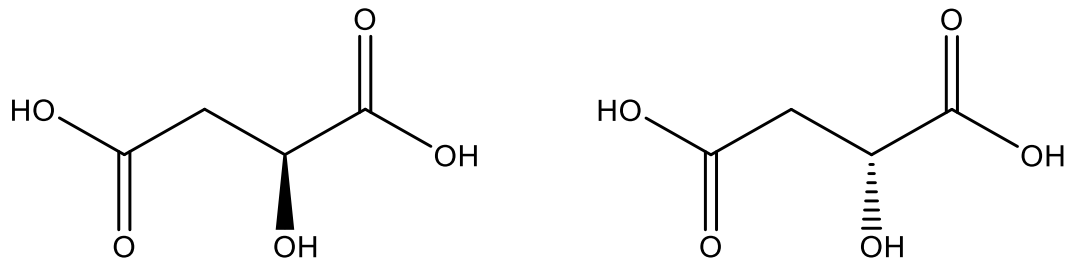

Figure 1-1: L-Malic acid (left) and D-malic acid (right)

The L-form of malic acid is the most common isomer and can be found in many different fruits. Under normal conditions, the organic acids from the metabolism are present as anions. Malate can be used in industry in wide range. In food industry, for example, it is used as a flavor enhancer or acidifier. In addition, malate is also included in some medications and infusions. Furthermore, metal cleaning and metalworking, paints, metallization and textile industry are more applications of malic acid (Goldberg *et al.* 2006). Malic acid can be produced in three different ways. Chemically by hydration of maleic acid or fumarate resulting in a racemic mixture (Miltenberger 2000; Lohbeck *et al.* 2005), enzymatically through conversion of fumarate by immobilized cells (Yamamoto *et al.* 1976) and by immobilized enzymes (Yamazaki, Maeda, and Kamibayashi 1982; Kajiwara and Maeda 1986)or fermentative with genetically modified organisms like *S. cerevisae* (Zelle *et al.* 2008) or *E. coli* (Moon *et al.* 2008; Zhang *et al.* 2011), and natural producers like *A. flavus* (Peleg *et al.* 1988). The commercial production of malate currently occurs mainly through the chemical synthesis of petroleum-based n-butane or benzene. In this case, a racemic mixture of D- and L-malate can be obtained via the hydration of maleic acid (Goldberg *et al.* 2006), but also the enzymatic conversion of fumarate e.g. using immobilized *Corynebacterium ammoniagenes* cells or *Corynebacterium glutamicum (Brevibacterium flavum)* cells using the enzyme fumarase to convert fumarate in malate. However, the yields are lower than in chemical synthesis because of subsequent purification processes required to obtain pure L-malate as a product. The fermentative approach using certain organisms like *Aspergillus* sp.or *S. cerevisiae* offers the advantage of enantioselective production of L-malic acid. Furthermore the sustainability, given by the renewability of the feedstock and the moderate parameters during the fermentation process makes is more environmental friendly. However, the missing economic feasibility due to low production rates and yield is still a drawback. The worldwide annual production of malate is about 40.000 tons per year and belongs to the intermediate-volume chemicals. Compared to the world citric acid production by industrial fermentation with *A. niger* of 1.4 million 2004 this is a low value (Goldberg *et al.* 2006).

1.1.2 Fumaric acid

A very important naturally occurring, organic raw material is fumaric acid. The structural formula is shown in **Figure 1-2**.

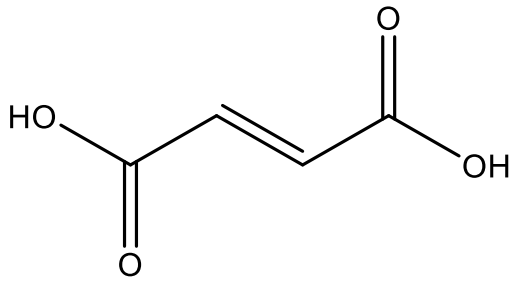

Figure 1-2: Fumaric acid

It is a C4 unsaturated dicarboxylic acid (trans-butenedioic acid). Due to the bifunctionality of the two caboxylic groups, fumarate can be easily esterified in synthetic chemistry or to produce polymers. Thus, this acid serves as an important platform chemical with many potential applications, ranging from the production of synthetic resins and intermediates for various chemical syntheses to the recovery of biodegradable polymers and bioplastics. In addition, fumarate is also used in the food industry as an acidifier and preservative and as a flavor enhancer (Goldberg *et al.* 2006).

In addition to L-malate and succinate, fumarate is one of the most important, high-quality basic chemicals that can be produced from renewable resources through microbial fermentation (Werpy and Petersen 2004; Sauer *et al.* 2008). In 2012, global fumaric acid demand was 225,200 t with demand expected to grow to 346,200 t by 2020 (Grand View Research 2015).

Naturally, fumaric acid is produced by many organisms in various biochemical pathways. Especially during the citrate cycle, larger amounts of fumaric acid are produced. Under growth conditions, however, the dicarboxylic acid is not accumulated and used in further reactions. In some filamentous fungi, when specific stress conditions are applied, a significant accumulation of citric acid intermediates, such as fumarate, L-malate and citric acid, occurs as end products (Goldberg *et al.* 1991).

1.2 Filamentous fungi as organic acid producers

Certain species of the genera *Aspergillus* and *Rhizopus* have been described as L-malate and fumarate producers (Abe *et al.* 1962; Battat *et al*. 1991; Bercovitz *et al.* 1990; Peleg *et al.* 1988; Peleg *et al.* 1989;). Accumulation of these organic acids occurs via a separate and unique route,

the reductive citrate cycle, which is localized in the cytosol (Goldberg *et al.* 1991). This pathway has a maximal theoretical yield of 2 mol L-malate or fumarate / mol glucose, if pyruvate was produced during the glycolysis (Brown *et al.* 2013).

1.2.1 Biosynthesis of malic and fumaric acid

The metabolic pathway resulting in the production and secretion of dicarboxylic acids of some filamentous fungi such as *Aspergillus oryzae* and *Rhizopus delemar* is the reductive tricarboxylic acid cycle localized in the cytosol. This unique metabolic pathway is the reverse reaction of the well-known oxidative citrate cycle (also called citric acid or Krebs cycle) which is localized in the mitochondria and is one of the major aerobic metabolic reactions and the oxidative degradation of organic substances for the purpose of energy production and the provision of intermediates for biosynthesis. Osmani und Scrutton (1983) proposed the existence of the reductive citrate cycle for the first time when they discovered that some filamentous fungi also express certain citrate cycle enzymes, like pyruvate carboxylase, malate dehydrogenase and fumarase, in the cytosol. The evidence that the reductive citrate cycle of filamentous fungi is involved in fumaric and L-malic acid production and that pyruvate from glycolysis is the starting material provided a 13C NMR experiment. Here, glucose labeled with the 13 C isotope on the first carbon atom was used as a substrate for the fermentation of *A. flavus* (Peleg *et al.* 1989) and *R. delemar* (Kenealy *et al.* 1986)

As shown in **Figure 1-3**, the reductive citrate cycle begins with pyruvate, which is produced in glycolysis.

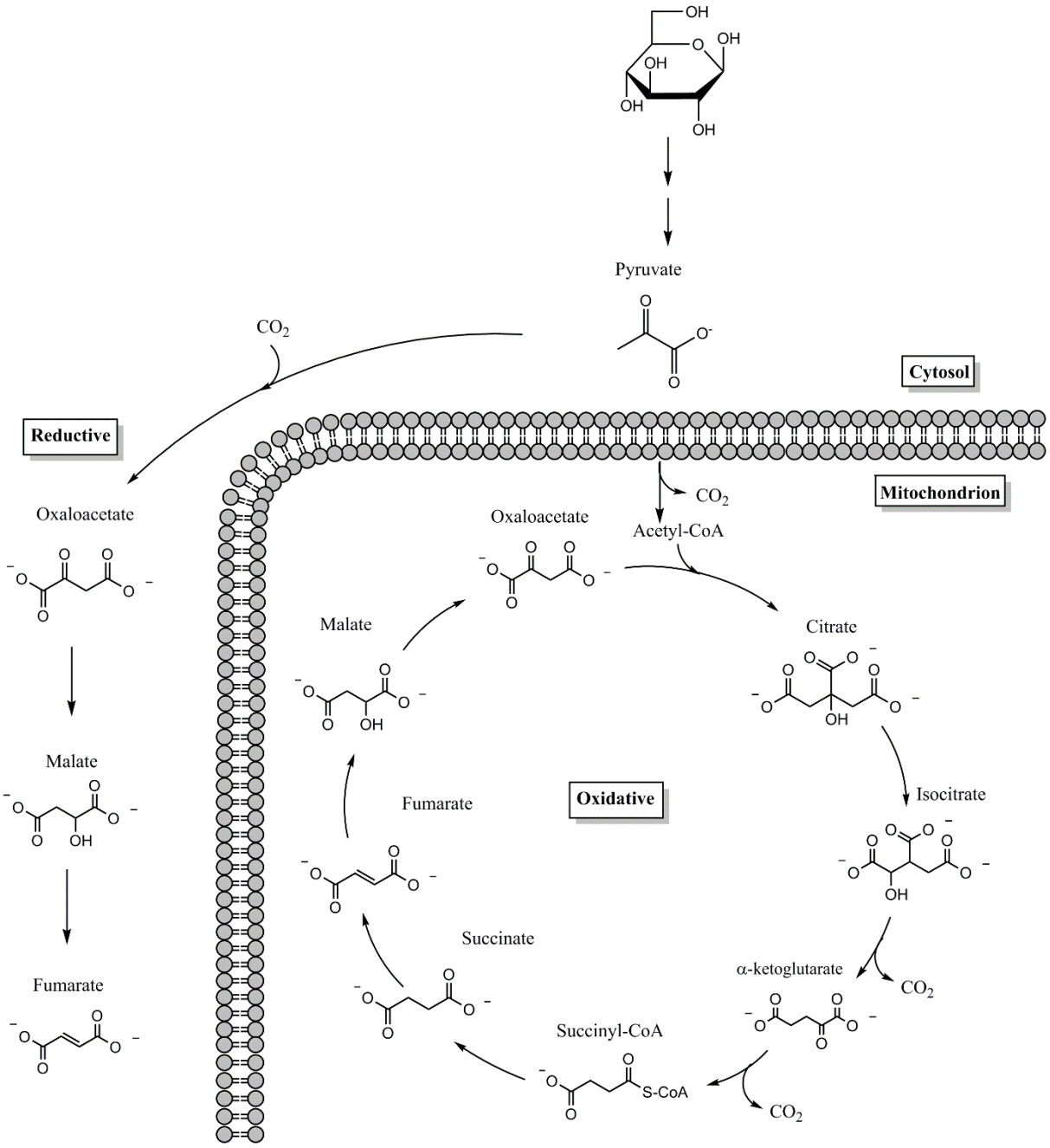

Figure 1-3: Reductive (rTCA) and oxidative tricarboxylic acid (TCA) postulated by Osmani and Scrutton (1985).

Pyruvate is converted to oxaloacetate by carboxylation with pyruvate carboxylase, followed by reduction to L-malate by the malate dehydrogenase. The reductive tricarboxylic acid (citric acid) cycle of the fungus *A. oryzae* seems to end here, as this organism increasingly produces L-malate. The fungus *R. delemar* increasingly accumulates fumaric acid, since the L-malate in the cytosol is dehydrated by the enzyme fumarase to form fumarate. From glucose to L-malate or fumarate, this pathway is ATP and NADH neutral and leads to the fixation of 1 mol CO_2/mol L-malate or fumarate with a maximum theoretical yield of 2 mol L-malate or fumarate/mol Glucose (Brown *et al.* 2013). For disaccharides 4 mol organic acid per mol carbon source and

for pentoses, metabolized through the pentose phosphate pathway, 1.67 mol organic acid per mol carbon source is possible (Ochsenreither *et al.* 2014).

1.2.2 Fermentation process

The ratio between both acids produced with the rTCA can vary with different production strains and due to process conditions (Peleg *et al.* 1989; Friedberg *et al.* 1995;Ding *et al.* 2011). The biosynthesis of both organic acids is carried out under aerobic conditions in a medium with high glucose concentration, a limiting amount of nitrogen and a pH neutralizer (e.g. $CaCO_3$). Nitrogen limitation is essential for acid accumulation, as the cells cannot produce biomass from the excess glucose under nitrogen limitation and thus switch to acid production (Peleg *et al.* 1988). For this reason, the fermentation of filamentous fungi takes place in two steps. The first step is a pre-fermentation in which the biomass production of the fungal cells is stimulated under the most suitable conditions. In the second step, biomass is transferred to the nitrogen deficient medium. The stress situation is initiated by the lack of nitrogen source and leads to acid production. Production processes have been further optimized in the last years, so that in fermentations with 120 g/L glucose as carbon source malic acid concentrations of 113 g/L were achieved with *A. flavus* resulting in a yield of 0.94 g/g (Battat *et al.* 1991). Because of the production of aflatoxins this fungus is not suitable for industrial production of malic acid. The production of malate by the close relative *A. oryzae*, which is not producing aflatoxins, has also been investigated (Knuf *et al.* 2013). Through metabolic engineering of *A. oryzae* strain NRRL 3488 malic acid concentrations of 154 g/L were produced from 160 g/L glucose (Brown *et al.* 2013). So far, a biotechnological production of malic acid is not industrially established due to the high process costs compared to the conventional chemical synthesis. However, keeping in mind the industrial production process of citric acid by *A. niger* with an annual production of 1.6 million tons in 2012 (Verhoff and Bauweleers 2012) a biotechnological process for malic acid seems to be feasible, if production and purification processes can be optimized to reduce the price, and if the demand can be increased due to further studies dealing with new applications. Overall, the sustainability of these kind of processes should be the main focus which makes it necessary to find alternative feedstocks.

1.3 Alternative Carbon Sources

In order to replace established chemical processes, production costs of biotechnological processes are the main factor. For future development, the sustainability should also be an important parameter. Modern industrial biotechnological production systems mostly use either starch or sugars as carbon sources, or waste products like glycerol or molasses. The "food or fuel" issue addresses the sustainability of carbon sources judged by the ability to act as food for humans. An increase of fermentative concepts for the production of chemicals makes the accessibility of new carbon sources necessary which are not competing with food. Lignocellulose as the most abundant biological material in the world can be the solution for this problem.

1.3.1 Lignocellulose

One of the biggest challenges for the establishment of a bio-based economy is, however, the naturally insufficient utilization due to low accessibility of lignocellulosic materials by a wide range of microorganisms. Therefore, many pretreatment methods have been developed to enable the accessibility of lignocellulosic materials in microbial processes. Lignocellulose is the main constituent of all woody plants. Cellulose accounts for 40-50% of lignocellulose, hemicellulose accounts for 25-30%, lignin is up to 15-20%. Cellulose is a linear homopolymer consisting of β-D glucose units linked by a β-1,4- glycosidic bond (**Figure 1-4**). A dimer of two β-D glucose units is called cellobiose. Hydrogen bonds and van der Waals forces bind long chains of cellulose polymers together.

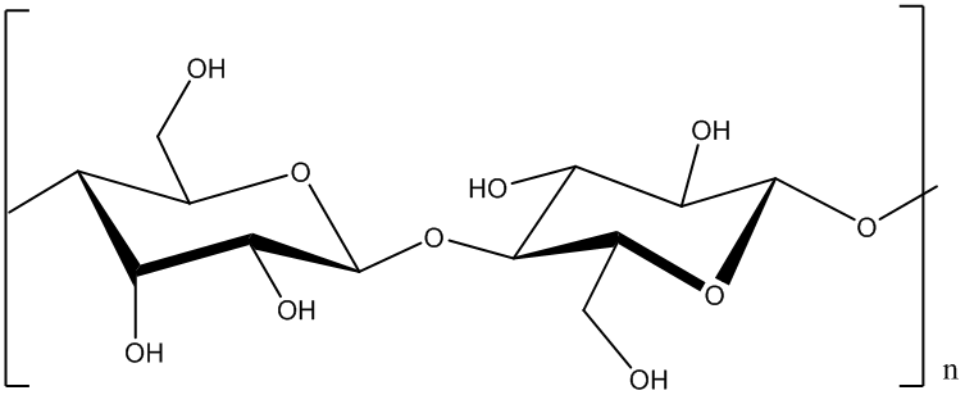

Figure 1-4: Cellulose fragment: β-1,4 glycosidic bond of several glucoe molecules

Hemicellulose, on the other hand, is a branched heterogeneous polymer, typically consisting of five different sugars. These include D - xylose and L - arabinose as pentoses and D - mannose, D - glucose and D - galactose as hexoses, as well as other components such as acetic acid, ferulic acid and glucuronic acid. The units are also linked to each other by β-1,4-glycosidic bonds; in some cases, β-1,3-glycosidic compounds can also occur.

Lignin is built up from aromatic compounds called phenylpropanoids and the major reason for the stability of lignocellulosic biomass. The lignin polymers are highly branched and covalent connected to each other resulting to one big lignin molecule per plant.

1.4 Pretreatments of Lignocellulose for fermentation

The success of a bio-based economy is based on biotechnological processes which enable the transformation of renewable resources to value-added products. In the organosolv process, for instance, the main components of lignocellulose are separated and cellulose and hemicellulose fractions are subsequently saccharified. The resulting fractions contain large amounts of easy metabolizable sugars and constitute therefore an ideal substrate for biotechnological processes. Lignin, however, remains largely inaccessible. A possible pretreatment to utilize lignocellulosic material completely by breaking all polymeric bonds, is pyrolysis resulting in pyrolysis oil. Pyrolysis oil is obtained by fast pyrolysis of wood or other lignocellulosic biomass. It offers a substrate, which can be generated from nearly every dry biomass, not competing with food or feed and which is much more independent from season and region than other biomass-based substrates for fermentation. The three key components of lignocellulose (lignin, cellulose and hemicellulose) are decomposed and depolymerized to monomeric sugars, small aromatic compounds and further low-molecular substances (Oasmaa and Czernik 1999) making pyrolysis oil a possible resource for fermentative processes.

1.4.1 Organosolv process

The organosolv process is used for the fractionation of lignocellulosic biomass like wood, agricultural and forestry waste to produce a mixture of low molecular weight compounds. During the process, lignocellulose is separated into its basic components, lignin, cellulose and hemicellulose. In primary refining, the pulping takes place at 36 bar and 200 °C. Afterwards, cellulose can be separated from hemicellulose and lignin (Laure *et al.* 2014). The precipitation of lignin splits off the hemicellulose. In secondary refining, saccharification of the cellulose into glucose and hemicellulose into xylose is carried out by enzymatic hydrolysis. The resulting products lignin, glucose and xylose serve as starting material for various industrial products (Johansson *et al.* 1987). Before purification, the individual fractions, especially the hemicellulose fraction, contain a variety of other substances such as ethanol, acetic and lactic acid, hydroxymethylfurfural (HMF), mannose, galactose, cellobiose, rhamnose and arabinose. The organosolv process is schematically shown in **Figure 1-5**.

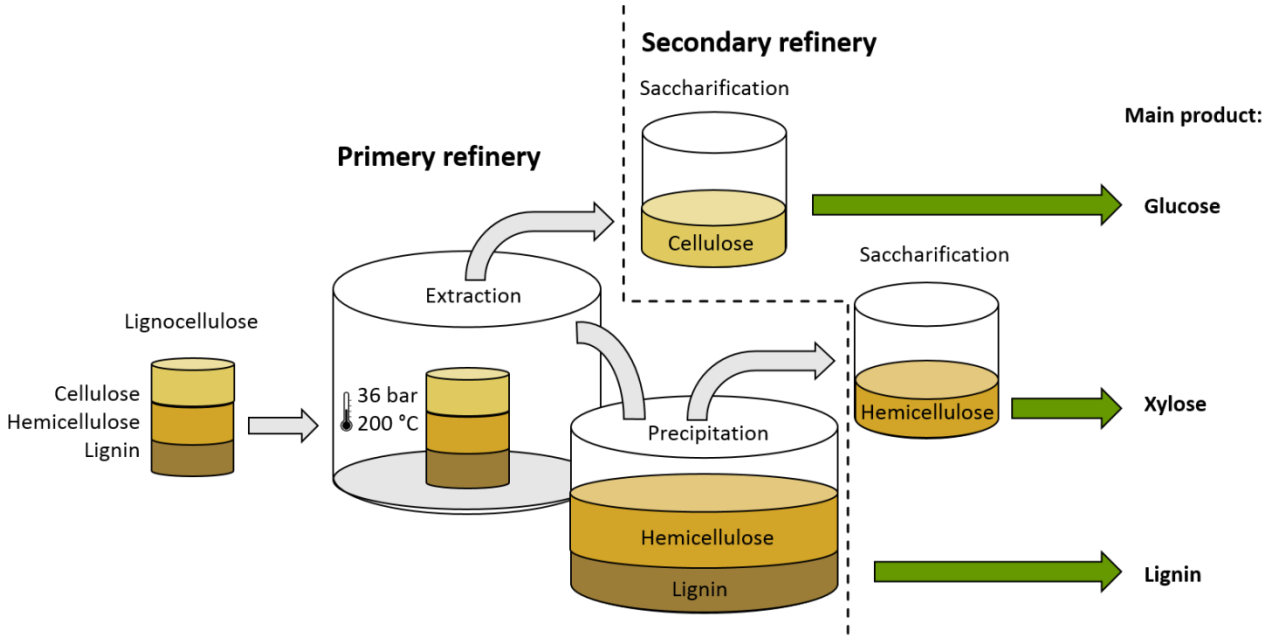

Figure 1-5: Schematic diagram of the organosolv process: In the primary refinery step, the three main components cellulose, hemicellulose and lignin are separated. In the secondary refinery step the carbohydrate polymers are saccharified to the resulting monomer solution.

The filamentous fungi *A. oryzae* and *R. delemar* are known to be able to convert various substrates such as xylose and glycerol. Other carbon sources such as maltose, fructose, sucrose and mannitol were shown to be a suitable carbon source for *R. delemar* (Carlsen and Nielsen 2001; Bai *et al.* 2004; Maas *et al.* 2006; Park *et al.* 2004). Combined with the known robustness of fungi, the resulted fractions from the organosolv process are promising substrate for a sustainable fermentative process for a future bioeconomy.

1.4.2 Pyrolysis

Preparation of pyrolysis products.

The pyrolysis products used in this study were prepared from wheat straw by fast pyrolysis. This process has been developed to convert biomass into a liquid fuel for various applications to produce heat, electricity and transportations fuels. Small and dry wood particles of a few mm size are rapidly heated up by a heat carrier (sand) in a pneumatically or mechanically fluidized bed at 500 ± 30 °C in the absence of oxygen (Henrich *et al.* 2016). The process is schematically shown in **Figure 1-6.**

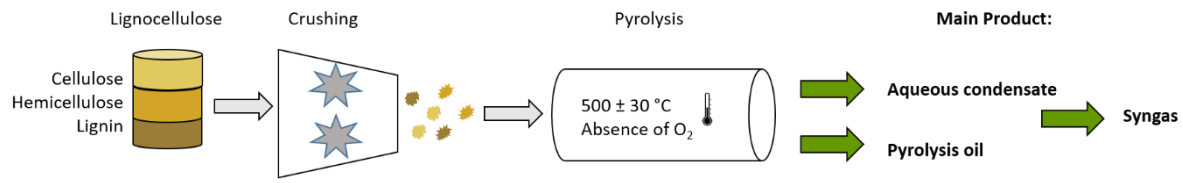

Figure 1-6: Schematic diagram of the pyrolysis process: The dry biomass gets crushed to particles with the size of a few mm and subsequently mixed with hot sand to a temperature of 500 °C for a few seconds in absence of oxygen. The resulting vapor is condensed to the two main products, pyrolysis oil and an aqueous condensate. Both pyrolysis products can further be gasified to syngas.

The fast pyrolytic decomposition takes place in only one or few seconds and liberates gases, vapors and fine entrained char and ash particles into the product gas stream. The organic and water vapors are recovered at ambient temperature by quench-condensation via recycle and injection of cooled condensate. The remaining gases, mainly CO_2, CO, some H_2 and C1-C5 alkanes and alkenes are combusted, together with some char to supply the process energy. The yields of both pyrolysis char and gas as well are 15-25 wt.%. Pyrolysis oil yields of 55-75 wt.% are typical for wood and only about 50 ± 10 wt.% for straw and straw-like herbaceous biomass. (Tröger *et al.* 2013). Pyrolysis oil contains also water originating from biomass humidity (typically around 10 % for air dry lignocellulose) and from chemical reactions during fast pyrolysis. In case of pyrolysis oils obtained from herbaceous biomass higher water amounts are formed which can cause phase separation. In this case, a phase rich in organic compounds (pyrolysis oil) and an aqueous phase consisting of up to 80 wt.% of water and water soluble organic compounds are established. The composition of both fractions used in this study is shown in the Appendix section (**Appendix 1** and **Appendix 2**). At KIT a fast pyrolysis process is developed in order to produce synthetic biofuels via synthesis gas production by gasification of pyrolysis products. The pyrolysis products itself as well as the syngas can be used for fermentation as well.

Pyrolysis oil

Pyrolysis oil is a mixture of organic compounds, formed during the high-temperature pyrolysis of organic substances like agricultural or forestry wastes. Because of its visual similarity to petroleum derived oils, i.e. its dark black color and its high viscosity, it is also called bio-oil. However, with regard to its chemical composition, fossil oil and pyrolysis oil can be clearly distinguished. **Figure 1-7** shows the viscous pyrolysis oil from bioliq® production plant in Karlsruhe.

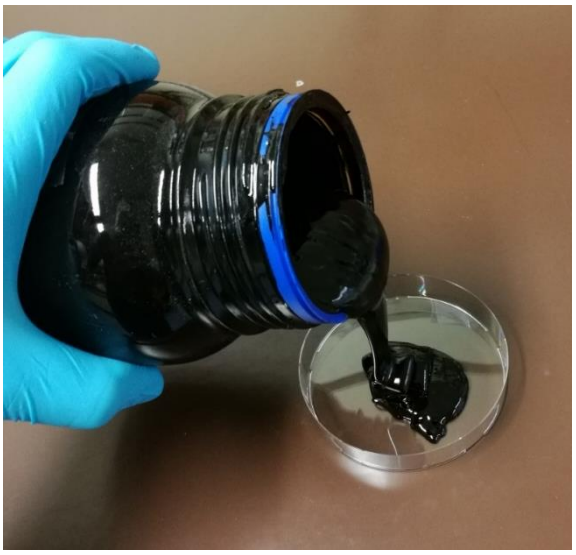

Figure 1-7: Pyrolysis oil from bioliq® production plant in Karlsruhe

The properties of pyrolysis oil depends highly on the used feedstock. An overview of the properties from different feedstocks is shown in **Table 1-1.**

Table 1-1: Properties of pyrolysis oil from different feedstocks.

Property	Forest residues	Straw	Pine
Water, wt%	24.1	19.9	16.6
C, wt%	56.6	55.3	55.8
O, wt%	36.9	37.7	38.2
H, wt%	6.2	6.6	5.8
N, wt%	0.1	0.4	0.1
S, wt%	0.03	0.05	0.02
Viscosity (50 °C), cP	29	11	31
HHV, MJ/kg	17.4	18.5	19.1
pH	2.9	3.7	2.6

(Demirbas and Balat 2007)

Biotechnologically relevant components of pyrolysis oil

The use of pyrolysis oil for microbial fermentation can be the solution of the accessibility problem of lignocellulosic material. In addition to known substances that can be used as a carbon sources, there are many critical and unknown components that can be problematic for microorganisms. Some organisms are known to be tolerant to these substances, and some are even able to metabolize them as a substrate for further metabolic pathways. In the following, the most important biotechnologically relevant components as well as their effects on organisms and the suitability as carbon source for fermentation are presented.

Organic acids

Organic acids are a substance class, highly present in pyrolysis oil. Theses acids, like valeric acid, formic acid, propionic acid and acetic acids are mostly responsible for the low pH in this mixture. Although many acids are valuable products as well, they can also be used as carbon sources for further fermentative production of platform and fine chemicals. Organic acids are typical intermediates in metabolism and therefore often metabolizable for many organisms. However, propionic acid and acetic acid have also antimicrobial properties and are therefore used as preservative in food industry.

For the production of ethanol by *Saccharomyces cerevisiae* it is reported that a concentration of more than 0.5 g/L of acetic acid inhibits the growth considerably, but did not affect the ethanol production. Increasing the acetate concentration to 1 g/L both, production and growth are completely inhibited (Pons *et al.* 1986). Further studies to investigate the inhibitory effects of propionic and acetic acid to *S. cerevisiae* were done by Ullah *et al.* (2012). When medium pH was constantly hold at pH 5 and 0.74 g/L propionic acid and 2.5 g/L acetic acid were added, a growth inhibition of 50% was observed. By adding both acids to growing cells, the intracellular pH-level decreased immediately which seemed to be the main reason for inhibition of growth by cultivation with these acids. For *Yarrowia lipolytica* UOFS Y-1701, the citric acid production from 30 g/L sunflower oil could be considerably increased from 0.5 g/L to 18.7 g/L if 10 g/L acetic acid was added to the medium (Venter *et al.* 2004). High concentrations of carbon sources are typical for fungal acid production. The well-known citric acid producer *Aspergillus niger* stops growing if the pH in medium decrease below 4.5. By cultivation of *A. niger* on paper discs containing 50 g/L acetic acid as sole carbon source, no citric acid production and no growth was observed. If mycelium was transferred from medium, containing good growth and citric acid production conditions with sucrose to medium containing 50 g/L acetic acid, both was inhibited immediately (Xu *et al.* 1989).

In contrast, many organisms are able to use acetic acid and propionic acid for production of value substances and growth. The archaeon *Methanosarcina* sp. is reported to be able to produce methane on acetic acid (Smith and Mah 1980). The photosynthetic bacterium *Rhodopseudomonas* sp. has the ability to grow on acetic acid and produce hydrogen. To achieve this, the cells were cultivated in enriched medium until the late lag phase was reached. The next step was the transfer to acetic acid containing hydrogen production medium. Thereby, hydrogen yields up to 72.8% were obtained (Barbosa *et al.* 2001). Another example is the bacterium *Cupriavidus necator (Alcaligenes eutrophus*), which is able to use propionic acid and acetic acid as suitable carbon source for growth and the production of polyhydroxyalkanoates (PHA)**,** especially polyhydroxybutyrate (PHB). In the two-step process, cells were cultivated in a nutrient rich medium and then transferred to a nutrient-free mineral medium containing acetic acid as sole carbon source. The transfer led to an increase of the PHA-polymer content from 0% to 51% of dry biomass. If medium was additionally enriched with small amounts of propionic acid, bio-polymer content was increased to 52% (Doi *et al.* 1987). **Table 1-2** gives an overview on biotechnologically produced substances using organic acids as carbon source, present in pyrolysis oil. The organic acid content, especially acetic acid, is therefore a promising carbon source for fermentation with fungi.

Sugars

This substance class is mostly present as anhydrosugars which develop during heat dissociation of cellulose in biomass, e.g. levoglucosan (1,6-anhydro-β-D-glucopyranose), 1,4:3,6-Dianhydro-β-D-glucopyranose and 1,5-anhydro-β-D-arabino-furanose, whereas levoglucosan constitutes the main part by far. In this form, the sugar molecule forms an intramolecular ether bond.

As sugars are the most preferred carbon sources for organisms, the sugar fraction of pyrolysis oil is the one with the highest potential for the production of biotechnologically based chemicals and products. All sugar polymers in biomass depolymerize ideally completely to monomeric anhydrosugar molecules during pyrolysis. Cellulose would depolymerize to levoglucosan which is the anhydride version of glucose and thus the main compound in this fraction (**Figure 1-8**).

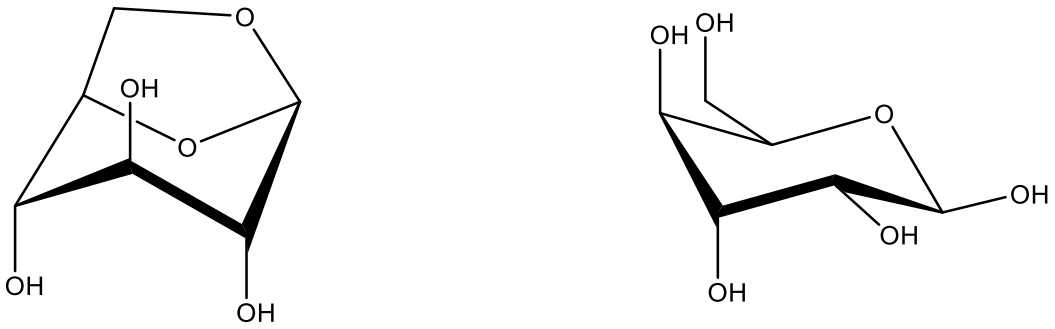

Figure 1-8: Levoglucosan/1,6-Anhydro-β-D-glucopyranose (left), D-Glucose/β-D-Glucopyranose (right)

However, the levoglucosan content in pyrolysis oil is usually low and does not resemble the cellulose content. The cellulose content in eucalyptus woody biomass for example ranges between 44.5 wt.% and 51.9 wt.% (Silva *et al.* 2010; Jeon *et al.* 2010). The levoglucosan content in pyrolysis oil resulting from this biomass ranges only between 3.39 wt.% and 6.49 wt.% depending on pyrolysis conditions (Garcia-Perez *et al.* 2008). It was discovered, that minerals and inorganic salts, which are contained in the biomass have a massive influence of the depolymerization process of cellulose during the pyrolysis and support the formation of smaller molecules derived from cellulose like glycolaldehyde (Piskorz *et al.* 1986; Piskorz *et al.* 1989).

The influence of the inorganic salts and ash as catalysts on the decomposition of cellulose during the pyrolysis process and the distribution of small molecules like formic acid, glycolaldehyde and hydroxyacetone, furan ring derivatives and anhydrosugars are analyzed and investigated by Patwardhan *et al*. (2010). The key step for the formation of levoglucosan is the first step during the pyrolysis process. The way in which the glycosidic bond is cleaved determines whether the glycan ring is decomposed, resulting in smaller molecules or the formation of levoglucosan. The effects of salts and minerals in concentrations of 5 µmol per g cellulose increases the production of lower molecular weight substances dramatically and consequently, the yield of levoglucosan is reduced. If the concentration of the inorganic ingredients can be controlled or removed with special pretreatment of the lignocellulose, the composition of the resulting bio-oil could be altered and the concentration of anhydrosugars (especially levoglucosan) could be increased (Patwardhan *et al.* 2009; Patwardhan *et al.* 2010).

Although levoglucosan is a sugar, it is not accessible to every organism due to its internal ether bond. A possible solution is an acidic treatment which results in the cleavage of the ether bond and the formation of glucose. Acid treated pyrolytic sugars can then be used for fermentation, like ethanol production with *S. cerevisiae*. In this case the sugar rich fraction was diluted with water and afterwards detoxified with different methods like overliming (treatment with

$Ca(OH)_2$), extraction with organic solvents (Chan and Duff 2010) or activated carbon *et al.* 2010). The detoxified fractions subsequently used for fermentation in shake flasks led to 0.24 g to 0.5 g ethanol per g glucose. Similarly, the hydrolytic sugar fraction was used for microbial lipid production leading to 0.089 g lipid per g glucose with *Rhodotorula glutinis* and 0.167 g lipid per g glucose with *Cryptococcus curvatus* (Lian *et al.* 2010).

Direct application of levoglucosan without pretreatment is possible when working with naturally levoglucosan metabolizing microorganisms. Levoglucosan is not only a product of pyrolysis, but develops also when biomass is burned and can therefore be detected after forest and bushfires. Several yeasts are able to grow on detoxified pyrolysis oil and many fungi of the genera *Penicillium* and *Aspergillus* can convert levoglucosan directly to glucose-6-phosphate, the first intermediate of the glycolysis (Prosen *et al.* 1993). In a study by Lian *et al.* (2013), the two oleaginous yeasts *Rhodosporidium glutinis* and *R. toruloides* were cultivated for the production of microbial lipids from non-hydrolyzed levoglucosan. Compared to cultivations with glucose, the obtained cell mass of both yeasts was considerably reduced in cultivations with levoglucosan. Under these conditions, 6.8 g/L of *R. glutinis* cells and 5.8 g/L of *R. toruloides* cells were grown. In cultivations with glucose, 8.1 g/L of *R. glutinis* cells and 6.5 g/L of *R. toruloides* cells were formed. However, the lipid accumulation was comparable. From levoglucosan, *R. glutinis* and *R. toruloides* accumulated a lipid concentration of 2.7 g/L and 2.0 g/L. From glucose, a maximum concentration of 2.9 g/L and 2.4 g/L of lipid were produced. When comparing the fatty acid composition resulting from glucose and levoglucosan, a slight increase of unsaturated fatty acids and a slight decrease of saturated fatty acids was observed for both yeasts. Additional experiments showed the suitability of detoxified levoglucosan from pyrolysis oil only for lipid production with *R. glutinis* with 0.78 g/L (Lian *et al.* 2013).

For the metabolism of levoglucosan an enzyme called levoglucosan kinase (LGK) is necessary. This inducible Mg^{2+}- and ATP- dependent enzyme enables usage of levoglucosan as sole source for energy and growth. It was firstly discovered, purified and characterized from the yeast *Sporobolomyces salmonicolor* (Kitamura and Yasui 1991). The genes for levoglucosan kinase have also been found and functionally characterized in *Lipomyces starkeyi* (Dai *et al.* 2009) and *Aspergillus niger* (X. Zhuang and Zhang 2002). Levoglucosan was also successfully used as carbon source for the fermentation of *Aspergillus terreus* K26 for the production of itaconic acid with comparable yields to fermentation with glucose (Nakagawa *et al.* 1984). The *A. niger* strain CBX-2 which produces citric acid with a high conversion rate of more than 90% from glucose or starch, converts pure and partially purified levoglucosan only with a low rate of

under 10% to citric acid. To increase the yield, conidia of this strain were radiated with γ-rays and a mutant strain (CBX-209) was identified, showing significantly increased citric acid conversion rate of 87.5%. The parental strain obtained only a rate of 5.63% when cultivated on pure levoglucosan. In addition, the citric acid conversion rates were also increased when fermenting the strain CBX-209 either on crude pyrolysis oil (pH adjusted with $CaCO_3$ to pH 6) from 0.88% to 2.38% and on pre-treated pyrolysis oil from 4.38% to 19.25% (X. Zhuang and Zhang 2002).

Concerning a bacterial strategy to metabolize levoglucosan, a primary dehydration of levoglucosan to 3-keto levoglucosan and in two following steps further to glucose is hypothesized. This hypothetical pathway was the first discovery of prokaryotic metabolism of levoglucosan and is still not further studied (Nakahara *et al.* 1994).

Organisms and products demonstrating the potential of sugar utilization from pyrolyzed biomass for the production of chemicals by fungal fermentation as well as the use of the key enzyme LGK for development of non-natural levoglucosan users by genetic engineering are summarized in **Table 1-2**.

Table 1-2: Biotechnologically produced substances from carbon sources present in pyrolysis oil

Carbon source	Organism	Product
Acetic acid, propionic acid	*Cupriavidus necator* (*Alcaligenes eutrophus*)	Polyhydroxyalkanoates (PHA)[1]
Acetic acid	*Rhodopseudomonas palustris* P4	Hydrogen[2]
Acetic acid	*Methanosarcina*	Methane[3]
Levoglucosan	*Rhodosporidium toruloides* and *Rhodotorula glutinis*	Triglycerides[4]
Levoglucosan	*Aspergillus niger* CBX 209	Citric acid[5]
Levoglucosan	*Aspergillus terreus* K26	Itaconic acid[6]
Levoglucosan	*Escherichia coli* KO11 + $LGK_{L.\text{-}starkeyi,}$ codon optimized	Ethanol[7]
Hydrolysed, detoxified pyrolysis oil	*Cryptococcus curvatus* and *Rhodotorula glutinis*	Triglycerides[8]

Hydrolysed, detoxified pyrolysis oil	*Saccharomyces cerevisiae*	Ethanol[8]

[1](Doi *et al.* 1987); [2](Barbosa *et al.* 2001);[3](Smith and Mah 1980); [4](Lian, *et al.* 2013); [5](Zhuang *et al.* 2001); [6](Nakagawa *et al.* 1984); [7](Layton *et al.* 2011); [8](Lian *et al.* 2010)

Other classes of organic compounds in pyrolysis oil are widely spread in composition and are present only in low concentrations. Due to their low content they are not considered as carbon sources, but are accounted for the high toxicity of pyrolysis oil.

Toxic compounds and challenges

Due to the antimicrobial characteristics of whole pyrolysis oil or fractions they can be used for example in wood preservation, especially the lignin-rich fraction containing monomeric and oligomeric phenolic compounds (Mohan *et al.* 2017). Therefore, some considerations regarding toxicity have to be done when proposing pyrolysis oil as a substrate for microbial fermentation. Some selected substances found in pyrolysis oil were tested for microbial inhibition properties and are shown in **Table 1-3** and **Table 1-4** for the two model organisms *E. coli* and *S. cerevisiae*, respectively.

Table 1-3: Some critical substances from pyrolysis oil and their inhibitory concentrations (IC, given in g/L) to *E. coli*

Substance	wt. % [a]	g/L[b]	*E. coli*[c]		Reference
			IC_{50}	IC_{100}	
Acetic acid	5.73	68.76	9	25	1
Furfuryl alcohol	0.02	0.24	4	20	2
Furfural	0.33	3.96	2.4	3.5	3
Vanillin	1.46	17.52	0.5	1.5	3
Guaiacol	0.12	1.44	0.6	3	2
Syringaldehyde	1.16	13.92	0.6	2.5	3
Hydroquinone	0.09	1.08	0.7	3	2

[1](Zaldivar and Ingram 1999); [2](Zaldivar *et al.* 2000); [3](Zaldivar *et al.* 1999)

[a] concentration in pyrolysis oil from fast pyrolysis of mallee woody biomass at 500°C (Garcia-Perez *et al.* 2008);

[b] calculated from wt.% using an average pyrolysis oil density of 1.2 kg/dm^3 (Oasmaa and

Czernik 1999)

[c] *E. coli* LY01 (Yomano *et al.* 1998) derivative of *E. coli* B

Table 1-4: Some critical substances in pyrolysis oils and their inhibition rate to *S. cerevisiae* ATCC 200062 (Lian *et al.* 2010)

Substance	wt.% in pyrolysis oil[a]	25% of concentration in pyrolysis oil (g/L)[b]	Growth inhibition (%)
Acetic acid	5.73	17.19	97.75
Propanoic acid	1.82	5.46	97.01
Furfuryl alcohol	0.02	0.06	5.94
Furfural	0.33	0.99	7.81
Phenol	0.57	1.71	76.95
Eugenol	0.51	1.53	97.04
Hydroxyacetone	2.61	6.48	44.17
2-Furanone	0.03	0.09	3.06
Stilbene	0.12	0.36	9.17
Vanillin	1.46	4.38	81.54
Syringaldehyde	1.16	3.48	71.68

[a]concentration in pyrolysis oil from fast pyrolysis of mallee woody biomass at 500°C (Garcia-Perez *et al.* 2008)

[b]calculated from wt.% using an average pyrolysis oil density of 1.2 kg/dm^3 (Oasmaa and Czernik 1999)

To investigate if these substances may cause problems when working with pyrolysis oil, their concentration in pyrolysis oil is given, too. Comparing the two tables, growth inhibition is mainly caused by phenolic compounds and organic acids, both in concentrations relevant when using pyrolysis oil as substrate. In contrast to *S. cerevisiae*, growth of *E. coli* is also strongly inhibited by furfural. Since only single substances were tested in the cited studies, predictions about synergistic effects cannot be made but have certainly to be considered. For some of the substances the mechanisms of the inhibitory effects have been studied. If using whole pyrolysis oil as a microbial carbon source is not possible due to toxicity, pretreatment or fractionation for enrichment of desired compounds and elimination or reduction of inhibitory substances may be an option. However, substances which are toxic for most microorganisms may not be toxic for

every microorganism as they have developed strategies for detoxification, inactivation or even metabolization of these critical compounds.

5-hydroxyfurfural and furfural are detoxified by microorganisms by modification to the less toxic compound furfuryl alcohol (Boopathy *et al.* 1993; Gutiérrez *et al.* 2006; Almeida *et al.* 2008). Two enzymes, DkgA and YqhD, which have NADPH-dependent furfural reductase activity, were discovered to be silenced in furfural-tolerant *E. coli* mutants (Miller *et al.* 2009). Both enzymes have low K_m values for NADPH, therefore, competition for NADPH with other enzymes may be the primary reason for growth inhibition by furfural, as suggested by Miller *et al.* (2009). The parental non-furfural tolerant *E. coli* strain continued to grow after complete furfural reduction. Silencing of the *yqh*D gene resulted in a tolerance to furfural and also to 5-hydroxyfurfural (Miller *et al.* 2010).

The antimicrobial activity of phenolic compounds can be explained with their hydrophobic character, which leads to permeabilization of the cell membrane (reviewed in Hyldgaard *et al.* (2012). As shown by Fitzgerald *et al.* (2004) vanillin acts bacteriostatic on different food-related bacteria due to its influence on cytoplasmatic membrane organization resulting in a loss of ion gradients, pH homeostasis and inhibition of respiratory activity. However, energy generation seems to be mainly unimpaired and the membrane damage appears to be non-lethal. Similarly, eugenol damages the cell membrane of gram negative and positive bacteria leading to protein leakage (Oyedemi *et al.* 2009). Antifungal activity of phenolic compounds also seems to be influenced by hydrophobicity. In a study about antifungal activity of oxygenated essential oil components which are also in large amounts present in pyrolysis oil, it was shown that the substances which indicated the highest antifungal activity against the wood-decaying fungi *Trametes versicolor* and *Coniophora puteana* are also the most hydrophobic ones. Consequently, less hydrophobic compounds showed less antifungal characteristics. Additionally, number and position of the substitution and its oxygen content influenced strongly the antifungal activity (Voda *et al.* 2003; Voda *et al.* 2004)

In 1993, an *Acinetobacter junii* strain was found, which is able to use guaiacol, catechol and syringol as its sole carbon sources (Gonzalez *et al.* 1993). Furthermore, lignin-degrading filamentous fungi are also able to metabolize many aromatic, lignin-derived substances like guaiacols, catechol and vanillin and even aromatic environmental pollutants like DDT, as shown for fungi of the *Phanerochaete* genus, e.g. *P. chrysosporium* (Bumpus and Aust 1987). Basidiomycetous white-rot wood-degrading fungi, for example *P. chrysosporium*, *T. versicolor*,

Pleurotus eryngii and *P. ostreatus*, secret lignin-modifying enzymes like laccases and peroxidases, which degrade the lignin-macromolecule by oxidation (reviewed in Baldrian 2005; Alfaro *et al.* (2014)). Beside degradation, the resulting oxidized phenolic compounds are far more hydrophobic, which leads to a low solubility in media and thus showed less antifungal activity (Voda *et al.* 2004). The examples show, that many organisms are able to deal with toxic compounds, but especially fungi show a high robustness against phenolic compounds.

Finding the toxic compounds within pyrolysis oil for organisms, producing value products and the inhibitory concentration limits as well as understanding the inhibitory mechanisms are important information for further processing and fractionations strategies to make pyrolysis oil a feasible carbon source. The gained results also support the utilization of the aqueous condensate of the pyrolysis process for fermentative processes. The study described in chapter 2, deals with a selection of 11 compounds from pyrolysis oil representing the portfolio of chemical substance classes in the pyrolysis products and their effects to growths and organic acid production of *A. oryzae* and *R. delemar*. The main compound in both pyrolysis products analyzed in this studies is acetic acid (about 4.5 to 5%) which could be discovered as a suitable carbon source for malic acid production (chapter 3 and 4).

1.5 Research proposal

As can be seen from the theoretical background, in a sustainable bioeconomy, renewable raw materials must be used as feedstock material for chemical and biotechnological processes. In In this thesis the challenge of using lignocellulose as substrate for fermentation will be addressed by evaluation of two different pretreatment methods. The pyrolysis process of wheat straw leads to two fractions, an organic (pyrolysis oil) and an aqueous condensate. Further gasification of a mixture of both condensates leads to the formation of syngas, which will be evaluated as carbon source as well. The organosolv-process of two different feedstock plants, *F. sylvatica* (beechwood) and *M. giganteus*, separates lignocellulose into its basic components, lignin, cellulose and hemicellulose. In secondary refining, saccharification of the cellulose into glucose and hemicellulose into xylose is carried out by enzymatic hydrolysis whereas lignin is used for chemical applications.

The evaluation processes is realized in the present work by addressing on the following aspects:

- Which fungi tolerate or are even able to consume pyrolysis oil as carbon source?
- Is pyrolysis oil or the aqueous condensate a suitable carbon source for organic acid production with fungi?
- Which substances from pyrolysis products are toxic for growth, which are toxic for organic acid production?
- Which compounds of pyrolysis products are usable as carbon source for the production of organic acids with fungi?
- Is synthesis gas suitable carbon source for organic acid production with fungi?
- Are the fractions from the organosolv process suitable carbon sources for organic acid production with fungi?

And finally:

- Which of the pretreatment methods are most promising method to prepare lignocellulosic material as carbon source for fungi?
- What are the advantages and disadvantages of the two different pretreatment methods for biological applications?

1.6 References of chapter 1

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2 Evaluation of pyrolysis oil as carbon source for fungal fermentation

This chapter is based on the publication

Evaluation of pyrolysis oil as carbon source for fungal fermentation

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Authors' contribution to this publication

Stefan Dörsam designed all experiments. Performed the growth experiments, performed the experiments of *A. oryzae* together with Jennifer Kirchhoff, and performed the *R. delemar* experiments together with Michael Bigalke. Evaluated the data, designed the table and graphics drafted the manuscript.

Jennifer Kirchhoff performed the experiments with *A. oryzae*.

Michael Bigalke performed the experiments with *R. delemar*.

Nicolaus Dahmen contributed to the production of the pyrolysis oil. Drafted the part of pyrolysis oil production.

Christoph Syldatk contributed to manuscript concept and advisory.

Katrin Ochsenreither contributed to the conception of the experiments and advised the lab work. Critically revised the final manuscript version.

2.1 Introduction

This chapter describes the evaluation of the organic condensate of the pyrolysis process from the bioliq® process at the KIT as carbon source for fungal growth and further more and especially for fungal fermentation for organic acid production. The known robustness, makes fungi the most promising organisms for pyrolysis oil utilization. The aim of this study is to evaluate the suitability of crude pyrolysis oil as a carbon source for fungal growth and fungal fermentation processes. Therefore a variety of fungi from all phyla were tested for their tolerance to crude pyrolysis oil. Tolerance and toxicity tests with representative model substances in several concentrations, were analyzed for their effect on growth and their effects of malic acid production of *A. oryzae* and fumaric acid production with *R. delemar* was studied. Pyrolysis oil is a complex mixture of several organic compounds, produced during flash pyrolysis of organic lignocellulosic material was evaluated for its suitability as alternative carbon source for fungal growth and fermentation processes. Therefore several fungi from all phyla were screened for their tolerance towards pyrolysis oil. Additionally *Aspergillus oryzae* and *Rhizopus delemar*, both established organic acid producers, were chosen as model organisms to investigate the suitability of pyrolysis oil as carbon source in fungal production processes.

It was observed that *A. oryzae* tolerates pyrolysis oil concentrations between 1 - 2% depending on growth phase or stationary production phase, respectively. To investigate possible reasons for the low tolerance level, eleven substances from pyrolysis oil including aldehydes, organic acids, small organic compounds and phenolic substances were selected and maximum concentrations still allowing growth and organic acid production were determined. Furthermore, effects of substances to malic acid production were analyzed and compounds were categorized regarding their properties in three groups of toxicity. To validate the results, further tests were also performed with *R. delemar*. For the first time it could be shown that small amounts of phenolic substances are beneficial for organic acid production. Regarding pyrolysis oil toxicity, 2-cyclopenten-1-on was identified as the most toxic compound for filamentous fungi; a substance never described for anti-fungal or any other toxic properties before and possibly responsible for the low fungal tolerance levels towards pyrolysis oil.

2.2 Material and Methods

2.2.1 Chemicals

All chemicals, including selected substances from pyrolysis oil were either purchased from Sigma-Aldrich (Munich, Germany) or Carl Roth (Karlsruhe, Germany).

2.2.2 Preparation of pyrolysis oil

The pyrolysis oil used in this study was prepared from wheat straw by fast pyrolysis in the bioliq plant at KIT (bioliq®). This process has been developed to convert biomass into a liquid fuel for various applications to produce heat, electricity and transportations fuels. Small and dry biomass particles of a few mm size are rapidly heated up by a heat carrier (e.g. sand) in a pneumatically or mechanically fluidized bed at 500 ± 30 °C in the absence of oxygen. This process is described by Heinrich *et al.* (2016). In case of pyrolysis oils obtained from herbaceous biomass, higher water amounts are formed, which can cause phase separation of the pyrolysis oil. A phase rich in organic compounds (which was used in this study) and an aqueous phase consisting of up to 80 wt.% of water and water soluble organic compounds are formed.

The analyzed monomeric substances are compiled as determined by Thünen Institute Hamburg by GC-MS in the appendix section (**Appendix 1**)

2.2.3 Fungi and media

The fungal strains used, *A. oryzae* DSM 1863 and *R. delemar* DSM 905, were obtained from the DSMZ strain collection (Deutsche Sammlung von Mikroorganismen und Zellkulturen, Braunschweig, Germany). *A. oryzae* was grown on minimal medium (MM) for *Aspergillus* spec. (Barratt *et al.* 1965): 6 g/L $NaNO_3$, 0.52 g/L KCl, 0.52 g/L $MgSO_4 \cdot 7H_2O$, and 1.52g/L KH_2PO_4. The pH was set to 6.5 with NaOH. 10 g/L glucose, 2 mL of 1000× Hutner's Trace Elements, and 15 g/L agar were added after autoclaving. 1000× 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 $(NH_4)_6Mo_7O_{24} \cdot 4H_2O$, pH 6.5 (Barratt *et al.* 1965). *R. delemar* was grown on modified supplemented agar (SUP) (modified from Wöstemeyer 1985): 10 g/L glucose, 0.5 g/L yeast extract, 4 g/L KH_2PO_4, 0.9 g/L K_2HPO_4, 4 g/L NH_4Cl, 0.25 g/L $MgSO_4$. 7 H_2O. The pH was set to 6.5 with NaOH.

For conidia collection, *A. oryzae* was grown on high-salt minimal medium (Song *et al.* 2001) which contains additionally 22.37 g/L KCl. For spore collection, *R. delemar* was grown on malt extract agar (MEA): 30 g/L malt extract, 3 g/L peptone, 15 g/L agar. The conidia and spores were harvested with 50% glycerol from plates that were incubated for five days at 30 °C and filtered through Miracloth (Calbiochem). The spore/conidia solution was diluted to a concentration of $1x10^7$ (spore/conidia)/mL and stored at -80 °C.

Fungi for pyrolysis oil tolerance tests were either obtained from DSMZ, ATCC (American Type Culture Collection), NRRL (Northern Regional Research Laboratory) or JMRC (Jena Microbial Resource Collection) and grown on MM (*Alternaria alternata* DSM 12633, *Aspergillus niger* NRRL 3, *Aspergillus terreus* DSM 5770, *Aspergillus nidulans* DSM 820, *Penicillium chrysogenum* ATCC 48271), yeast minimal medium (*Aureobasidium pullulans* DSM 2404, *Candida bombicola* ATCC 22214, *Saccharomyces cerevisiae* DSM 11285, *Yarrowia lipolytica* DSM 1345, *Cryptococcus curvatus* ATCC 20508, *Phanerochaete chrysosporium* DSM 1547, *Pleurotus ostreatus* DSM 11191, *Trametes versicolor* DSM 3086, *Mucor circinelloides* SF 006299) or modified SUP (*Backusella circina* SF 000941, *Mortierella elongate* SF 009721, *Phycomyces blakesleeanus* SF 018907, *Rhizopus microspores* STH 00427, *Umbelopsis ramanniana* SF 011341). Yeast minimal medium (YMM) contains 20 g/L glucose and 6.7 g/L yeast nitrogen base. All media were sterilized by autoclaving.

Organic acid production was accomplished in a two-step process with a pre-culture and a main culture. The pre-culture medium for *A. oryzae* consists of 40 g/L glucose monohydrate, 4 g/L $(NH_4)_2SO_4$, 0.75 g/L KH_2PO_4, 0.98 g/L $K_2HPO_4 \cdot 3H_2O$, 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 $FeSO_4 \cdot 7H_2O$. Main culture medium for *A. oryzae* contains 120 g/L glucose monohydrate, 1.2 g/L $(NH_4)_2SO_4$, 0.1 g/L KH_2PO_4, 0.17g/L $K_2HPO_4 \cdot 3H_2O$, 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 $FeSO_4 \cdot 7H_2O$.

The pre-culture medium for *R. delemar* consists of 30 g/L glucose, 2.0 g/L urea, 0.6 g/L KH_2PO_4, 0.5 g/L $MgSO_4 \cdot 7H_2O$, 0.11 g/L $ZnSO_4$, 8.8 mg/L $FeSO_4 \cdot 7H_2O$. The pH was set to 4.5 with 10 M HCl after autoclaving to support growth in form of pellets. Main culture medium for *R. delemar* consists of 100 g/L glucose, 0.2 g/L urea, 0.6 g/L KH_2PO_4, 0.5 g/L $MgSO_4 \cdot 7H_2O$, 0.11 g/L $ZnSO_4$, 8.8 mg/L $FeSO_4 \cdot 7H_2O$. The media were sterilized by autoclaving. To keep the pH above 5.5 during fermentation, 90 g/L $CaCO_3$ was added to both main culture media. For inhibition experiments main culture medium was mixed with the indicated amount of chemicals or pyrolysis oil.

2.2.4 Germination and growth inhibition analysis

To prepare testing plates, different concentrations of the respective substances were added to the agar containing MM for *A. oryzae* or modified SUP for *R. delemar* directly after autoclaving. To determine the inhibitory concentration of pyrolysis derived substances on growth and germination, agar plates were inoculated onto the middle of the plate with $4x10^4$ conidia/spores. After incubation for three days at 30 °C the diameter of the colony was determined every day

over five days using a ruler. For the inhibitory concentration of pyrolysis oil, conidia/spores were streaked onto MM/SUP agar plates with different amounts of pyrolysis oil. For pyrolysis oil tolerance tests with all other fungi, spores, conidia or mycelium fragments were transferred on agar plates containing pyrolysis oil and incubated for five days.

To promote agar solidification after addition of pyrolysis oil, the pH was set to 6 by titration with NaOH.

2.2.5 Organic acid production

For *A. oryzae* pre-culture, 100 mL of pre-culture medium was filled into 500 mL Erlenmeyer shake flasks and inoculated with $2x10^7$ conidia. The flasks were incubated at 100 rpm and 30 °C for 24 hours in a rotary shaker. To remove the pre-culture medium, fungal pellets were washed twice with distilled water. 100 mL of main culture was transferred to 500 mL Erlenmeyer shake flasks and mixed with 9 g/L sterile $CaCO_3$. The flasks were inoculated with 10% (*v*/*v*) of washed pre-culture and incubated at 120 rpm and 32 °C for seven days.

For *R. delemar* pre-culture, 100 mL of pre-culture medium was filled into 500 mL Erlenmeyer shake flasks and inoculated with $1x10^7$ spores. The flasks were incubated at 100 rpm and 35 °C for 30 hours in a rotary shaker. To remove the pre-culture medium, fungal pellets were washed twice with distilled water. 100 mL of main culture was transferred to 500 mL Erlenmeyer shake flasks and mixed with 9 g/L sterile $CaCO_3$. The flasks were inoculated with 10% (*v*/*v*) of washed pre-culture and incubated at 120 rpm and 35 °C for seven days.

For both fungi, the first sample was taken after 72 hours and subsequently every 48 hours.

2.2.6 Organic acid analytics

For malic and fumaric acid quantification by HPLC, fermentation broth samples were pre-treated and analyzed as described in Ochsenreither *et. al.* (2014) with minor modifications. To re-dissolve the precipitated calcium malate/fumarate, 1 mL of well-mixed sample was mixed with 1 mL of 3 M H_2SO_4 and 3 mL of distilled water and incubated at 80 °C for 20 min. 1 mL of the mixture was transferred to a 1.5 mL Eppendorf tube and centrifuged in a table top centrifuge for 5 minutes at 20,000×g. The supernatant was used for HPLC analysis, which was performed with a standard HPLC device (Agilent 1100 Series, Agilent, Germany) prepared with a 15 cm reversed phase column (Synergi™4 µm Fusion-RP 80 Å, LC Column 150×4.6 mm, Phenomenex, Aschaffenburg, Germany) at 30 °C. Mobile phase solution A was 100% methanol, and solution B was 20 mM KH_2PO_4, pH 2.5. The flow rate was 1 mL/min and a gradient was used for the separation of organic acids: 0–0.5 min 100% eluent B, 0.5–10-min

increase of eluent A from 0 to 10%, 10–12-min a further increase of eluent A from 10 to 70%, 12-14 min a decrease of eluent A from 70 back to 0%, and 14–18 min again 100% eluent B. The increase of eluent A to 70% from 10-12 min was applied to elute and analyze the tested hydrophobic substances which were added to the medium. The injection volume was 10 µL and the detection was performed by a UV detector at 220 nm. Standards were used for peak identification and calibration. The linear detection range went from 0.1 to 5 g/L malic acid and 0.02 to 0.5 g/L fumaric acid.

2.3 Results

2.3.1 Pyrolysis oil as carbon source for fungi

To determine the pyrolysis oil tolerance limits of fungi, fungal species of all phyla (Ascomycota, Basidiomycota, and Zygomycota) were either streaked out, or mycelium fragments were transferred on agar plates containing in addition to glucose different concentrations of pyrolysis oil. The results are summarized in **Table 2-1**.

Table 2-1: Growth of fungi on minimal agar plates depending on addition of different concentrations of pyrolysis oil from 0 to 3%.

Phylum	Organism	Pyrolysis oil content (% w/v)[a]				
		0	**0.5**	**1**	**2**	**3**
Ascomycota	*Alternaria alternata*	+	+	-	-	-
	Aspergillus niger	+	+	-	-	-
	Aspergillus terreus	+	+	+	-	-
	Aspergillus nidulans	+	+	-	-	-
	Aspergillus oryzae	+	+	+	+	-
	Aureobasidium pullulans	+	+	+	-	-
	Candida bombicola	+	+	+	-	-
	Penicillium chrysogenum	+	-	-	-	-
	Saccharomyces cerevisiae	+	+	+	-	-
	Yarrowia lipolytica	+	+	-	-	-
	Trigonopsis variabilis	+	-	-	-	-
Basidiomycota	*Cryptococcus curvatus*	+	-	-	-	-
	Phanerochaete chryso-sporium	+	+	+	-	-
	Pleurotus ostreatus	+	-	-	-	-
	Trametes versicolor	+	+	+	+	+
Zygomycota[b]	*Backusella circina*	+	+	-	-	-
	Mortierella elongata	+	-	-	-	-
	Mucor circinelloides	+	+	+	-	-
	Phycomyces blakesleeanus	+	+	-	-	-
	Rhizopus microsporus	+	-	-	-	-
	Rhizopus delemar	+	+	+	-	-
	Umbelopsis ramanniana	+	-	-	-	-

[a]Media contain 10 g/L glucose

[b]Former phylum contains: *Entomophthoromycotina, Kickxellomycotina, Mucoromycotina, Zoopagomycotina*

Most of the analyzed fungi tolerated a pyrolysis oil content of 0.5%. Only *P. chrysogenum, T. variabilis, C. curvatus, P. ostreatus, M. elongata, R. microspores* and *U. ramanniana* were not able to grow under these conditions. 1% is above the upper tolerance limit of *A. alternata, A. niger, A. nidulans, Y. lipolytica, B. circina* and *P. blakesleeanus*. Higher concentrations were only tolerated by *A. oryzae*, which grew up to a pyrolysis oil content of 2% and *T. versicolor* which grew on all tested concentrations. Additionally colonies of *T. versicolor* showed a dark/black halo on pyrolysis oil containing agar plates.

The results showed a great range of tolerance across the kingdom of fungi. Beside tolerance, the metabolization of pyrolysis oil is necessary for it to be used as carbon source for fungal fermentation. Because of the ability to produce a value added product and the highest tolerance limit for pyrolysis oil, further tests were conducted with *Aspergillus oryzae* and additionally with *Rhizopus delemar*.

A. oryzae was able to grow on sugar free medium containing up to 1% of pyrolysis oil as sole carbon source. Therefore, *A. oryzae* is able to metabolize substances within pyrolysis oil for biomass production. However, on plates containing more than 1% of pyrolysis oil as sole carbon source, growth was not observed (data not shown). Due to the fact that organic acid production takes place during the stationary growth phase, the fermentation process is substantially different to active growth. Therefore, the effect of pyrolysis oil on malic acid production has to be investigated separately. Consequently, malic acid production was tested in the presence of 0 - 3% pyrolysis oil. By using pyrolysis oil as sole carbon source, malic acid production was not observed.

The comparison between tolerance tests (medium contains glucose and pyrolysis oil in various concentrations) and utilization tests (medium contains only pyrolysis oil in various concentrations) indicates that pyrolysis oil can be tolerated in higher concentrations by fungi when glucose is the main carbon source. However, with increasing pyrolysis oil content fungal growth is more and more restrained and the production of malic acid and fumaric acid by *A. oryzae* and *R. delemar*, respectively, is strongly reduced even in the presence of glucose (**Figure 2-1**).

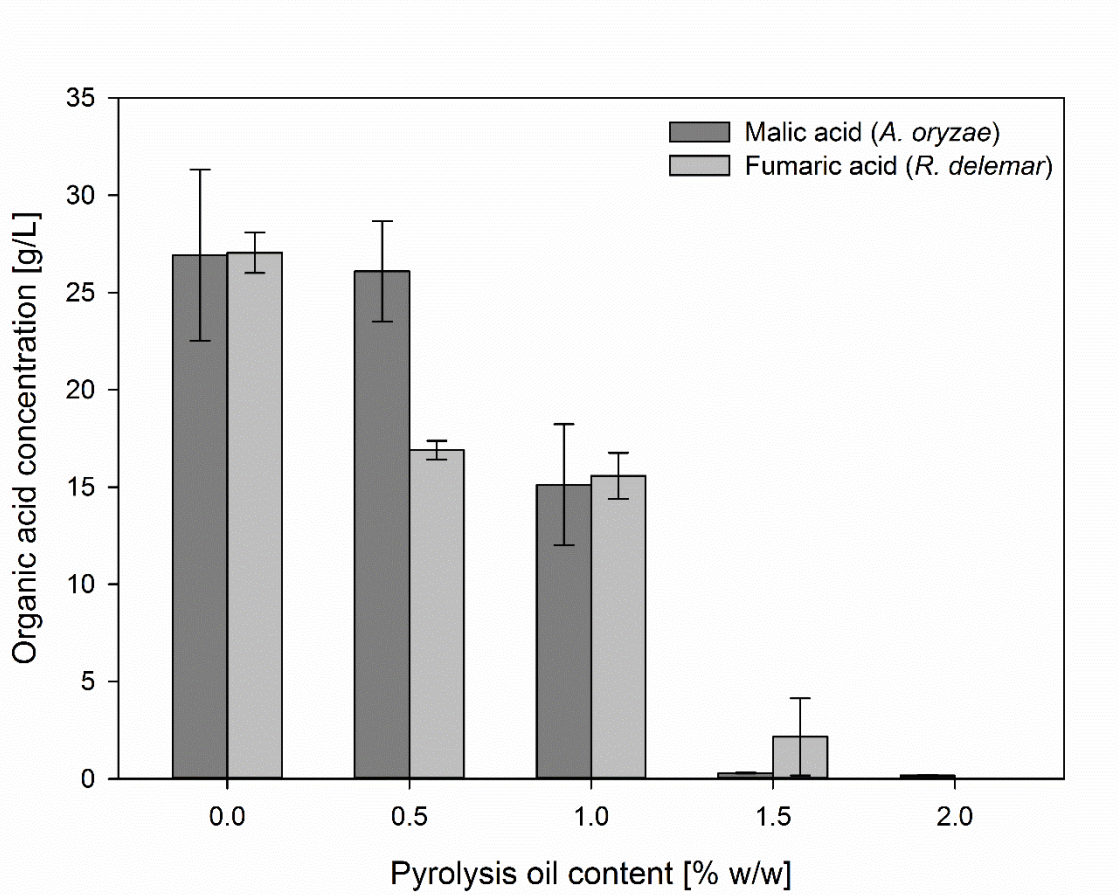

Figure 2-1: Influence of different pyrolysis oil concentrations in production medium on malic acid production by *A. oryzae* DSM 1863 and fumaric acid production by *R. dele-mar* DSM 905. The experiments were done in shake flasks cultivated at 120 rpm and 32

With respect to malic acid production by *A. oryzae*, the addition of 0.5% of pyrolysis oil to organic acid production medium has no influence compared to medium without pyrolysis oil (26.09 ± 2.59 g/L to 26.92 ± 4.40 g/L), whereas fumaric acid production by *R. delemar* is strongly influenced in the presence of 0.5% of pyrolysis oil (27.04 ± 1.04 g/L to 16.90 ± 0.43 g/L). Higher concentrations led to a successive decrease of malic acid production with *A. oryzae* from 15.12 ± 3.11 g/L at 1%, to finally 0.3 ± 0.03 g/L and 0.18 ± 0.03 g/L with 1.5% and 2% pyrolysis oil content, respectively. Between 0.5% and 1% there was only a small decrease in fumaric acid production with *R. delemar* to 15.58 ± 1.18 g/L. Higher concentration led also to a successive decrease of final organic acid concentration to 2.15 ± 1.98 g/L at 1.5% and 0 with 2%.

2.3.2 Toxicity analysis of pyrolysis oil derived substances

Results of the tolerance and utilization tests showed that crude pyrolysis oil is in principle suitable as carbon source for fungi; however, applicable concentrations are too low for most processes and for organic acid production in particular. The elucidation of substances problematic for growth and organic acid production is therefore a prerequisite for further application of

pyrolysis oil in biotechnology. By avoiding the formation of the identified substances during fast pyrolysis by adjusting process parameters of by decreasing their content below the critical concentration by fractionation might be a practical solution. For this, eleven representative and commercially available substances which are present in higher concentrations in the oil, were selected and tested for the more tolerant *A. oryzae*. A selection of these were also tested for *R. delemar*. The chosen concentrations were based on the amount found in pyrolysis oil as the upper limit. Some of the analyzed substances showed no inhibition effects, whereas some had a great impact even in low concentrations. An overview of the analyzed chemicals and their inhibitory concentrations for *A. oryzae* are shown in **Table 2-2** and for *R. delemar* in **Table 2-3**.

Table 2-2: Overview of growth and malic acid production limits of *A. oryzae* depending on different concentrations of pyrolysis oil derived substances.

Tested substances	Concentration in pyrolysis oil [% w/w]	Growth limit [% w/w][a]	Malic acid production limit [% w/w][a]
Propionic acid	1.302	0.07	> 1.3
Ethylene glycol	1.258	> 1.25	> 1.25
γ-Butyrolactone	0.335	> 0.335	> 0.335
Hydroxyacetone	4.4631	1.5	2.5
Syringol*	0.556	0.27	0.3
Guaiacol	0.469	0.1	0.1
Furfural	0.281	0.03	0.07
Phenol	0.384	0.07	0.07
Isoeugenol*	0.524	0.03	0.06
o-,m,-p-Cresol	0.17	0.05	0.03
2-Cyclopenten-1-on	0.308	0.00625	0.0125

[a] Values represent the highest tested concentrations where *A. oryzae* was still able to grow/ produce malic acid. In the next higher tested concentration growth/production was not observed.

*Analysis indicates a degradation of substances during cultivation.

‘>’ Limit is above highest tested concentration

Table 2-3: Overview of growth and fumaric acid production limits of *R. delemar* depending on different concentrations of pyrolysis oil derived substances.

Tested substances	Concentration in pyrolysis oil [% w/w]	Growth limit [% w/w][a]	Fumaric acid production limit [% w/w][a]
Propionic acid	1.302	0.1	> 1.3
Hydroxyacetone	4.4631	1.5	1.5
Isoeugenol	0.524	0.025	0.005
2-Cyclopenten-1-on	0.308	0.005	0.005

[a] Values represent the highest tested concentrations where *R. delemar* was still able to grow/ produce fumaric acid. In the next higher tested concentration growth/production was not observed.

'>' Limit is above highest tested concentration

2.3.3 Growth limits

When observing the influence of the selected compounds on the growth behavior of *A. oryzae* compared to control, the obtained results could be divided in two groups. The first group comprises of substances with very low influence to the growth of *A. oryzae* in the analyzed concentrations. This group contains ethylene glycol and γ-butyrolactone. It was concluded that the maximum tolerance levels are probably much higher than the concentrations in pyrolysis oil, so that these substances will not be accounted as critical. The second group contains all other analyzed substances. These substances showed a considerable inhibition to fungal growth when added in concentrations relevant to their content in pyrolysis oil. Typically, growth is reduced even at the lowest tested concentration when compared to the control. The inhibition curves are shown in **Figure 2-2** using the examples γ-butyrolacton and guaiacol as representatives for the two groups. For *R. delemar* all substances in the tested concentration could be classified to the second group.

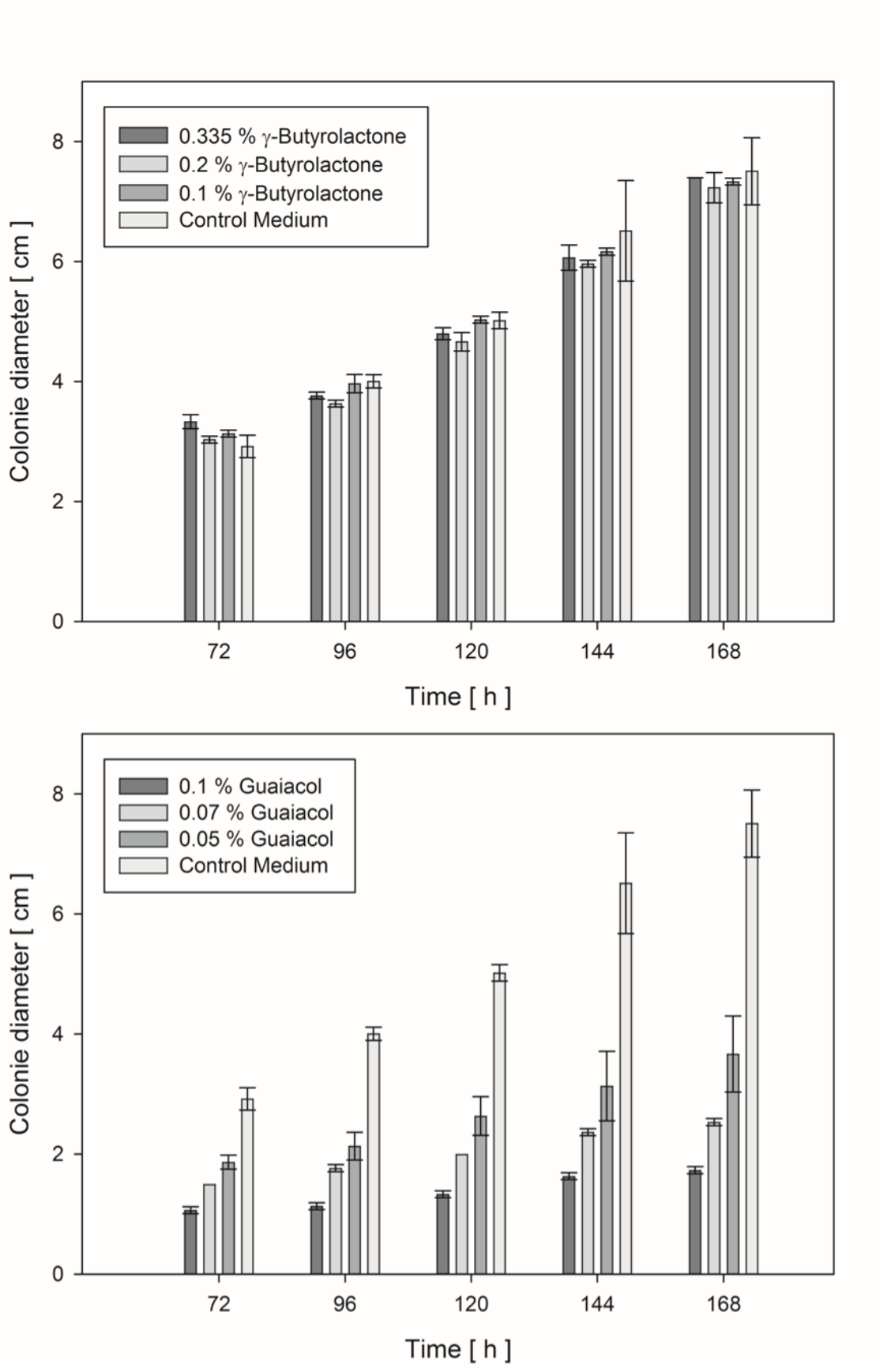

Figure 2-2: Growth of *A. oryzae* on Agar plates containing γ-butyrolacton or guaiacol. Plates were incubated at 30 °C and the diameter of the colony was measured with a ruler every 24 h. All values are given as average of three independent experiments ± standard.

In the growth experiments with *A. oryzae*, agar medium containing syringol, a yellow/orange colored substance, was decolorized around the fungal colony indicating for degradation or derivatization of syringol.

2.3.4 Organic acid production limit

Several substances from pyrolysis oil were tested for their effects on the organic acid production of *A. oryzae* and *R. delemar* and their inhibition limits were detected. The analyzed concentrations and the resulting yields in relation to the respective organic acids are shown in **Table 2-4** and **Table 2-5**.

Table 2-4: Overview of tested substances from pyrolysis oil and tested concentrations and their effects to malic acid production yields of *A. oryzae*.

Tested substances	Concentrations in main culture medium [%]	$Y_{P/S}$ [g/g]
Control (10% Glucose)		0.64
Propionic acid	1.3	0.66
	1	0.65
	0.5	0.67
	0.4	0.69
	0.3	0.74
	0.1	0.59
	0.07	0.58
	0.05	0.61
Hydroxyacetone	2.5	0.42
	2	0.37
	1.5	0.39
	1	0.54
Isoeugenol	0.06	0.35
	0.05	0.60
2-Cyclopenten-1-on	0.0125	0.40
	0.00625	0.38
	0.003125	0.46
Ethylene glycol	1.25	0.56

	1.2	0.48
	1	0.62
	0.7	0.51
	0.5	0.65
	0.3	0.66
γ-Butyrolactone	0.335	0.59
	0.3	0.57
	0.25	0.55
	0.2	0.56
	0.15	0.52
	0.1	0.49
Syringol	0.3	0.04
	0.27	0.06
	0.25	0.06
	0.23	0.09
	0.2	0.17
	0.17	0.20
Guaiacol	0.1	0.82
	0.07	0.69
	0.005	0.65
Furfural	0.07	0.67
	0.05	0.54
	0.03	0.55
	0.02	0.56
	0.01	0.57
Phenol	0.07	0.25
	0.05	0.52
	0.03	0.86
o-,m,-p-Cresol	0.03	0.53
	0.02	0.62
	0.01	0.74

Tested substance concentrations with no observed production are not shown.

Table 2-5: Overview of tested substances from pyrolysis oil and tested concentrations and their effects to fumaric acid production yields of *R. delemar*.

Tested substances	Concentration in main culture medium [%]	$Y_{P/S}$ [g/g]
Control (10% Glucose)		0.38
Propionic acid	1.3	0.51
	1	0.10
	0.7	0.22
	0.5	0.21
	0.25	0.26
Hydroxyacetone	1.5	0.15
Isoeugenol	0.005	0.02
2-Cyclopenten-1-on	0.005	0.10

Tested substance concentrations with no observed production are not shown.

In contrast with the growth inhibition experiments, malic acid production is affected in a more complex way by the added substances. In the control approach, a yield of malic acid production with *A. oryzae* of 0.64 g/g could be achieved. Based on the production curves appearance, the chemicals tested can be divided into three groups. The first one contains propionic acid, cresol, ethylene glycol, 2-cyclopenten-1-on, furfural, guaiacol and γ-butyrolactone. Regarding γ-butyrolactone, ethylene glycol and propionic acid all tested concentrations showed no influence on malic acid production compared to their absence. This is also valid for the yields, with a range from 0.48 g/g to 0.66 g/g with ethylene glycol and 0.49 g/g to 0.59 g/g with γ-butyrolactone. 0.3% of propionic acid in the medium even led to a substantially higher yield for malic acid production (0.74 g/g). For furfural (0.54 - 0.67 g/g), guaiacol, cresol and 2-cyclopenten-1-one (0.38 - 0.46 g/g) no inhibition of malic acid production was observed until a certain critical concentration of substances was added. In fact, malic acid production was even promoted in the presence of cresol and guaiacol compared to the control approach, also with higher yields (up to 0.82 g/g with guaiacol and 0.74 g/g with cresol) until the critical concentration was reached. However, the transition from no influence to total inhibition of production is very abrupt. Selected production curves of furfural are shown in **Figure 2-3** as representative of this group.

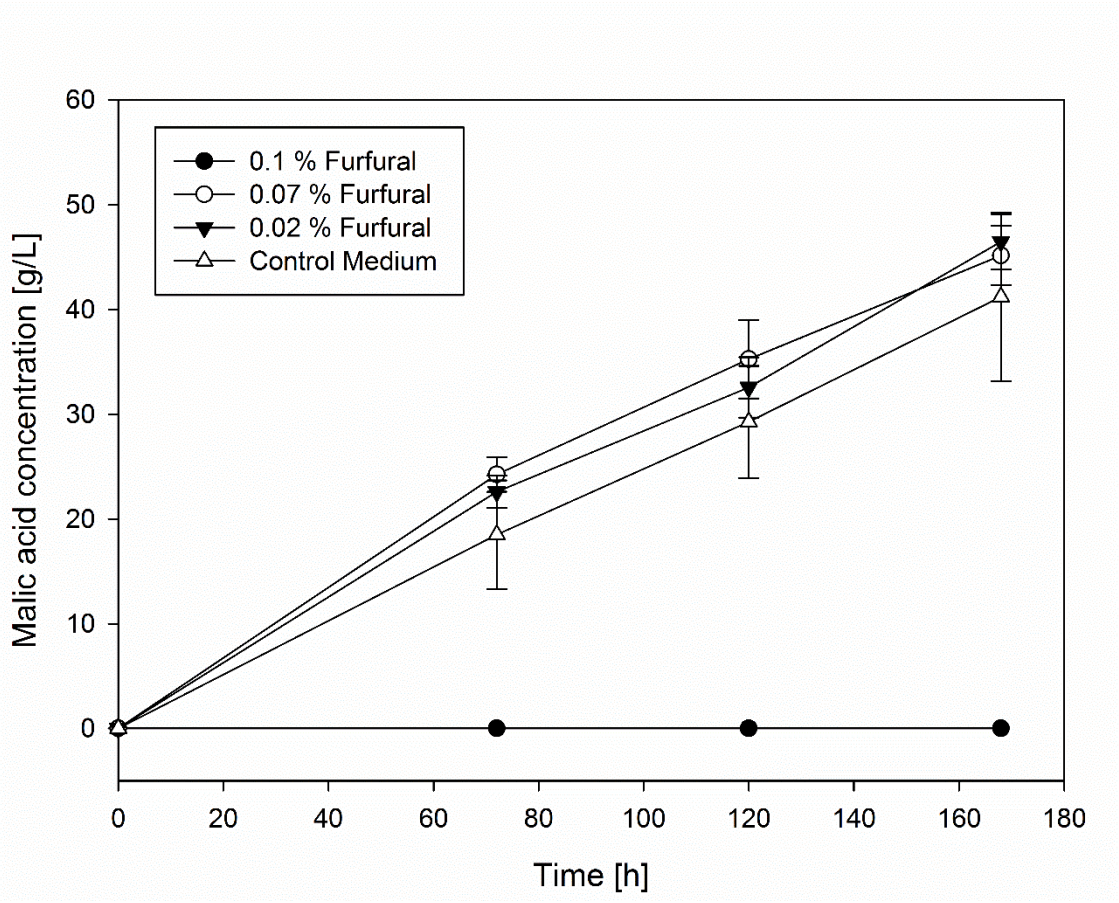

Figure 2-3: Selected production curves of malic acid depending on different furfural concentration added to the main culture medium. As reference main culture medium without furfural was used. Shaking flasks were incubated at 32 °C for seven days.

The produced concentration of malic acid after seven days of fermentation with furfural addition ranged between 43.59 ± 2.69 g/L and 46.48 ± 2.63 g/L, with ethylene glycol addition between 36.04 ± 3.26 g/L and 44.59 ± 2.36 g/L, with propionic acid addition between 33.66 ± 2.47 g/L and 44.41 ± 4.29 g/L. With 2-cyclopenten-1-on addition the concentration ranged between 32.91 ± 7.45 g/L and 39.05 ± 9.2 g/L and with cresol addition between 40.09 ± 2.37 g/L and 67.07 ± 14.49 g/L. For guaiacol addition the produced final malic acid concentration ranged between 43.66 ± 27.09 and 61.45 ± 20.91. The control approach resulted in a malic acid concentration of 41.21 ± 8.06 g/L. Except 2-cyclopenten-1-on, substances from this group are therefore considered as moderately critical in concentrations relevant to their content in pyrolysis oil but have also been shown to promote malic acid production below a certain threshold.

The second group includes most of the remaining substances. Regarding hydroxyacetone, phenol and syringol, malic acid production correlates directly with their concentration in the main culture medium. For these substances the transition from no influence to total inhibition is

smooth. As an example of this group a selection of the production curves of phenol is shown in **Figure 2-4**.

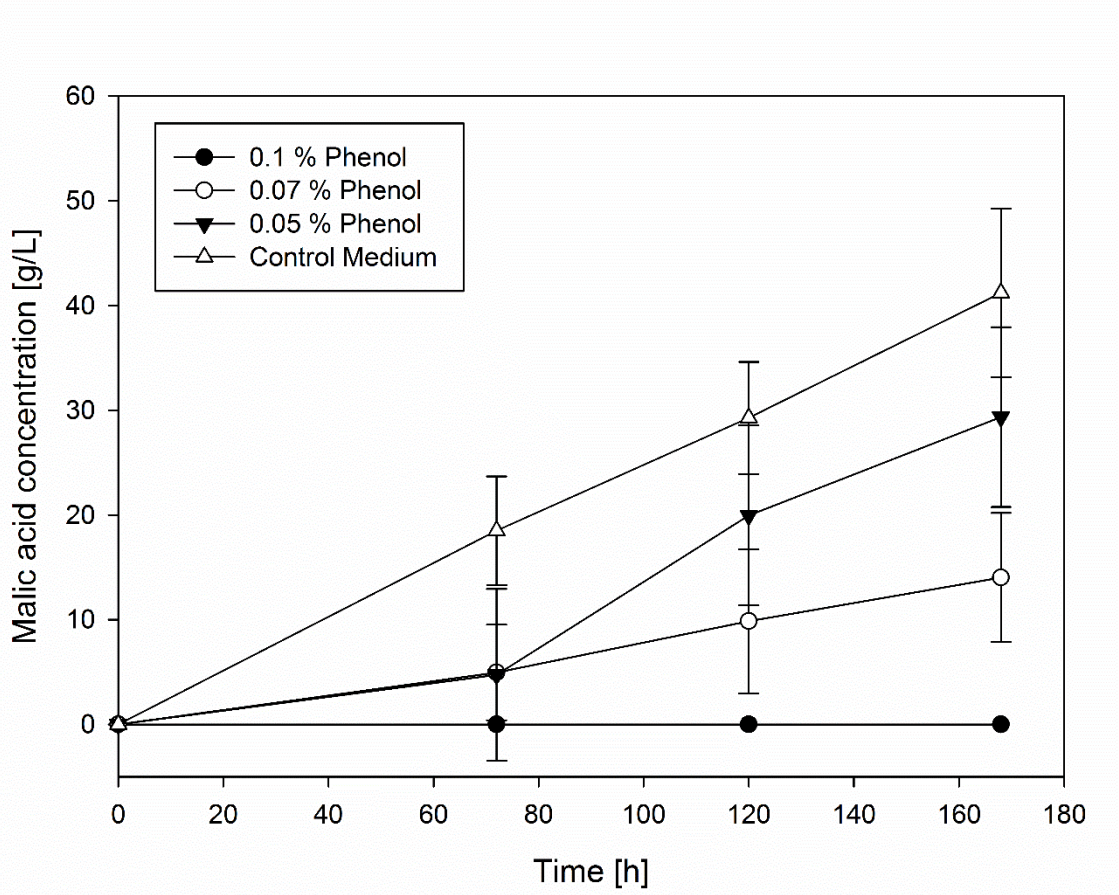

Figure 2-4: Selected production curves of malic acid depending on different phenol concentration added to the main culture medium. As reference main culture medium without phenol was used. Shaking flasks were incubated at 32 °C for seven days.

It was observed that lower concentrations of phenol increased malic acid production when compared to the absence of phenol. By adding 0.03% of phenol, malic acid concentration raised to 53.79 ± 1.25 g/L after 168 hours compared to the control approach with 41.21 ± 8.06 g/L. Above this limit concentration of phenol decreased the production. This is also valid for the yields, where 0.03% of phenol leads to the highest yield of 0.86 g/g. In contrast, higher concentrations of phenol decreased the yield until 0.25 g/g with 0.07%. Similarly, the addition of 1% hydroxyacetone led to the production of 46.19 ± 8.09 g/L malic acid which is slightly higher than in the control approach but with a lower yield (0.54 g/g). Lower concentrations of added hydroxyacetone had no influence on malic acid production, whereas higher concentrations of hydroxyacetone decreased the production. Yields ranged from 0.37 g/g to 0.54 g/g in tested concentrations. However, the lowest tested concentration (0.17%) of syringol resulted in a much lower concentration of malic acid (21.83 ± 5.13 g/L) than in the control approach. The

resulting yields for all syringol concentrations were in a very long range, between 0.04 g/g (with 0.3%) and 0.2 g/g (with 0.17%), increasing the yield with the decreasing syringol concentration.

The last group of malic acid production curves contains only one member. Isoeugenol showed considerable evidence of degradation by the fungus during the fermentation process. A selection of the production curves of isoeugenol are shown in **Figure 2-5**.

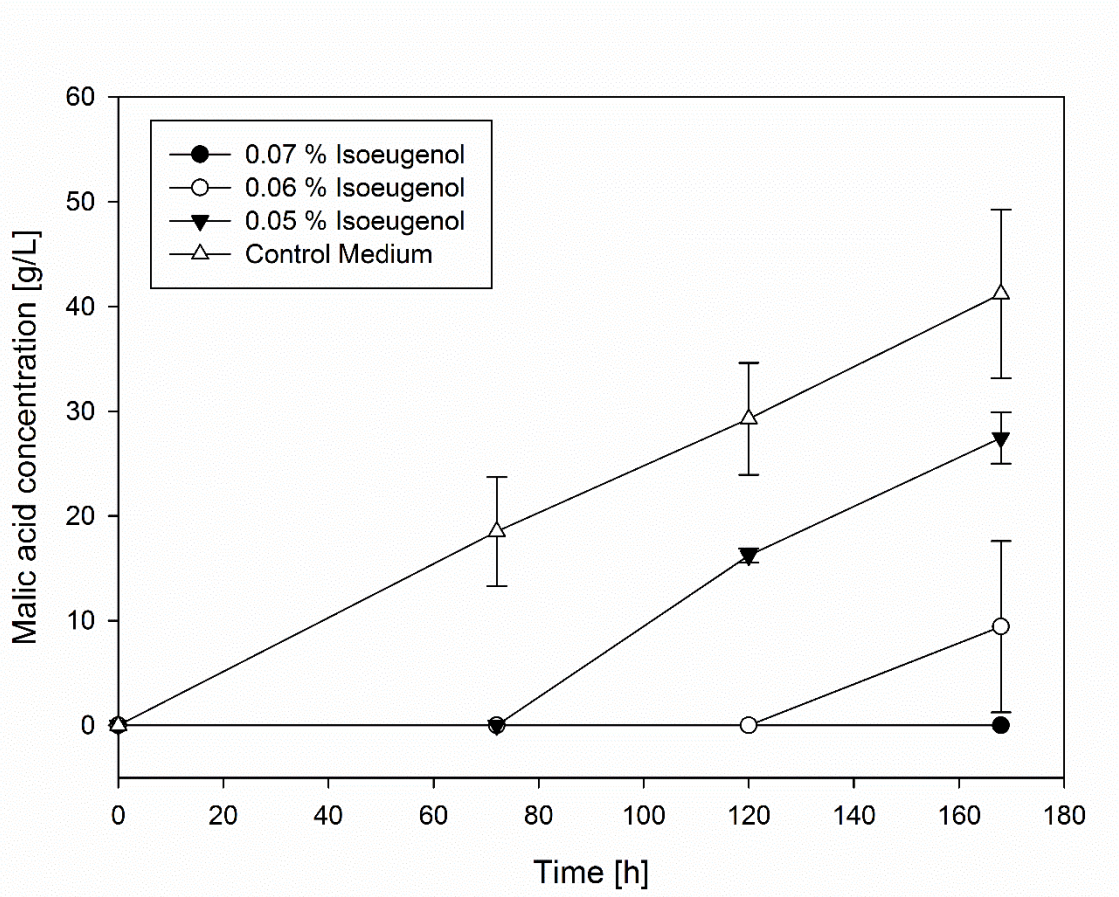

Figure 2-5: Selected production curves of malic acid depending on different isoeugenol concentration added to the main culture medium. As reference main culture medium without isoeugenol was used. Shaking flasks were incubated at 32 °C for seven days.

For all tested concentrations, malic acid production was delayed depending on initial isoeugenol concentration. At a concentration of 0.07% production was not observed during cultivation. The lowest tested isoeugenol concentration (0.05%) resulted in much lower malic acid production (27.46 ± 2.45 g/L) than in the control approach, but with a very similar yield of 0.6 g/g. This was lower for 0.6% isoeugenol (0.35 g/g), which could be a hint for degradation of isoeugenol during fermentation **(Table 2-2**).

The categorization into several groups of fumaric acid production inhibition of *R. delemar* is not possible because of the small amount of tested substances and larger concentration intervals.

The yields were in general very low compared to the control approach with 0.38 g/g, except the yield for 1.3% propionic acid, which is higher than the control (**Table 2-3**). By decreasing the propionic acid concentration to 0.25%, fumaric acid yield increased to 0.26 g/g. Only one single, very low concentration could be found for hydroxyacetone (0.15 g/g), 2-cyclopenten-1-on (0.1 g/g) and isoeugenol (0.02 g/g) where product formation could be observed.

2.4 Discussion

Pyrolysis oil as a complex mixture of organic compounds is an interesting but challenging substrate for fermentation. Besides sugars and organic acids, which are easily assessable as carbon sources, many substances are present which have not been studied for their influence on microorganisms and which might be critical for growth or production. So far, the main focus of biotechnological application of pyrolysis oil lies on the carbohydrate components, in particular levoglucosan. Levoglucosan was either converted to glucose or used directly for biotechnological processes, e.g. ethanol production and itaconic acid production (Luque *et al.* 2014; Nakagawa *et al.* 1984). *Aspergillus niger* CX-209 was cultivated for citric acid production both, on pure levoglucosan and on a cotton based pyrolysis oil (Zhuang *et al.* 2001). Using pure cellulose as a feedstock results in a levoglucosan rich pyrolysis oil containing low amounts of other organic and lignin-derived compounds making a comparison to the pyrolysis oil used in this study impossible. Tests with similar crude pyrolysis oil are rare. Yang *et. al*. (2011) analyzed the growth of 6 fungi based on either pure pyrolysis oil or pyrolysis oil added to potato medium in concentration between 0 - 0.3%. Two of the tested fungal species, *Aspergillus niger* and *Phanerochaete chrysosporium*, were also tested in this study and found to tolerated up to 0.5% and 1% pyrolysis oil, respectively (**Table 2-1**). We tested fungi of all phyla and showed that most fungi are able to tolerate much higher concentrations of pyrolysis oil than tested before. Furthermore the high tolerance level of *T. versicolor* and the ability of growth with up to 1% pyrolysis oil of *A. oryzae* makes crude pyrolysis oil to a possible carbon source for biomass formation, but not for organic acid production. The tolerance of higher amounts of pyrolysis oil of *T. versicolor* is attributed to the fact that these organism is a lignin degrader and can probably handle aromatic compounds in the oil. However, the results also give an insight into the complex toxic effects of the different compounds within pyrolysis oil. The eleven chosen substances were analyzed as representatives of the different chemical groups, like organic acids, phenolic compounds and lactones. Because glucose was used as carbon source in all tolerance experiments the main focus of this work was to discover and describe the toxicity of the chemical compounds in pyrolysis oil and the reason for the observed production and growth limits. These

results are also important for biotechnological application of other pretreated biomass containing similar substances as contaminants. Additionally, some of the analyzed chemicals are also relevant as environmental pollutants, and therefore this work could be helpful in the field of fungal bioremediation.

Following, the effects of the single substances and possible inhibition mechanisms are discussed. With regard to organic acids, the results show that addition of propionic acid has a major influence on the growth of *A. oryzae* and *R. delemar*. Even in the presence of low propionic acid concentration, i.e. 0.1%, growth was only observed for *R. delemar*. This observation is consistent with numerous studies that report a growth inhibition of *A. flavus*, a close relative to *A. oryzae*, with increasing concentration of propionic acid (Ghosh and Häggblom 1985). Interestingly, propionic acid had a minor influence on malic acid production as 33.66 ± 2.47 g/L malic acid was produced in the presence of 1.3% propionic acid concentration, which corresponds to its content in pyrolysis oil. This is also valid for the yields. Propionic acid has a higher impact to the fumaric acid production with *R. delemar*, where a rising concentration leads to a lower production in total but does not influence the yields, being the highest at 1.3% propionic acid. For every other concentration the yields are lower than in the control. However, acetic acid, which is the main component of pyrolysis oil with approx. 5%, didn´t show any toxic effects, and in contrast, it can be used as carbon source for *A. oryzae* (Oswald *et al.* 2016). With respect to small molecular compounds, the addition of ethylene glycol had only a minor influence on growth. A slight decrease in the formed malate concentrations and the colony diameter with increasing concentrations of ethylene glycol was observed. Regarding malic acid production, ethylene glycol had also only minimal effects to the yields. Alcohol oxidase, which was discovered and described in *A. ochraceus* (Isobe *et al.* 2007), and is responsible for the degradation of ethylene glycol, might be accountable for the observed tolerance towards ethylene glycol in this study. However, a decrease of ethylene glycol during cultivation time could not be verified.

One of the most critical substances tested is 2-cyclopenten-1-one, with which growth and malic acid production are only possible at very low concentrations. The presence of this substance had a greater influence on the growth of *A. oryzae* than on malic acid production, but in general it is toxic for both fungi in very low concentrations. In contrast to *A. oryzae*, it leads with *R. delemar* also to a very low yield of 0.1 g/g. Due to the low inhibitory concentration, a hormonal effect of 2-cyclopenten-1-one might be conceivable. In cell culture it could be shown that 2-cyclopenten-1-one induces the production of the heat shock protein 70 (HSP 70) in human cells,

and interferes with protein expression (Rossi *et al.* 1996). Another possible reason for the high inhibitory potential of 2-cyclopenten-1-one might be the inhibition of important metabolic pathways, which are not yet described for this substance. Similarly, when adding furfural to agar plates, growth was strongly inhibited for all tested concentrations. Even at a furfural concentration of 0.05% fungal growth was inhibited. The growth inhibition could, as known for *E. coli*, be caused by a low availability of sulfur-containing amino acids. The inhibition of the synthesis of these amino acids could be observed in presence of furfural (Miller *et al.* 2009). Another possible reason for a growth inhibition is the chemical reactivity of the aldehyde furfural. This reactivity has been suggested to be the reason for the toxicity or furfural (Zaldivar, Martinez, and Ingram 1999). However, the influence of furfural on malic acid production was less pronounced. In contrast, hydroxyacetone was tolerated in very high concentration compared to other tested substances for both fungi. Because of the solvent properties of hydroxyacetone, a change in the ambient conditions due to hydroxyacetone addition might possibly be leading to increased cell membrane disorganization, especially in the higher concentration ranges, resulting in an inhibition of growth and malic acid production. Surprisingly, the impact of the product yield was considerably higher for *R. delemar* with a yield of 0.15 g/g, in contrast to minor effect on malic acid production with *A. oryzae*.

Although γ-butyrolactone is also used as a solvent, it shows apparently no effects to the cells at the tested concentrations. Yet, in the presence of all tested γ-butyrolactone concentrations inhibition of growth and malic acid production of *A. oryzae* was not observed. Furthermore, it might be possible that *A. oryzae* is able to metabolize this lactone as a carbon source. The ability to degrade THF is described for some Ascomycota like *Aureobasidium pullulans* (Patt and Abebe 1995). This could be an indication for possible degradation by *A. oryzae*. However, degradation of γ-butyrolactone was not observed by the used analysis methods and the slightly lower yield for malic acid would present a disqualification for its possible usage as carbon source.

The addition of the phenolic compounds phenol, o-, m-, p-cresol, guaiacol, syringol, and isoeugenol, resulted in a strong inhibition of growth even at low concentrations. Phenol is a well-known biocide, o-, m-, p-cresol is frequently used as fungicide. Its toxicity is based on its membrane activity (Mörsen and Rehm 1990). Similar effects of guaiacol on *A. parasiticus* have been published (Pillai and Ramaswamy 2012). At the lowest tested concentration of phenol, cresols and gauaiacol, more malic acid was detected than in the control approach, indicating either a promoting effect of phenol on malic acid production or a better release of the organic acids from the cell. The possibility of an enhanced release of malate and fumarate in the culture medium could be explained by a potentially occurring permeabilization of the cell membrane by

the presence of phenol. It is known that one bottleneck of the malic acid production is the export from cytoplasm to medium (Brown *et al.* 2013) and therefore the permeabilization of the membrane could lead to an accelerated transport by the concentration gradient. With increasing concentrations of phenol, malic acid production drops off quickly due to the toxic effects of increasing cell membrane permeabilization. Compared to that, the yields of malic acid rises with an increase of phenol except the lowest phenol concentration where the yield is extremely high (0.86 g/g) which supports the permeabilization theory.

Although it is known that *A. oryzae* has genes encoding for enzymes enabling the degradation of m-cresol (Machida *et al.* 2005), it seems that *A. oryzae* only tolerates cresol in very low concentrations (growth limit 0.05%; production limit 0.03%). In a study dealing with the degradation of phenol, p-cresol, m-cresol and o-cresol by various fungal species it was shown that *Aspergillus* sp. is less capable of degrading phenol and cresol compared to other fungi (Atagana 2004). The reason for the very sharp transition from less influence to total inhibition, which leads to the classification of cresol to the first group in contrast to phenol, could be that a mixture of o-, m- and p-cresol was tested. Contrary to the other substances, the cresol mixture shows lower toxicity limits to malic acid production than to growth.

Even at low concentrations of syringol inhibition of growth and malic acid with *A. oryzae* production was observed with a correlation between decreasing malic acid production and increasing concentrations of syringol. During growth tests on agar plates, as well as during production in liquid culture medium, decolorization of former violet medium was observed indicating for a possible degradation or derivatization of syringol. The yield of malic acid increased from 0.04 g/g with 0.3% syringol to 0.2 g/g with 0.17% guaiacol, which supports the hypothesis of degradation. However, this kind of degradation or derivatization seems to happen simultaneously to growth or production and does not result in a delay of malic acid production as observed for isoeugenol. The antifungal effect of isoeugenol to the surface growth of various *Aspergillus* species was already described in 1996. In these studies, strong inhibition of growth was observed on agar plates with 0.02% isoeugenol and finally a total inhibition at the same concentration as in this study (0.03%) (Mansour *et al.* 1996). Regarding the malic acid production profile in the presence of isoeugenol, it is fundamentally different to the production curves of other analyzed substances. The start of the malic acid production is delayed: the higher the concentration of isoeugenol, the later the production started, until no production was observed during cultivation time. A possible reason for this phenomenon could be the degradation or derivatization of isoeugenol until a more tolerated concentration is reached. This process needs

energy, which is produced during cell respiration, so as long as energy is needed for the derivatization of the toxic substance, malic acid production does not take place. This theory is supported by consideration of the malic acid yields: In the presence of 0.6% isoeugenol, the yield of fumaric acid with *R. delemar* is the lowest measured. In general, fungal mycelium appears to tolerate higher amounts of toxic substances if it does not grow as during the organic acid production phase. Because of the novelty of this observation, the reasons can only be speculated. During the production phase, the fungal growth is limited by nitrogen deficiency, leading to a decrease of metabolic activity and a shift of resources to acid production. This might bring a lower susceptibility to toxic effects.

2.5 Conclusion

In summary, it was observed that *A. oryzae* and *R. delemar* tend to be more tolerant towards toxic compounds during the acid production phase, when less biomass is formed than during the active growth phase. Therefore, using pyrolysis oil or fractions thereof for fermentation processes might be possible when the growth phase and the production phase are separated and production takes place during stationary growth. Although this applies to the individual substances *A. oryzae* tolerates higher concentrations of pyrolysis oil during growth phase (2%) than during malic acid production phase (1%) during the observed cultivation time. One compound was identified to be a main reason for the low tolerance level of pyrolysis oil: 2-cyclopenten-1-one, which is present in a concentration of 0.308%. In growth tolerance tests with *A. oryzae*, the growth limit of this substance was observed at a concentration of 0.00625% corresponding to approx. 2% of the content in pyrolysis oil. This is consistent to the growth limit with pyrolysis oil (2%). In tolerance tests the growth and also fumaric acid production limit of *R. delemar* is even lower at 0.005%, which corresponds to approx. 1.6%. However, for malic acid production, the limit was at a concentration of 0.0125% corresponding to approx. 4% of the content in pyrolysis oil. Therefore, this substance alone is not responsible for the acid production limit of pyrolysis oil (1%). The fumaric acid production limit in presence of isoeugenol (0.005%) corresponds with to approx. 1% pyrolysis oil and for growth the limit is near 5%. Possible synergistic effects of the analyzed and also not analyzed substances could not be tested because of the large number of possible combinations and low availability of chemicals. Strong synergistic effects of furfural in combination with other aldehydes were described in former studies (Zaldivar *et al.* 1999). Moreover, chemical reactions between the components also be possible, which would lead to new unknown substances. However, even by testing single substances, the results give an idea of the complex nature of pyrolysis oil with many possible and different inhibition mechanisms of its compounds.

2.6 References Chapter 2

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3 Evaluation of the aqueous condensate from pyrolysis as carbon source for fungal fermentation

3.1 Introduction

In this chapter, the aqueous condensate of the bioliq® pyrolysis process at the KIT is evaluated as carbon source for fungal fermentation for organic acid production. A method to break down lignocellulosic materials into smaller components is the flash pyrolysis of dry biomass. This method is primarily used for generation of a flowable, energy-dense suspension for biofuel production. Thereby an organic (pyrolysis oil) and an aqueous condensate is formed containing monomeric sugars, small aromatic compounds and other low-molecular substances (Oasmaa and Czernik 1999). The organic condensate is already evaluated as possible C-source for fermentative processes with microorganisms (Dörsam *et al.* 2016). During the pyrolysis process, chopped biomass like wheat straw is heated up to 500 °C in the absence of oxygen (combustion air ratio $\lambda = 0$) which leads to pyrolysis vapors and fine coke. The coke is separated and the vapors are liquefied by a two-stage condensation leading to an organic condensate, pyrolysis oil, and an aqueous condensate. The pyrolysis coke and the pyrolysis condensates are mixed to a suspension ("Biosyncrude"). In the following gasification it can be quickly converted to synthesis gas (Dahmen *et al.* 2007). The aqueous condensate has not yet been studied as a substrate for filamentous fungi. It consists of a variety of non-aromatic components such as acids, alcohols, aldehydes and ketones (**Appendix 2**). Acetic acid is one of the main ingredients (4.5%). For evaluating the aqueous condensate as carbon source, shake flask cultivations with diluted and undiluted fractions, as well as several detoxifying pretreatments were done to reduce the amount of phenolic and solvent like compounds.

3.2 Material and Methods

3.2.1 Chemicals

All chemicals, were either purchased from Sigma-Aldrich (Munich, Germany) or Carl Roth (Karlsruhe, Germany).

3.2.2 Preparation of aqueous condensate

The aqueous condensate used in this study was prepared from wheat straw by fast pyrolysis in the bioliq plant at KIT (bioliq®). Small and dry biomass particles of a few mm size are rapidly heated up by a heat carrier (e.g. sand) in a pneumatically or mechanically fluidized bed at 500 ± 30 °C in the absence of oxygen. This process is described by Heinrich *et al.* (2016). In case of pyrolysis oils obtained from herbaceous biomass, higher water amounts are formed, which can cause phase separation of the pyrolysis oil. A phase rich in organic compounds and an aqueous phase consisting of up to 80 wt.% of water and water soluble organic compounds are formed. This aqueous condensate was used in this study.

The analyzed monomeric substances are compiled as determined by Thünen Institute Hamburg by GC-MS in the appendix section (**Appendix 2**).

3.2.3 Fungi and media

The fungal strain *A. oryzae* DSM 1863 was obtained from DSMZ strain collection (Deutsche Sammlung von Mikroorganismen und Zellkulturen GmbH, Braunschweig, Germany) and treated like described in Dörsam *et al*. (2016). *A. oryzae* was grown on minimal medium (MM) for *Aspergillus* spec. (Barratt *et al.* 1965): 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. The pH was set to 6.5 with NaOH. 10 g/L glucose, 2 mL of 1000× Hutner's Trace Elements, and 15 g/L agar were added afterwards. 1000× 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 $(NH_4)_6Mo_7O_{24} \cdot 4H_2O$, pH 6.5 (Barratt *et al.* 1965).

For conidia collection, *A. oryzae* was grown on high-salt minimal medium (Song *et al.* 2001) which additionally contains 22.37 g/L KCl. The conidia were harvested with 50% glycerol from plates that were incubated for 5 days at 30 °C and filtrated with Miracloth (Calbiochem). The conidia solution was diluted to a concentration of 1×10^7 (conidia)/mL and stored at -80 °C.

Malic acid production was accomplished in a two-step process with a pre-culture and a main-culture. The pre-culture medium consists of 40 g/L glucose monohydrate, 4 g/L $(NH_4)_2SO_4$,

0.75 g/L KH_2PO_4, 0.98 g/L $K_2HPO_4 \cdot 3H_2O$, 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 $FeSO_4 \cdot 7H_2O$. Main-culture medium contains different carbon sources as indicated in the results section. The control medium contains 4.5% Acetic acid, 1.2 g/L $(NH_4)_2SO_4$, 0.1 g/L KH_2PO_4, 0.17 g/L $K_2HPO_4 \cdot 3H_2O$, 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 $FeSO_4 \cdot 7H_2O$. To keep the pH-Level above 5.5 during fermentation, 90 g/L $CaCO_3$ powder was added to the main-culture medium. All media were sterilized by autoclaving.

3.2.4 Germination and growth tests

To prepare testing plates, different concentrations of aqueous condensate were added to the agar containing MM directly after autoclaving. Agar plates were inoculated onto the middle of the plate with $4x10^4$ conidia and incubated for 168 h at 30 C. To promote agar solidification after addition of aqueous condensate, the pH was set to 6 by titration with NaOH.

3.2.5 Organic acid production

100 mL of pre-culture medium in a 500 mL baffled Erlenmeyer shake flasks was inoculated with $2x10^7$ conidia. The flasks were incubated at 100 rpm and 30 °C for 24 hours in a rotary shaker. After incubation, pre-culture medium was removed by washing the fungal pellets twice and resuspending in 100 mL water. 100 mL of the main-culture was transferred to 500 mL Erlenmeyer shake flasks and 9 g/L $CaCO_3$ powder was added. The flasks were inoculated with 10% (*v*/*v*) of washed pre-culture and incubated at 120 rpm and 32 °C for 7 days. To remove the pre-culture medium, fungal pellets were washed twice and resuspended in 100 mL water. Each cultivation was done in triple approach.

3.2.6 Organic acid analytics

For malic and acetic acid quantification by HPLC, fermentation broth samples were pretreated and analyzed as described in Ochsenreither *et. al.* (2014) with minor modifications described in chapter 2. 1 mL of well-mixed sample was mixed with 1 mL of 3 M H_2SO_4 and 3 mL of distilled water and incubated at 80 °C for 20 min. 1 mL of the mixture was transferred to a 1.5 mL Eppendorf tube and centrifuged in a table top centrifuge for 5 minutes at 20,000×g. The supernatant was used for HPLC analysis, which was performed with a standard HPLC device (Agilent 1100 Series, Agilent, Germany) prepared with a 15 cm reversed phase column (Synergi™4 µm Fusion-RP 80 Å, LC Column 150×4.6 mm, Phenomenex, Aschaffenburg, Germany) at 30 °C. Mobile phase solution A was 100% methanol, and solution B was 20 mM KH_2PO_4, pH 2.5. The flow rate was 1 mL/min and a gradient was used for the separation of organic acids: 0–0.5 min 100% eluent B, 0.5–10-min increase of eluent A from 0 to 10%, 10–

12-min a further increase of eluent A from 10 to 70%, 12-14 min a decrease of eluent A from 70 back to 0%, and 14–18 min again 100% eluent B. As described in chapter 2, the increase of eluent A to 70% from 10-12 min was applied to elute and analyze the tested hydrophobic substances which were added to the medium. The injection volume was 10 μL and the detection was performed by a UV detector at 220 nm.

3.3 Results

3.3.1 Preliminary Experiments

In contrast to pyrolysis oil, analyzed in chapter 2, the aqueous condensate from the pyrolysis process contains mostly the water soluble components resulting from degradation of the three main compartments of lignocellulose, cellulose, hemicellulose and lignin. Most of the organic substances only occur in traces. The main components are acetic acid with 4.5%, hydroxyacetone with 3.5%, methanol with 1.7%, ethylene glycol with 0.5% and propionic acid with 0.4%. Acetic acid as the main constituent of pyrolysis oil and the aqueous condensate was identified as possible carbon source for fermentation with *Aspergillus oryzae*. An approach with 4.5% acetic acid acts as control experiment for cultivation. Except methanol, the other main components were tested for their toxicity to growth and malic acid production in chapter 2 and published in (Dörsam *et al.* 2016). The toxic concentration limits were above 1.3% for propionic acid (P), above 1.25% for ethylene glycol (EG) and 1.5% for hydroxyacetone (HA). To determine the effects of methanol in main-culture medium during malic acid production, several concentrations of methanol were added and the malic acid production over seven days was analyzed using glucose as carbon source. A selection of malic acid production curves are shown in **Figure 3-1**.

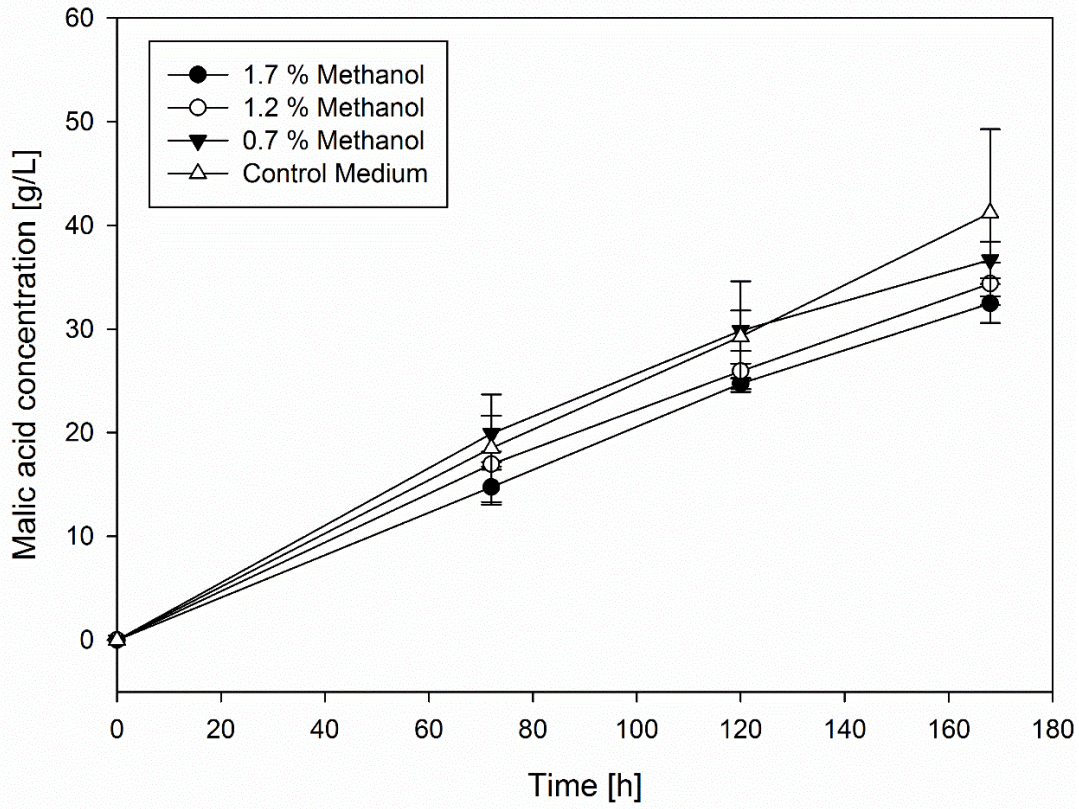

Figure 3-1: The effect of different methanol concentrations on malic acid production added to the main culture medium. As reference main culture medium without methanol was used. Shaking flasks were incubated at 32 °C for seven days.

It was observed that the methanol concentration in main culture medium up to the highest tested concentration of 1.7% has only minor effects on malic acid production. The final malic acid concentration ranged between 32.48 ± 1.89 g/L and 39.67 ± 2.31 g/L with a slight decrease with increasing methanol concentrations. The control approach resulted in a malic acid concentration of 41.21 ± 8.06 g/L.

With respect to this information, hydroxyacetone is the only main component with an inhibitory concentration below the concentration in the aqueous condensate. The next logical next step was to use mixtures of the main components as models of aqueous condensate to analyze possible synergetic effects. For this, 4 different models of aqueous condensates were designed. Model 1 contained all main components except hydroxyacetone (4.5% Ac, 0.4% P, 1.7% MeOH and 0.5 % EG), Model 2 contains all main components (4.5% Ac, 0.4% P, 1.7% MeOH and 0.5 % EG, 3.5% HA), model 3 contained all main components with only half the concentration of hydroxyacetone (4.5% Ac, 0.4% P, 1.7% MeOH and 0.5 % EG, 1.75% HA) and model 4 was a 1:1 dilution of all substances (2.25% Ac, 0.2% P, 0.85% MeOH and 0.25 % EG, 1.75% HA). Subsequently the medium salts were added and the model mixtures were used as fermentation medium. The production curves are shown in **Figure 3-2.**

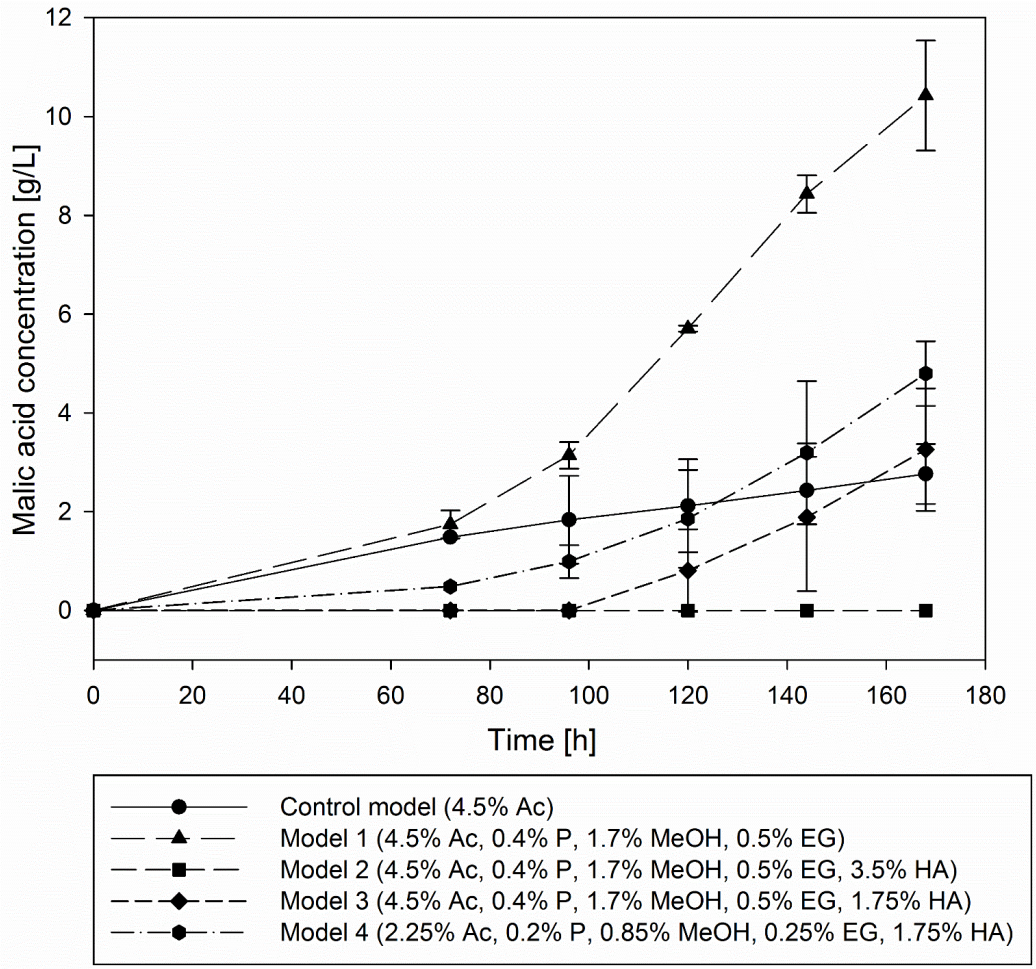

Figure 3-2: Malic acid concentration during cultivation of *A. oryzae* in 4 different mixture models of aqueous condensate of the pyrolysis process. Ac= Acetic acid; P=Propionic acid; MeOH= Methanol; EG= Ethylene glycol; HA= Hydroxyacetone

Except model 2, all tested model approaches resulted in a higher malic acid concentration after 168 hours of cultivation than the control approach. Cultivation in model 1 led to 10.42 ± 1.11g/L malic acid. Using Model 2 no product formation could be detected. Cultivation in model 3, 3.25 ± 1.24 g/g malic acid was produced and in model 4 a titer of 4.8 ± 0.66 g/L was observed. The control approach resulted in a malic acid concentration of 2.76 ± 0.61 g/L. In contrast to the control, malic acid concentration of the models showed an exponential/sigmoidal curve. With model 1, which is the model without hydroxyacetone, the product concentration is very similar to the control until 72 hours of cultivation. After this, the concentration increased fast to the maximum after 168 hours. Each of the models 2 and 3 contained half of the hydroxyacetone concentration and the product formation started later than in the control. At the end of cultivation, the concentration is similar to the control. The decrease of acetic acid during cultivation correlates to malic acid production. The final malic acid concentrations and yields are shown in **Table 3-1**

Table 3-1: Final malic acid concentration and yield by cultivating *A. oryzae* with different aqueous condensates model mixtures.

Aqueous condensate models	1	2	3	4	Control
Malic acid concentration (g/L)	10.42	0	3.25	4.8	2.76
$Y_{P/S}$(g/g)	0.4	-	0.37	0.28	0.12

The preliminary experiments showed the possible suitability of the aqueous condensate when diluted 1:1 as shown for model 4.

3.3.2 Evaluation of aqueous pyrolysis condensate as carbon source for fungal fermentation

Compared to model mixtures, the original aqueous condensate contains a lot more organic substances in traces. The pH level of untreated fraction is about 3. Because of this, several approaches were tested for fermentation. For cultivation, the fractions were diluted 1:1, 1:2, 1:3 and 1:4 and mixed with the media salts. With untreated fractions no malic acid production was observed. Same dilutions were additionally neutralized with NaOH. This neutralization resulted in formation of a thin organic phase which was discarded. The aqueous condensate was used for cultivation. This approach also did not result in any product formation.

To reduce possible oxidative stress, and to support the reaction of reactive substances in the fractions, two further pretreatments were tested. After neutralization, the fraction was heated to 80 °C for 20 minutes. This procedure led to the formation of an organic phase on top of the fraction, which was removed by decantation. Another method was the addition of 0.5 g/L yeast extract to reduce oxidative stress. Both solutions were diluted to 1:1, 1:2, 1:4 and used for cultivation, but did not result in malic acid production.

For further pretreatment, the fraction was treated like described in Lian *et al.* (2012). Three different approaches were analyzed:

(I) the aqueous condensates were neutralized by addition of NaOH to pH 6, subsequently filtered four times with a bottle-top filter and incubated for 90 minutes at 80°C. The resulted fraction was used for fermentation.

(II) Volatile compounds were evaporated at 80°C (90 rpm) in several steps. After 5 minutes of evaporation at atmospheric pressure, the pressure was reduced in 10 steps to 100 mbar following by 10 steps to 30 mbar and 2 minutes evaporation at this pressure. After bottle-top filtration (4x), the resulted fraction was used for fermentation.

(III) To reduce the amount of impurities and organic substances, the fractions were further mixed with 1 g activated carbon per 5 mL of fraction, incubated for 3 hours and subsequently filtrated. The resulted fraction was used for fermentation.

The acetic acid concentration in the first fraction was 45 g/L, in the second 70 g/L and in the third 135 g/L due to the evaporation and filtration steps. For cultivation the fractions were diluted to an acetic acid concentration in medium of 45 g/L, 40 g/L, 35 g/L, 30 g/L, 25 g/L 20 g/L and 15 g/L. 45 g/L corresponds to the acetic acid concentration in untreated aqueous condensate and 15 g/L is the determined minimal amount of acetic acid necessary for malic acid production. None of the tested approaches resulted in any product formation during fermentation.

Additionally to this, growth of *A. oryzae* on agar plates containing different amounts of aqueous condensates was tested. The plates were prepared like described in the material and method section with a content of 25%, 10% and 5% of aqueous condensate. No growth could be observed on all plates.

3.4 Discussion

3.4.1 Preliminary Experiments

The effects of methanol on malic acid production are very low. For *A. niger* and the citric acid production, the influence of methanol in concentrations between 1% to 4 % is reported even to support the acid production (Navaratnam *et al.* 1998; Maddox *et al.* 1986), but seems not to be transferable to *A. oryzae* which can be justified with the very different metabolic pathways of acid production. The tolerance of *Aspergilli* was investigated by Eloff *et al.* (2007), and found to be up to 40% methanol for *A. fumigatus*. As already shown in chapter 2, the negative influence of hydroxyacetone on malic acid production was confirmed in this experiments. The results indicated, that *A. oryzae* might be able to metabolize and convert one or more of the other main compounds beside acetic acid. Especially when comparing the control with model 1, malic acid concentration is more than three times higher. It is also possible that the other components are not convertible, but support malic acid production. In chapter 2 it was discovered that the presence of small amounts of phenolic substances slightly support the acid production. The membrane disordering properties of solvents like methanol is on the one hand the reason for the toxicity, but on the other hand it could be the reason for the increased malic acid production in some model mixtures, because of the better transport of products outside of the cell into the medium. The delayed production in the presence of hydroxyacetone, is possibly caused by evaporation of the substance over time or adaptation of the organism. With respect to the toxicity results from chapter 2 in this approach the concentration of hydroxyacetone is too high.

3.4.2 Evaluation of aqueous pyrolysis condensate as carbon source for fungal fermentation

Comparing the two fractions of pyrolysis, the organic (pyrolysis oil) and the aqueous condensates, some similarities and some differences can be observed. The main ingredients of both is acetic acid with 5% and 4.5 % followed by hydroxyacetone with 4.6% and 3.5%. The other main ingredients differ in their concentration: Propionic acid (1.3% pyrolysis oil; 0.4% aqueous condensate), ethylene glycol (1.3% pyrolysis oil, 0.4%) and methanol which is only present in the aqueous condensate with 1.7%. Highly hydrophobic compounds like isoeugenol are not present in the aqueous condensate. Furthermore 2-cyclopenten-1-one, which was discovered as toxic in chapter 2 is with 0.262% (0.308% in pyrolysis oil) still present in a concentration which is problematic. The toxic concentration to malic acid production with *A. oryzae* was 0.00625% which corresponds to about 2% aqueous condensate. Because of the needed high amount of

carbon source for the fermentation process, concentration in this range was not tested. The idea of the pretreatment of the aqueous condensate was to reduce the amount of solvents and organic substances as much as possible, so the most critical substances are the one with similar physicochemical properties like water. The protocol was adapted from the publication of (Lian *et al.* 2012) in which 6.9 g/L lipids were produced with *Cryptococcus curvatus* from detoxified aqueous condensate. In contrast to the aqueous condensate used in this study where wheat straw was used as feedstock, Lian *et al.* (2012) used pelletized wood from waste. The resulting fraction contained mostly: 5.7% acetic acid, 3.5% hydroxyacetone and 0.7% formic acid. About other substances only a value for lignin derived compounds (8.3%) is given, which makes the comparison between these two aqueous condensates difficult. The used organism *C. curvatus* was also tested in chapter 2 for its tolerance towards pyrolysis oil and did not grow even at a concentration of 0.5% oil content. By comparing these results, the pyrolysis products in this study seem to contain much more water soluble critical compounds which could not be removed by pretreatment and makes the aqueous condensate from wheat straw to a still interesting, but not applicable carbon source for fungi.

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4 Sequential mixed cultures: From Syngas to malic acid

This chapter is mostly based on the publication

Sequential mixed cultures: From syngas to malic acid

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Authors' contribution to this publication

Florian Oswald designed the experiments with *C. ljundahlii*. Performed together with Michaela Zwick the experiments with *C. ljundahlii* and the anaerobic part. Drafted as co-first author, the syngas part of the manuscript and parts of introduction and discussion. Designed **Figure 4-1**.

Stefan Dörsam design of the experiments with *A. oryzae*. Performed together with Nicolas Veith the experiments with *A. oryzae* and the aerobic part. Drafted as co-first author, the fungal part of the manuscript and parts of introduction and discussion.

Nicolas Veith performed together with Stefan Dörsam the experiments with *A. oryzae*.

Michaela Zwick performed together with Florian Oswald the experiments with *C. ljundahlii*.

Anke Neumann contributed to the conception of the syngas fermentation and critically revising of the final version to be published.

Katrin Ochsenreither contributed to the conception of the fungal fermentation and critically revising of the final version to be published.

Christoph Syldatk contributed to the idea of the coupling fermentation and critical revision of the work for important intellectual content

4.1 Introduction

This chapter describes the evaluation of the so called Syngas (synthesis gas) as a possible carbon source for fermentation with the filamentous fungus *A. orzae*. Because this fungus is not able to metabolize gases directly, the idea of a coupled process where one organism produces a product from gas which can also be the carbon source for the fungus was implemented. Synthesis gas fermentation using acetogenic bacteria is an approach for production of bulk chemicals like acetate, ethanol, butanol or 2,3-butandiol avoiding the "food or fuel" dilemma by using carbon monoxide, carbon dioxide and hydrogen from gasification of biomass or industrial waste gases. Suffering from energetic limitations, yields of C_4-molecules produced by syngas fermentation are quite low compared with fermentation using sugars as a substrate. On the other hand, fungal production of malic acid has high yields of product per gram metabolized substrate but is currently limited to sugar containing substrates. In this study, it was possible to show that *Aspergillus oryzae* is able to produce malic acid using acetate as sole carbon source which is a main product of acetogenic syngas fermentation. Bioreactor cultivations were conducted in 2.5 L stirred tank reactors. During the syngas fermentation part of the sequential mixed culture, *Clostridium ljungdahlii* was grown in modified Tanner medium and sparged with 20 mL/min of artificial syngas mimicking a composition of clean syngas from entrained bed gasification of straw (32.5 vol-% CO, 32.5 vol-% H_2, 16 vol-% CO_2 and 19 vol-% N_2) using a microsparger. Syngas consumption was monitored via automated gas chromatographic measurement of the off-gas. For the fungal fermentation part gas sparging was switched to 0.6 L/min of air and a standard sparger. Ammonia content of medium for syngas fermentation was reduced to 0.33 g/L NH_4Cl to meet the requirements for fungal production of dicarboxylic acids. Malic acid production performance of *A. oryzae* in organic acid production medium and syngas medium with acetate as sole carbon source was verified and gave $Y_{P/S}$ values of 0.28 g/g and 0.37 g/g respectively. Growth and acetate formation of *C. ljungdahlii* during syngas fermentation were not affected by the reduced ammonia content and 66% of the consumed syngas was converted to acetate. The overall conversion of CO and H_2 into malic acid was calculated to be 3.5 g malic acid per mol of consumed syngas or 0.22 g malic acid per gram of syngas.

4.2 Material and Methods

4.2.1 Microorganisms and medium

If not stated differently all chemicals were purchased from Carl-Roth (Germany) or Sigma-Aldrich (Germany).The organism used for the syngas fermentation part of the study was *C. ljungdahlii* DSM13528 which was kindly provided by the group of Peter Dürre, University

of Ulm. Medium used for cultivation of *C. ljungdahlii* for bioreactor cultivation was based on Tanner (2007). Medium for maintenance and pre-culture cultivation contained: 20 g/L 2-(*N*-morpholino)ethansulfonic acid (MES), 0.5 g/L yeast extract (BD, USA), 2 g/L NaCl, 2.5 g/L NH_4Cl, 0.25 g/L KCl, 0.25 g/L KH_2PO_4, 0.5 g/L $MgSO_4 \cdot 7\ H_2O$, 0.1 g/L $CaCl_2 \cdot 2\ H_2O$, 10 mL trace element solution (composition see below), 10 mL vitamin solution (composition see below) and 0.001 g/L resazurin and was prepared using strict anaerobic techniques. The pH was adjusted to 5.9 using KOH before bottling. Bottles were anaerobized using a gas mixture containing 20 vol-% carbon dioxide in nitrogen (Air Liquide, France). After autoclaving at 121 °C, 1 g Cysteine-HCl·H_2O and 10 g fructose per liter were added. Trace element solution contained: 2 g/L nitrilotriacetic acid, 1 g/L $MnSO_4 \cdot H_2O$, 0.567 g/L $FeSO_4 \cdot 7\ H_2O$, 0.2 g/L $CoCl_2 \cdot 6\ H_2O$ (Riedel-de Haën, Germany), 0.2 g/L $ZnSO_4 \cdot 7\ H_2O$, 0.02 g/L $CuCl_2 \cdot 2\ H_2O$, 0.02 g/L $NiCl_2 \cdot 6\ H_2O$, 0.02 g/L $Na_2MoO_4 \cdot 2\ H_2O$, 0.02 g/L $Na_2SeO_3 \cdot 5\ H_2O$ and 0.022 g/L $Na_2WO_4 \cdot 2\ H_2O$. Vitamin solution used for all anaerobic medium in this work contained: 0.002 g/L biotin, 0.002 g/L folic acid, 0.01 g/L pyridoxine (Alfa Aesar, Germany), 0.005 g/L thiamine-HCl, 0.005 g/L riboflavin, 0.005 g/L niacin, 0.005 g/L Ca-pantothenate, 0.005 g/L cobalamin, 0.005 g/L 4-aminobenzoic acid and 0.005 g/L liponic acid (Cayman Chemical, USA). Maintenance cultures were cultivated at 37 °C without shaking and inoculated every four days using the latest maintenance culture.

The *A. oryzae* DSM1863 strain was received from DSMZ strain collection (Deutsche Sammlung von Mikroorganismen und Zellkulturen, Braunschweig, Germany) and was grown on minimal medium (MM) for *Aspergillus* species (Barratt *et al*. 1965): 6 g/L $NaNO_3$, 0.52 g/L KCl, 0.52 g/L $MgSO_4 \cdot 7\ H_2O$, and1.52 g/L KH_2PO_4. The pH was set to 6.5 with 4M NaOH. 2 mL of 1000× Hutner's Trace Elements, 10 g/L glucose, and 15 g/L agar were added, and the medium was sterilized by autoclaving. 1000× Hutner's Trace Element solution contained 5 g/L $FeSO_4 \cdot 7\ H_2O$, 50 g/L EDTA-Na_2, 22 g/L $ZnSO_4 \cdot 7\ H_2O$, 11 g/L H_3BO_3, 5 g/L $MnCl_2 \cdot 4\ H_2O$, 1.6 g/L $CoCl_2 \cdot 6\ H_2O$, 1.6 g/L $CuSO_4 \cdot 5\ H_2O$, and 1.1 g/L $(NH_4)_6Mo_7O_{24} \cdot 4\ H_2O$, pH 6.5 (Barratt *et al.* 1965). For conidia collection, the fungus was grown on high-salt minimal medium (Song *et al.* 2001) which additionally contained 22.37 g/L KCl. Conidia were harvested with 50% glycerol solution from plates that were incubated for 5 days at 30 °C and filtered through Miracloth (Calbiochem). The conidia solution was diluted to a concentration of 10^7 conidia/mL and stored at -80 °C. Sequential malic acid production was accomplished in a two-step process with a pre-culture and a main culture. The main culture was either the fermentation broth from *C. ljungdahlii* syngas-fermentation (see above) or main culture medium for fungal malic acid

production (Ochsenreither *et al.* 2014). The pre-culture medium contained 40 g/L glucose monohydrate, 4 g/L $(NH_4)_2SO_4$, 0.75 g/L KH_2PO_4, 0.98 g/L $K_2HPO_4 \cdot 3H_2O$, 0.1 g/L $MgSO_4 \cdot 7 H_2O$, 0.1 g/L $CaCl_2 \cdot 2 H_2O$, 5 mg/L NaCl, and 5 mg/L $FeSO_4 \cdot 7 H_2O$ and was sterilized by autoclaving for 20 min at 121 °C. Main culture medium contained 120 g/L glucose monohydrate, 1.2 g/L $(NH_4)_2SO_4$, 0.1 g/L KH_2PO_4, 0.17 g/L $K_2HPO_4 \cdot 3H_2O$, 0.1 g/L $MgSO_4 \cdot 7 H_2O$, 0.1 g/L $CaCl_2 \cdot 2 H_2O$, 5 mg/L NaCl, and 60 mg/L $FeSO_4 \cdot 7 H_2O$. To keep the pH above 5.5 during acid production 90 g per liter $CaCO_3$ were added.

4.2.2 Fermentation setup and operation

Fermentations were carried out in Minifors bench-top stirred tank reactors from Infors-HT (Switzerland) with a total volume of 2.5 L and a liquid volume of 1.5 L leaving a 1 L headspace. **Figure 4-1** shows a basic scheme of the process setup for both anaerobic syngas fermentation and aerobic fungal fermentation.

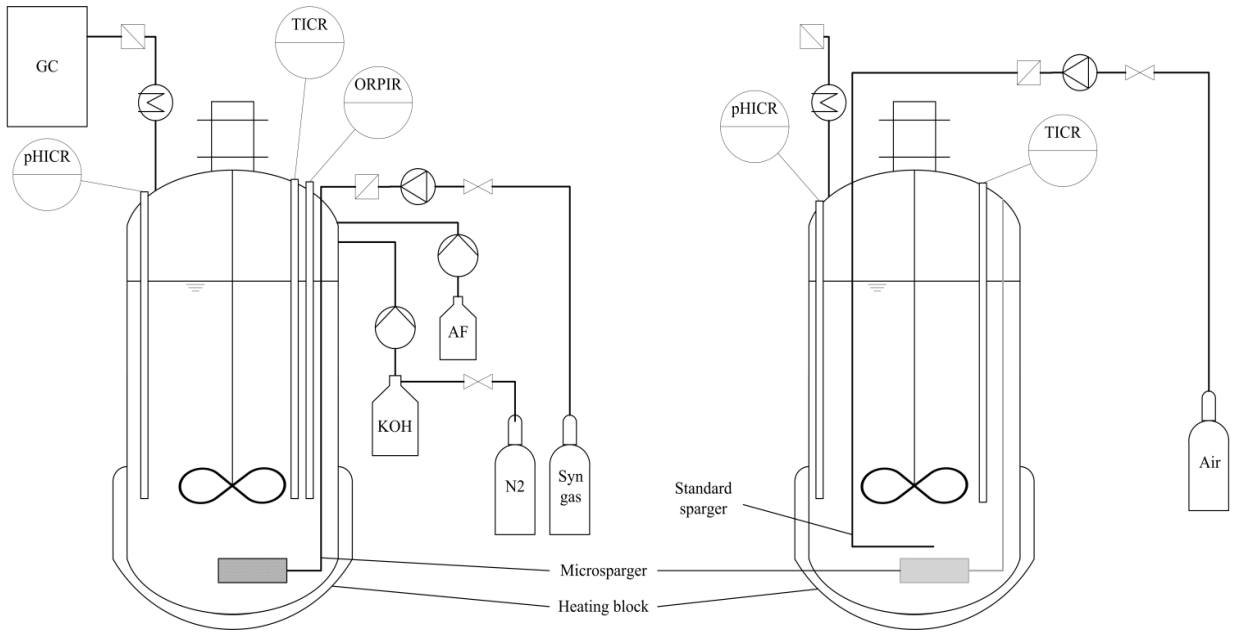

Figure 4-1: Basic scheme of process setup for anaerobic syngas fermentation (left) and aerobic fungal fermentation (right). pHICR, pH indicate, control and record; TICR, temperature indicate, control and record; ORPICR, ORP indicate, control and record; AF, anti-foam; GC, gas chromatograph.

For anaerobic syngas fermentation with *C. ljungdahlii*, each bioreactor was equipped with a Pt-100 temperature probe (Infors-HT, Switzerland), pH-probe (Mettler-Toledo, U. S. A.) and an EasyFerm Plus ORP-probe (Hamilton, Switzerland) for measurement and recording of temperature, pH and redox potential during fermentation. The pH was regulated to 5.9 by addition of 4 M KOH solution which was kept under a nitrogen atmosphere to ensure anaerobic conditions. Temperature of the broth was maintained at 37 °C using the heating block of the bioreactor

housing. To prevent foaming, bioreactors were fitted with a foam probe (Infors-HT, Switzerland) using Contraspum A 4050 HAC (Zschimmer und Schwarz, Germany*)* as an anti-foaming agent. Each bioreactor was equipped with a microsparger for creation of microbubbles to enhance mass transfer between gaseous and aqueous phase (Bredwell 1998). The gas flow rate of 20 mL/min into the bioreactors was controlled using red-y smart series mass-flow-controller (MFC) from Vögtlin Instruments (Switzerland). Composition of the syngas used in this work was 32.5 vol-% H_2, 32.5 vol-% CO, 16 vol-% CO_2 and 19 vol-% N_2 (Air Liquide, France), mimicking a composition of purified syngas from entrained bed gasification of straw. The headspace of the bioreactor was at atmospheric pressure. Gas-liquid mixing was achieved at 800 rpm using a stirrer setup for vortex formation (**Figure 4-1**). No baffles were used.

Medium for bioreactor cultivations was prepared under aerobic conditions with the same composition as the maintenance- and pre-culture medium except for fructose which was omitted and cysteine-HCl which was reduced to 0.53 g/L. After autoclaving at 121 °C for 20 min the redox potential of the medium was lowered to about -200 mV by sparging with syngas and addition of the above stated amount of cysteine-HCl. Pre-cultures of *C. ljungdahlii* for reactor experiments were grown for 48 h with fructose as carbon and energy source. Bioreactors were inoculated with 10% of their final volume using sterile silicon tubing with cannulas at both ends. After 96 h of growth on syngas the broth was either harvested for preliminary tests with *A. oryzae* or switched to aerobic conditions for the main coupling experiment.

For *A. oryzae* fermentation, 100 mL pre-culture medium was filled in 500 mL baffled shake flasks and inoculated with 2×10^7 conidia. The culture was incubated for ca. 24 h at 30 °C and 100 rpm in a rotary shaker. Fungal pellets were washed twice with distilled water to remove pre-culture medium components before inoculation of the main culture. For shake flask cultivation, 100 mL of main-culture was transferred to 500 mL baffled shake flasks and mixed with 9 g sterile $CaCO_3$. The flasks were inoculated with 10 vol-% of washed pre-culture and incubated at 120 rpm and 32 °C for 7 days. Fermentation in bioreactor was done in small scale bioreactor Minifors (vessel volume 2.5 L) (Infors, Switzerland). To generate the conditions for malic acid production, some modifications of the reactor were necessary. The antifoam probe was replaced by a standard sparger to avoid clogging of the microsparger by the fungus and the redox potential probe was removed to enable inoculation with fungal preculture (fungal biomass of two preculture flasks) and $CaCO_3$ was added (90 g per bioreactor). The microsparger was twisted sideways. Before inoculation, aeration was changed from syngas to air for approx. 30 minutes to remove all CO in solution and 200 µL of antifoam reagent (Contraspum A 4050

HAC, Zschimmer und Schwarz) was added. After inoculation, the fermentation took place at 35 °C with an aeration rate of 0.6 L/min and a stirrer speed of 300 rpm. Approximately every 24 h 5 mL samples were taken.

4.2.3 Analytical Methods

The concentrations of malic and acetic acid during cultivation with *A. oryzae* were quantified by HPLC. Fermentation broth samples were pretreated and analyzed as described in Ochsenreither *et al.* (2014). To resolve as calcium salt precipitated organic acids, 1 mL of well-mixed sample was mixed with 1 mL of 3 M H_2SO_4 and 3 mL of distilled water and incubated at 80 °C for 20 min. 1 mL of the mixture was transferred to a 1.5 mL Eppendorf tube and centrifuged in a bench top centrifuge for 5 minutes at 20,000×g. The supernatant was used for HPLC analysis. The analysis was performed at 30 °C with a standard HPLC device (Agilent 1100 Series, Agilent, USA) prepared with a 15 cm reversed phase column (Synergi™4 µm Fusion-RP 80 Å, LC Column 150×4.6 mm, Phenomenex, Germany). Mobile phase solution A was 100% methanol, and solution B was 20 mM KH_2PO_4, pH 2.5. The flow rate was 1 mL/min and a gradient was used for the separation of organic acids: 0–0.5 min 100% eluent B, 0.5-10 min increase of eluent A from 0 to 10%,10-12 min a decrease of eluent A from 10 back to 0%, and 12–14 min again 100% eluent B. The injection volume was 10 µL and the detection was performed by a UV detector at 220 nm. Malic acid standard was purchased from Sigma-Aldrich (Germany), acetic acid standard from Carl-Roth (Germany). Both were used for peak identification and calibration. The linear detection range went from 0.1 to 5 g/L malic acid and acetic acid.

4.3 Results

4.3.1 Preliminary Experiments

Optimization of bioreactor medium for sequential production of malic acid from acetic acid

Since malic acid is produced by *A. oryzae* only under nitrogen limited conditions (Knuf *et al.* 2013) it was necessary to reduce the ammonia content of the medium to ensure nitrogen limitation after syngas fermentation. Initial cultivations, using the above mentioned medium for syngas fermentation, were conducted to determine the amount of ammonia consumed by *C. ljungdahlii* during 96 h of growth on synthesis gas. Based on the consumed amount of ammonia, the NH_4Cl content of the medium for all following pre-cultures and bioreactor experiments was reduced to 0.33 g per liter. Ion chromatography for NH_4^+-detection was kindly conducted by the section for Bioprocess Engineering of the Institute of Process Engineering in Life Sciences, KIT. Results of medium optimization and preliminary cultivations with NH_4-reduced medium are shown in. Measurement of ammonia concentration after 96 h of syngas fermentation using NH_4-reduced medium was not possible due to high amounts of potassium ions in the broth.

After 96 h of fermentation on syngas using syngas fermentation medium (see above) with 2.5 g/L ammonia chloride, *C. ljungdahlii* consumed 386.7 mg/L ammonia. Nevertheless fermentation under nitrogen reduced conditions yielded an average of 17.08 g/L acetic acid and 1.14 g/L ethanol compared to 15.27 g/L acetic acid and 0.57 g/L ethanol when *C. ljungdahlii* was cultivated with an excess of ammonia. In ammonia rich medium *C. ljungdahlii* consumed 0.75 mol/L of hydrogen and 0.77 mol/L of carbon monoxide and in NH_4-reduced medium they consumed 0.64 mol/L of hydrogen and 0.74 mol/L of carbon monoxide. The values for consumed carbon dioxide were around zero for all fermentations in syngas fermentation medium with an average of 0.01 mol/L whereas in NH_4-reduced medium in some cultivations the bacteria released CO_2 to the off-gas, thus the negative mean value of -0.09 mol/L. For both medium types the ratio of products (acetic acid and ethanol) to consumed substrates ($Y_{P/S}$) is roughly the same, with averages of 0.68 g/g for syngas medium and 0.67 g/g for NH_4-reduced medium.

Preliminary experiment for fungal fermentation

Large quantities of organic acids are produced by certain fungi generally under nitrogen limiting conditions and a simultaneous excess of carbon source. For the production of malic acid with *Aspergillus oryzae* these requirements are met in a special production medium as published by (Battat *et al.* 1991). However, this production medium is considerably different from the

syngas fermentation medium, a general microbial cultivation medium. Therefore, preliminary experiments were conducted to determine the suitability of acetic acid and ethanol as carbon source, which was never shown before, and the influence of other medium ingredients on malic acid production. These experiments are summarized in **Table 4-1**.

Table 4-1: Results of preliminary experiments with *A. oryzae* regarding the influence of different medium components and carbon sources on malic acid production.

Medium	Carbon source (g/L)	Modification/ Pretreatment	Malate (g/L)	$Y_{P/S}$[b] (g/g)
Organic acid production medium[a]	Glucose 109	-	47.84 ± 3.49	0.8
Organic acid production medium	Glucose 109	0.533 g/L Cysteine	44.2 ± 5.85	0.64
Organic acid production medium	Glucose 109	0.533 g/L Sodium sulfide	54.04 ± 14.16	0.65
Organic acid production medium	Acetic acid 50	Exchange of carbon source	8.62 ± 1.15	0.28
Organic acid production medium	Acetic acid, ethanol 33.33, 16.66	Exchange of carbon source	11.68 ± 1.27	0.55
Organic acid production medium	Ethanol 50	Exchange of carbon source	0	0
Syngas fermentation medium[a]	Acetic acid 50	Exchange of carbon source	2.69 ± 0.81	0.09
Syngas fermentation medium	Acetic acid 50	Exchange of carbon source, without ammonium	4.11±0.50	0.37
Syngas fermentation medium: fermented	Acetic acid 9.80 ± 0.21	Removal of *C. ljungdahlii* biomass	0	0
Syngas fermentation medium: fermented	Acetic acid 15.84 ± 1.55	Reduced ammonium	4.34±0.10	0.27

Syngas fermentation medium: fermented	Acetic acid 8.88 ± 3.42	Reduced ammonium Removal of *C. ljungdahlii* biomass	0	0

[a]control approach, [b]yield is given after 168 h cultivation ($Y_{P/S}$)

All concentrations are given as average of three independent experiments ± standard deviation

Influence of major medium components on malic acid production was evaluated in shake flask experiments and fermentation experiments in bioreactors as indicated. The biggest differences of the established process were the carbon source and the nitrogen concentration. It could be shown that acetic acid is an appropriate carbon source for malic acid production in established malic acid production medium with an $Y_{P/S}$ of 0.28 g/g and a final product concentration of 8.62 ± 1.15 g/L (**Figure 4-2)**

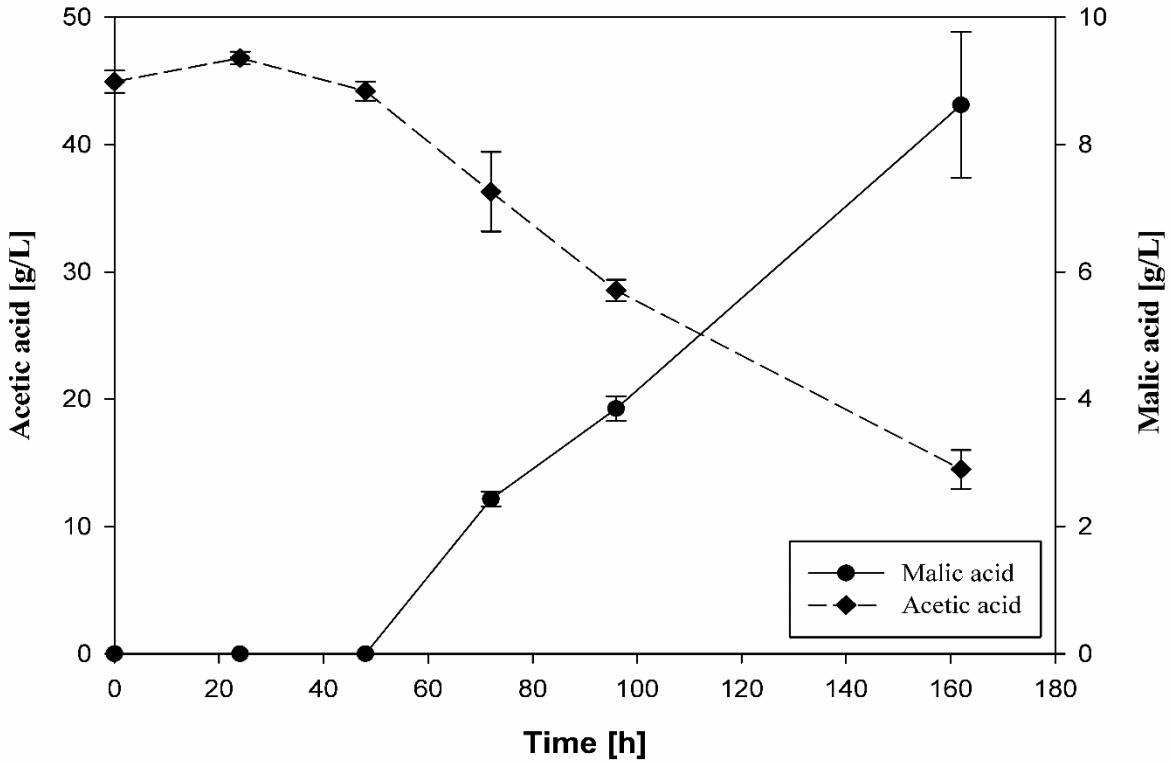

Figure 4-2: Malic acid production using acetic acid as carbon source in fungal organic acid production medium. All concentrations are given as average of three independent experiments ± standard deviation.

The most important byproduct of syngas fermentation is ethanol. Various concentrations of both, acetate and ethanol, could be achieved in different fermentations. To analyze the influence on or the suitability of these molecules as substrates for malic acid production, ethanol was added to production medium. With a yield of 0.55 g/g the acetic acid/ethanol-approach reached a final product concentration of 11.68 ± 1.27 g/L (**Table 4-1**). When ethanol was used as sole carbon source, acid production was not detected. In the second round of experiments the reduction agent cysteine and sodium sulfide were tested. These agents are used during syngas fermentation to reduce contaminating oxygen. Because cysteine is also a potential nitrogen source for *A. orzyae*, tests of the effects on malic acid production are necessary. The results showed a minor influence of the preferred reduction agent cysteine with a final malic acid concentration of 44.2 ± 5.85 g/L ($Y_{P/S}$ of 0.64 g/g), as well as the alternative reduction agent sodium sulfide with a $Y_{P/S}$ of 0.65 g/g (54.04 ± 14.16 g/L) (**Figure 4-3**) compared to the control approach, where no modifications were done, with 47.84 ± 3.49 g/L where a higher $Y_{P/S}$ of 0.8 g/g could be achieved (**Table 4-1**).

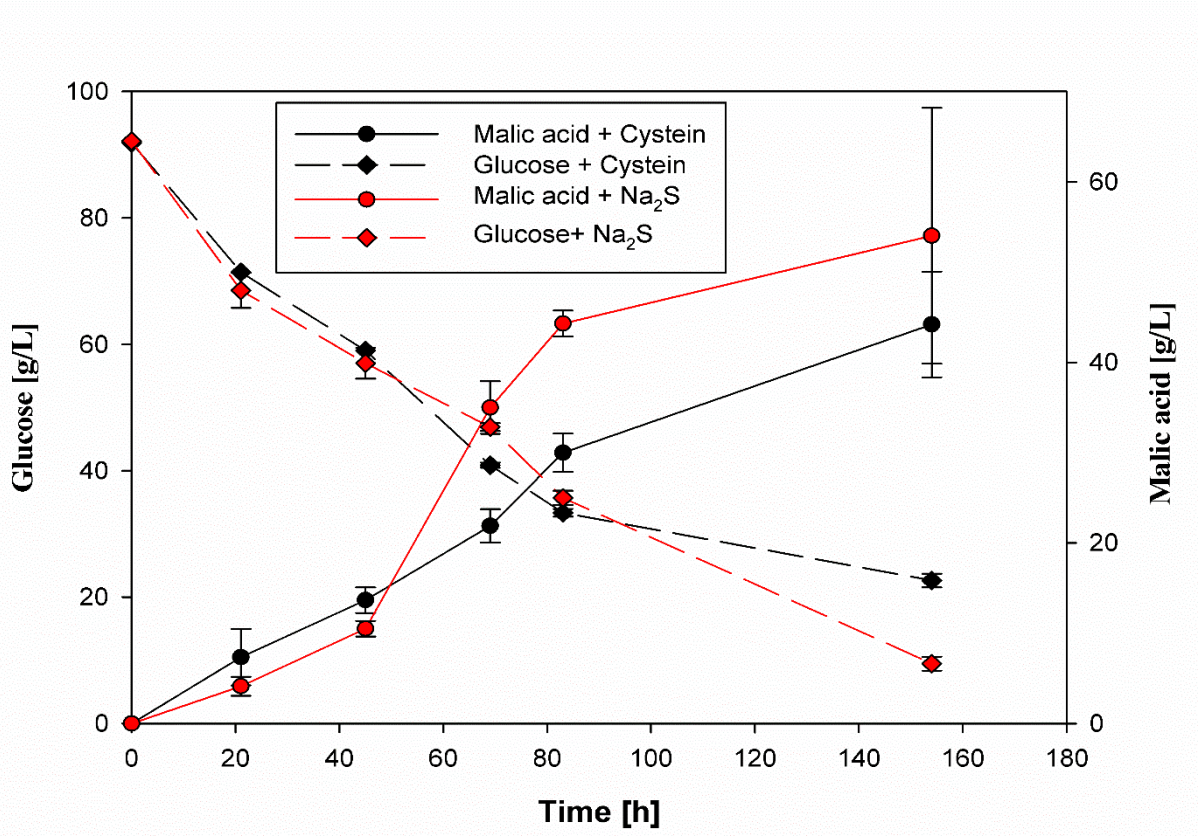

Figure 4-3: Malic acid production and glucose consumption in main-culture medium by addition of 0.533 g/L the reduction agent cysteine or sodium sulfide. All concentrations are given as average of three independent experiments ± standard deviation.

It could be shown that the reduction agents are not problematic and acetic acid is an appropriate carbon source for malic acid production. The syngas fermentation medium, which has a significant different composition compared to the malic acid production medium, was a further challenge. Especially the initial ammonium concentration proved to be problematic as ammonium was not completely consumed during syngas fermentation, so that considerable amounts of ammonium remained for the subsequent fungal fermentation medium. Therefore, to prove if syngas fermentation medium is in general suitable for malic acid production, fungal cultivations in shake flasks with normal concentration and without nitrogen were conducted. Syngas fermentation medium was mixed, autoclaved and enriched with acetic acid as carbon source. Normal ammonium concentration leads to a final malic acid concentration of 2.69±0.81 g/L with a yield of 0.09 g/g. If no nitrogen source was added 4.11±0.50 g/L and a yield of 0.37 g/g could be achieved (**Table 4-1**). Therefore, the syngas fermentation medium is a suitable medium for malic acid production, but nitrogen concentration must be limited to a minimum level.

However, in the sequential mixed culture fermentation, the syngas fermentation medium might possibly undergo unknown modifications as a result of *Clostridia* cultivation. To analyze the effects to the *A. oryzae* fermentation, an authentic already fermented medium was used including *Clostridia*-produced acetic acid. On the one hand, the medium containing biomass is a possible nitrogen source; on the other hand biomass could contain some important medium ingredients. To test the influence of biomass on malic acid production, bacterial biomass was either removed by centrifugation or left inside in nitrogen rich or reduced medium. For the first experiments in the bioreactor, a nitrogen rich medium was used. To reduce the nitrogen concentration, biomass was removed by centrifugation. In this approach, no malic acid production could be measured. Further tests in shake flasks were done with ammonium reduced medium, with either removed or not removed biomass. With microbial biomass a malic acid concentration of 4.34 ± 0.10 g/L was produced from 15.84 ± 1.55 g/L acetic acid corresponding to a yield of 0.27 g/g (**Figure 4-4**). The removal of microbial biomass prevented product formation.

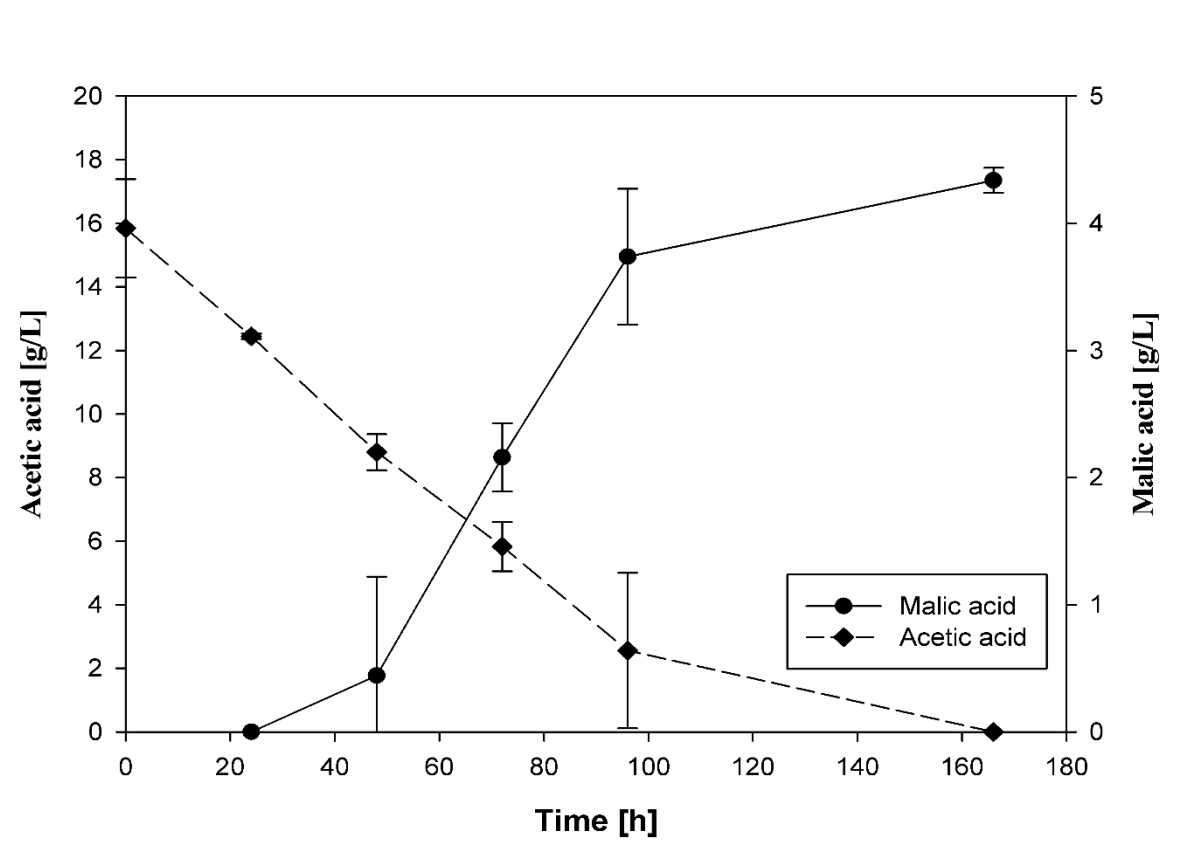

Figure 4-4: Malic acid production using acetic acid as carbon source in fermented syngas fermentation medium. All concentrations are given as average of three independent experiments ± standard deviation.

Because initial shake flask experiments were promising, the sequential mixed culture approach was tested under realistic conditions, i. e. syngas fermentation followed by fungal fermentation without medium removal and/or delay in between.

4.3.2 Coupling experiment for sequential production of malic acid from acetic acid.

Syngas fermentation

For the main experiment, NH_4-reduced medium was used for syngas fermentation to ensure nitrogen limited conditions after 96 h. Syngas was delivered into the broth with a starting rate of 20 mL/min and was increased after 42 h to 25 mL/min. Directly following the syngas fermentation, the reactor was changed to fungal fermentation as stated above. Microbial biomass was not removed.

Sequential mixed culture

The sequential mixed culture was accomplished in three replicates in the described fermentation setup in a bioreactor. Results for malic acid fermentation are shown in **Figure 4-5.**

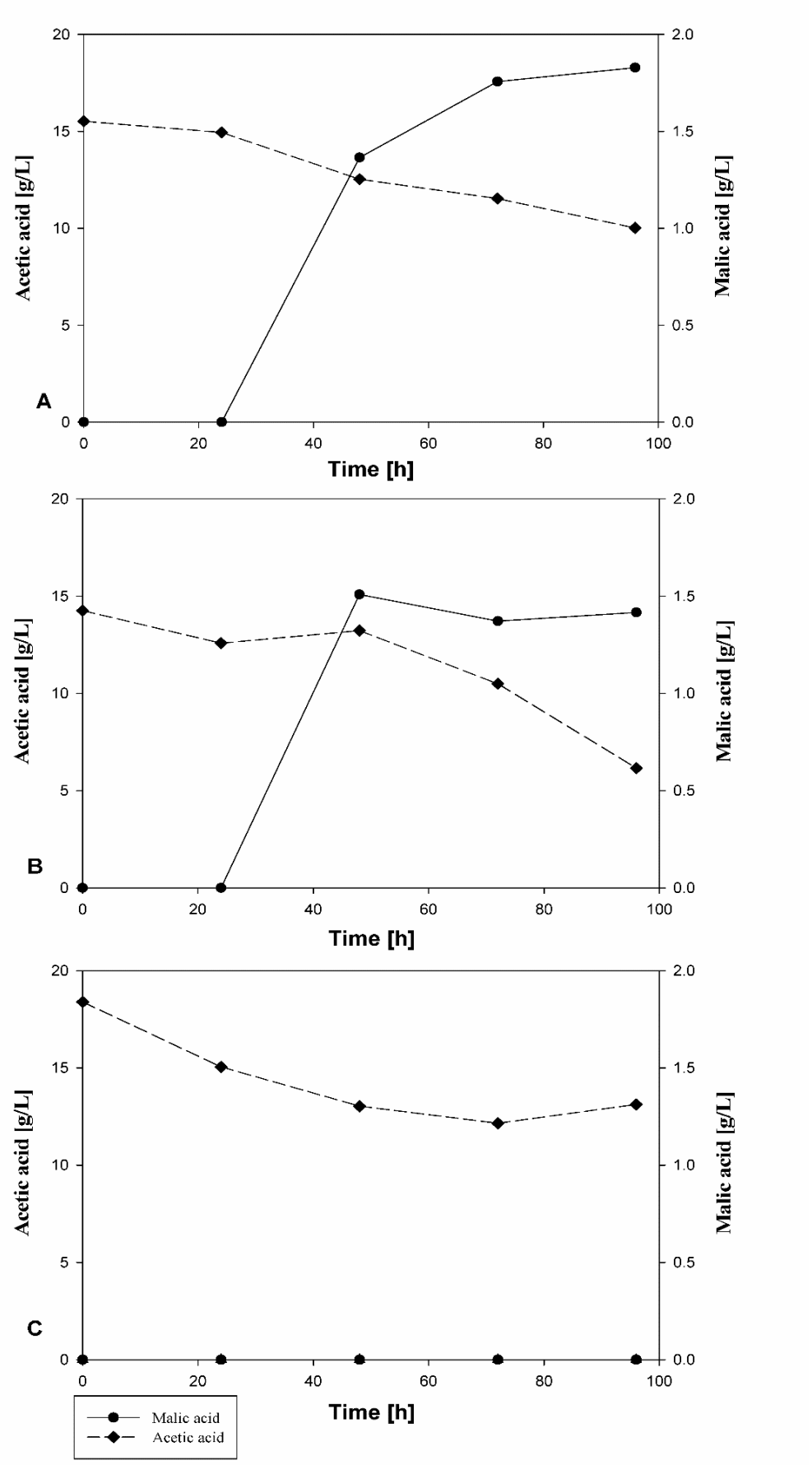

Figure 4-5: Malic acid production, and acetic acid consumption in the three bioreactors A, B and C from triple approach for syngas fermentation after 96 h of fermentation.

In two of the three bioreactor runs, malic acid production was detected. In one bioreactor, acetic acid was partly metabolized, but no product was formed. In reactor **A** acetic acid decreased from 15.53 g/L to 10.02 g/L during malic acid production of 1.83 g/L corresponding to a yield of 0.33 g/g. In reactor **B**, the initial acetic acid concentration of 14.26 g/L was reduced during fermentation process to 6.15 g/L by simultaneous production of 1.42 g/L malic acid resulting in a yield of 0.18 g/g. During cultivation in reactor **C**, 5.39 g/L acetic acid was metabolized in total, from initially 18.39 g/L to 13.12 g/L, but without product formation. After 96 hours of fermentation, the three reactors differed greatly. In reactor **A** the malic acid concentration decreased to 0**.** In reactor **B** malic acid concentration increased to 2.02 g/L and in reactor **C** further no malic acid production could be observed (data not shown).

4.4 Discussion

For the sequential mixed culture fermentation from syngas to malic acid, the main challenges were the requirements of the involved microorganisms in terms of reactor set-up, medium composition and product synthesis. Optimizing product yield and productivity for a certain process usually addresses the needs of the organism involved. Since sequential mixed culture fermentation uses at least two different organisms the key aspect for sequential mixed culture fermentation is either a medium compromise for both organisms or the compatibility of the first (optimized) medium for the second organism in terms of product synthesis. Furthermore, the second organism has to be able to use the product of the first process as a carbon source. The combination of both aspects must be fulfilled to achieve an optimal value added chain from syngas to malic acid.

For the first time, this study shows that *A. oryzae* is able to use acetic acid, the main product of syngas fermentation, as a proper carbon source for malic acid production. The metabolic pathways and mechanisms are largely understood, when using carbohydrates as carbon source. A partly reductive TCA cycle following on glycolysis and malic acid is synthesized from pyruvic acid and oxaloacetic acid to malic acid (Osmani *et al.* 1983; Peleg *et al.* 1988; Peleg *et al.* 1989; Bercovitz *et al.* 1990). Fermentative mechanisms for malic acid production from other carbon sources are still not fully known and therefore speculative. For growing on acetic acid, acetyl-CoA synthetase (ACS) was described as a key enzyme for the metabolism of ethanol and acetic acid, which converts acetic acid to acetyl-CoA in *C. albicans* (Carman *et al.* 2008). Acetyl-CoA may then enter the glyoxylate cycle which is partly located in the peroxisome and is done for gluconeogenesis. Malic acid occurs in this pathway as an intermediate. This process may be

the pathway for malic acid production from acetic acid. This metabolic flux is summarized by Strijbis and Distel (2010).

4.4.1 Preliminary experiments

Cultivation of *C. ljungdahlii* in medium containing 2.5 g/L ammonia chloride for 96 h resulted in consumption of 0.131 g/L ammonia. This equals to a concentration of 0.39 g/L ammonia chloride. To ensure that after 96 h of syngas fermentation no ammonia is left, a total of 0.5 g ammonia chloride was used for 1.5 L of medium. The results of preliminary experiments indicate that reducing the ammonia concentration in the medium does not negatively affect product formation, substrate consumption and overall product yield, although biomass concentration is slightly lower in NH_4-reduced medium. This is consistent with experimental data from Xu *et al.* (2011) reporting slight differences in biomass concentration in this range of ammonia concentrations.

Comparing the fungal fermentation on acetic acid in the two different media, it could be seen that a reduction of the nitrogen concentration is mandatory. If ammonium is omitted in syngas fermentation medium a similar yield was achieved for malic acid production medium ($Y_{P/S}$ of 0.28 g/g) and syngas fermentation medium ($Y_{P/S}$ of 0.37 g/g) using acetic acid despite the nitrogen present in malic acid production medium. Although observed yields were similar, malic acid concentration in syngas fermentation medium is approximately half as high as in optimized organic acid production medium (8.62 ± 1.15 g/L against 4.11 ± 0.50 g/L) after 168 hours. There is also a lack in malic acid production depending on presence of microbial biomass. In medium, without microbial biomass and with reduced ammonium, malic acid production was firstly detected after 72 hours of cultivation and decreased to 0 afterwards. If microbial biomass was not removed malic acid concentration reached the detection limit already after 48 hours. Because biomass itself could be used as source of minerals, nutrients and vitamins, it might be helpful for the adaption of the fungus to acetic acid as carbon source. Nevertheless, for this sequential mixed culture approach it is a good result that biomass has a positive effect on malic acid production and does not need to be removed. A further challenge was the side product of syngas fermentation, ethanol. As reported in several studies, various concentrations of this alcohol could be produced during cultivation of acetogenic bacteria. With *C. ljungdahlii* ethanol concentrations of 48 g/L could be achieved using syngas from coal as energy and carbon source (Klasson *et al.* 1993). The experiments with ethanol as sole carbon source for fungal fermentation showed no malic acid production, so that it can be assumed that ethanol alone in those high concentrations is not a suitable carbon source for *A. oryzae*. However, the acetic acid/ethanol

mixed approach showed the highest yield for malic acid compared to the other acetic acid fermentations ($Y_{P/S}$ of 0.55 g/g). It was shown that stress conditions for the fungus are beneficial for malic acid production, due to an up-regulation and overexpression of the genes involved in the malic acid production pathway (Knuf *et al.* 2013). Ethanol as a solvent may lead to stress for *A. oryzae* during cultivation which enhances the product synthesis. It is also possible that ethanol in low concentrations could be metabolized and serves as possible carbon source for malic acid production on the described pathway. In this case the ethanol amounts, produced in this process (0.75 g/L to 1.14 g/L) would not be problematic. All in all the results of preliminary experiment led to the assumption that a sequential mixed culture from syngas to malic acid is a promising approach.

4.4.2 Sequential mixed culture from syngas to malic acid

For the main experiment, average acetate concentration after 96 h was about one gram per liter lower and standard deviation was 0.5 g per liter higher compared to preliminary bioreactor experiments. The differences in acetate concentration between the three bioreactors might be due to different rates of decreasing hydrogen consumption.

The malic acid production in the three bioreactors with *A. oryzae* varied widely. Bioreactor **A** and **B** showed both malic acid production after 48 hours as expected from shake flask experiments. The curve of organic acid production is similar, but reactor **B** had already reached a plateau after 48 hours, whereas reactor **A** reached the plateau after 96 hours. There is also a spread of 0.15g/L of the yield between bioreactor **A** and **B**. The reason for the lack of malic acid production in reactor **C** and the different yields in both other reactors is very difficult to discuss due to the high complexity of the medium composition after syngas fermentation. It seems that small differences in syngas fermentation may have large effects on the following fungal fermentation. Because of that fact, this should not be seen as a triplicate but as three different batches from syngas fermentations. Despite the fact that interpretation of the results is difficult and the failure in one bioreactor it was clearly shown that malic acid production from syngas by sequential mixed culture fermentation is possible. The overall conversion efficiency of syngas into acetate and ethanol for the syngas fermentation part can be expressed as $Y_{P/S}$ of 0.66 g acetic acid and ethanol per gram of consumed syngas. Combined with the $Y_{P/S}$ for the conversion of acetic acid into malic acid an overall $Y_{P/S}$ for the conversion of CO and H_2 into malic acid of 0.22 g/g (3.5 g malic acid per mol syngas consumed) for reactor **A** and 0.12 g/g (1.9 g malic acid per mol syngas consumed) for reactor **B** was achieved. This was achieved with complete conversion of CO and H_2 into products. Since there are no reported processes

for production of malic acid from syngas the yields were compared with anaerobic production of other C_4 molecules. Anaerobic processes for production of butanol from sugars described in literature gave $Y_{P/S}$ values between 0.1 g/g and 0.3 g/g when using *C. beijerinckii* or *C. acetobutylicum* and sugar from lignocellulosic substrates as a carbon source (Schiel-Bengelsdorf *et al.* 2013). Using syngas for production of butanol as did Lewis *et al.* (2007) yielded 0.08 g butanol per gram of consumed carbon monoxide. Other processes for production of C_4 molecules using anaerobic organisms and syngas as a substrate described in literature do not state $Y_{P/S}$ values which prevents proper comparison.

Although sequential mixed cultures have been used for centuries in food industry, e.g. sake production, applications for production of value added chemicals is rare. It could be shown, that this kind of biotechnological process is suitable for the production of low price chemicals like single cell oils for biofuel production (Hu *et al.* 2016). There are also some approaches for interlinking cultivations, like co-cultivating a homoacetogen (e. g. *C. ljungdahlii*) and an anaerobic organism that is able to grow on syngas, ethanol or acetate and produces butyrate or butanol as described by Datta *et al.* (2014), a combination of algae and yeast fermentation (Dillschneider *et al.* 2014), dextran fermentation (Kim and Day 1994) and biogas production, but sequential fermentations are rare.

4.5 Conclusion

The possibility of the production of high-value L-malate from syngas was successfully shown. Further increase of yield is feasible as the process medium was neither optimized for acetic acid production nor for malic acid production and only wild type strains of *C. ljungdahlii* and *A. oryzae* were used. Both strains are available at the DSMZ. The advantage of this kind of biotechnological process is the extension of the product portfolio of anaerobic syngas fermentation. Because of the toxicity of oxygen to *Clostridia*, there is no further step necessary than changing the sparging from syngas to air, to prepare the medium for fungal fermentation. The work at hand demonstrates that *A. oryzae* is able to use acetic acid as a substrate for malic acid formation. Moreover it shows that it is possible to link anaerobic syngas fermentation and aerobic malic acid production using sequential mixed cultures of *C. ljungdahlii* and *A. oryzae*. In doing so, this study not only broadened the feedstock for malic acid production from glycerol and sugars to the whole feedstock of gasification processes but also reported the highest yields to date for the production of C_4 components from syngas.

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5 Evaluation of organosolve based carbon sources from lignocellulose

This chapter is based on the publication

Sustainable carbon sources for microbial organic acid production with filamentous fungi

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Authors' contribution to this publication

Stefan Dörsam designed the experiments together with Katrin Ochsenreither. Performed the pure sugar conversion and shake flask cultivation with *M. giganteus* lignocellulose fractions. Performed together with Jana Fesseler the shake flask experiments and bioreactor cultivation with *A. oryzae* and beech-wood lignocellulose fractions. Performed together with Olga Gorte the bioreactor cultivation with *R. delemar* and beech-wood lignocellulose fractions. Analyzed the results and designed the graphics and tables. Drafted the manuscript

Jana Fesseler performed together with Stefan Dörsam the shake flask experiments and bioreactor cultivation with *A. oryzae* and beech-wood lignocellulose fractions.

Olga Gorte performed together with Stefan Dörsam the bioreactor cultivation with *R. delemar* and beech-wood lignocellulose fractions.

Thomas Hahn provided the lignocellulose fractions, drafted the part of the production of the lignocellulose fractions. Critically revised the final manuscript version.

Susanne Zibek provided the lignocellulose fraction.

Christoph Syldatk contributed to manuscript concept and advisory. Critically revised the final manuscript version.

Katrin Ochsenreither contributed to the conception of the experiments and advised the lab work. Critically revised the final manuscript version.

5.1 Introduction

This chapter describes the evaluation of lignocellulose fractions, gained by the separation of the three main compartments of lignocellulose during the organsolov process. The enzymatic saccharification of cellulose leads to a glucose-rich fraction whereas a xylose-rich fraction results from hemicellulose (Laure *et al.* 2014). The challenge of using lignocellulose hydrolysates is on one hand the potential formation of toxic compounds during the fractionation process, mainly phenols from lignin, furfural and hydroxymethylfurfural (HMF) from cellulose and hemicellulose (Jönsson *et al.* 2013). On the other hand, the pretreatment process results in the formation of xylose oligomers in the supernatant which cannot be adequately enzymatic hydrolyzed afterwards due to unfavorable conditions and degradation products present. Both aspects make especially the xylose-containing fraction to the more challenging substrate. The aim of this study is the evaluation of pyrolytic sugar, different pretreated and post-treated fractions from the organosolv process from beechwood (*Fagus sylvatica)*, and *Miscanthus giganteus* as substrate for the fermentative malic acid production with the filamentous fungi *A. oryzae* and *R. delemar*. The fractions were produced by cooperation partners from Fraunhofer IGB in Stuttgart. Before lignocellulose hydrolysate fractions were tested as substrates, experiments with several mono- and disaccharides, possibly present in pretreated biomass, were conducted for their suitability for malic acid production with *A. oryzae.* This includes levoglucosan, glucose, galactose, mannose, arabinose, xylose, ribose and cellobiose as well as cheap and easy available sugars, e.g. fructose and maltose. *A. oryzae* is able to convert every sugar investigated to malate, albeit with different yields. Based on the promising results from the pure sugar conversion experiments, fractions of the organosolv process from beechwood (*Fagus sylvatica*) and *Miscanthus giganteus* were further analyzed as carbon source for cultivation and fermentation with *A. oryzae* for malic acid and *R. delemar* for fumaric acid production.

5.2 Material and Methods

5.2.1 Chemicals

All chemicals were either purchased from Sigma-Aldrich (Munich, Germany) or Roth (Karlsruhe, Germany).

5.2.2 Hydrolysate preparation

Different fractions were obtained by the organosolv process incubating the chopped raw material at high temperatures (> 140 °C) in aqueous ethanol solution with small amount of H_2SO_4 as catalyst. The fiber fraction, mainly containing the cellulose and a part of hemicellulose, was directly subjected to enzymatic hydrolysis after washing. The supernatant of the organosolv process was further processed to isolate the lignin and to utilize the carbohydrates from hemicellulose for fermentative purposes. Carbohydrate, acid and toxic compound content of the resulting solution was quantified via HPLC (see Sluiter *et al.* (2008) for further description). Processing of the residual fractions was carried out as follows.

Beechwood

Fiber (cellulose) fraction

Enzymatic hydrolysis of the fiber was performed at a temperature of 50 °C with a 10% (w/v) suspension for 24 h. For stirrer description see Ludwig *et al*. (2014).The pH of the suspension was adjusted to pH 4.8 during hydrolysis using a concentrated NaOH solution. Enzyme addition (0.06 g Cellic® CTec3 and 0.0025 g Cellic® HTec3 per g cellulose) started the reaction. The solid material was afterwards removed applying an extruder press. The successive evaporation of the filtrate resulted in the mono- and disaccharide concentrations shown in **Table 5-1**. 108.7 g of this fraction was used for fermentation purposes.

Hemicellulose fraction

After removal of the biomass, evaporation of the residual ethanol was performed to precipitate the lignin and to concentrate the carbohydrates. Enzymatic hydrolysis was not performed with this fraction. The compounds shown in **Table 5-1** could be identified by total hydrolysis and subsequent chromatographic analysis. 99.5 g of this fraction was used for fermentation.

Miscanthus fiber

Enzymatic hydrolysis of the fiber was performed at a temperature of 50 °C with a 10% w/v suspension for 24 h. For stirrer description see Ludwig *et al*. (2014). pH of the suspension was adjusted to pH 4.8 using a concentrated NaOH solution during hydrolysis. Enzyme addition

(0.06 g Cellic® CTec2 per g cellulose, 0.006 g Cellic® HTec2 per g cellulose) started the reaction. Residual solid material was removed after hydrolysis by centrifugation for 15 min at 4696 g. Succeeding concentration of the supernatant via evaporation resulted in the concentrations shown in **Table 5-1**. For shake flask cultivation, the solution was diluted do 100 g/L carbon sources and the salts for main-culture medium were added.

Table 5-1: Composition of the different Lignocellulose fractions from beechwood and Miscanthus.

	Beechwood Hemicellulose fraction (g/L)	Beechwood Fiber (Cellulose) fraction (g/L)	*Miscanthus* Fiber (Cellulose) fraction (g/L)
Ethanol	1	0	0
Acetic acid	15	0	0.2
Cellobiose	0	67	0
Glucose	20	609	102
Xylose monomer	100	179	25
Xylose oligomer	310	0	0
Rhamnose	27	0	0
Arabinose	18	0	0.2

5.2.3 Fungi and media

The fungal strains *A. oryzae* DSM 1863 and *R. delemar* DSM 905 were obtained from DSMZ strain collection (Deutsche Sammlung von Mikroorganismen und Zellkulturen GmbH, Braunschweig, Germany) and treated like described in Dörsam *et al*. (2016). *A. oryzae* was grown on minimal medium (MM) for *Aspergillus* spec. (Barratt *et al.* 1965): 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. The pH was set to 6.5 with NaOH. 10 g/L glucose, 2 mL of 1000× Hutner's Trace Elements, and 15 g/L agar were added afterwards. 1000× 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 $(NH_4)_6Mo_7O_{24} \cdot 4H_2O$, pH 6.5 (Barratt *et al.* 1965). *R. delemar* was grown on modified supplemented agar (SUP): 10 g/L glucose, 0.5 g/L yeast extract, 4 g/L KH_2PO_4, 0.9 g/L K_2HPO_4, 4 g/L NH_4Cl, 0.25 g/L $MgSO_4 \cdot 7H_2O$. The pH was set to 6.5 with NaOH.

For conidia collection, *A. oryzae* was grown on high-salt minimal medium (Song *et al.* 2001) which additionally contains 22.37 g/L KCl. For spore collection, *R. delemar* was grown on malt

extract agar (MEA): 30 g/L malt extract, 3 g/L peptone, 15 g/L agar. The conidia and spores were harvested with 50% glycerol from plates that were incubated for 5 days at 30 °C and filtrated with Miracloth (Calbiochem). The spore/conidia solution was diluted to a concentration of 1×10^7 (spore/conidia)/mL and stored at -80 °C.

Organic acid production was accomplished in a two-step process with a pre-culture and a main-culture. For *A. oryzae* the pre-culture medium consists of 40 g/L glucose monohydrate, 4 g/L $(NH_4)_2SO_4$, 0.75 g/L KH_2PO_4, 0.98 g/L $K_2HPO_4\cdot3H_2O$, 0.1 g/L $MgSO_4\cdot7H_2O$, 0.1 g/L $CaCl_2\cdot2H_2O$, 5 mg/L NaCl, and 5 mg/L $FeSO_4\cdot7H_2O$. Main-culture medium contains the corresponding carbon source, in equivalent carbon amounts as in control medium. The control contains 120 g/L glucose monohydrate, 1.2 g/L $(NH_4)_2SO_4$, 0.1 g/L KH_2PO_4, 0.17 g/L $K_2HPO_4\cdot3H_2O$, 0.1 g/L$MgSO_4\cdot7H_2O$, 0.1 g/L $CaCl_2\cdot2H_2O$, 5 mg/L NaCl, and 60 mg/L $FeSO_4\cdot7H_2O$. To keep the pH-Level above 5.5 during fermentation, 90 g/L $CaCO_3$ powder was added to the main-culture medium.

The pre-culture medium for *R. delemar* consists of 30 g/L glucose, 2.0 g/L urea, 0.6 g/L KH_2PO_4, 0.5 g/L $MgSO_4\cdot7H_2O$, 0.11 g/L $ZnSO_4$, 8.8 mg/L $FeSO_4\cdot7H_2O$. The pH was set to 4.5 with 10 M HCl after autoclaving to support growth in form of pellets. Main-culture medium contains the corresponding carbon source, in equivalent carbon amounts as 100 g/L glucose. Further 0.2 g/L urea, 0.6 g/L KH_2PO_4, 0.5 g/L $MgSO_4\cdot7H_2O$, 0.11 g/L $ZnSO_4$, 8.8 mg/L $FeSO_4\cdot7H_2O$. All media were sterilized by autoclaving.

5.2.4 Organic acid production

For *A. oryzae* pre-culture, 100 mL of pre-culture medium in a 500 mL baffled Erlenmeyer shake flasks was inoculated with 2×10^7 conidia. The flasks were incubated at 100 rpm and 30 °C for 24 hours in a rotary shaker. After incubation, pre-culture medium was removed by washing the fungal pellets twice and resuspending in 100 mL water. 100 mL of the main-culture was transferred to 500 mL Erlenmeyer shake flasks and 9 g/L sterile $CaCO_3$ powder added. The flasks were inoculated with 10% (*v*/*v*) of washed pre-culture and incubated at 120 rpm and 32 °C for 7 days.

For *R. delemar* pre-culture, 100 mL of pre-culture medium was filled into 500 mL baffled Erlenmeyer shake flasks and inoculated with 1×10^7 spores. The flasks were incubated at 100 rpm and 35 °C for 30 hours in a rotary shaker. To remove the pre-culture medium, fungal pellets were washed twice and resuspending in 100 mL water.

Every cultivation was done in triple approach.

For the bioreactor cultivations, 1.5 L of main culture medium was used. Additionally, 120 g $CaCO_3$ powder for pH regulation and 200 µL of antifoam reagent (Contraspum A 4050 HAC, Tschimmer und Schwarz) were added before autoclaving. The bioreactor was inoculated with the fungal biomass of two pre-culture flasks (suspended in 100 mL water) for *A. oryzae* and with the biomass of five pre-culture flasks (suspended in 100 mL water) for *R. delemar*. Every fermentation was done in double approach. The fermentation was carried out in a small-scale bioreactor (vessel volume 2.0 L) Minifors (Infors, Switzerland) at 35 °C, an aeration rate of 0.5 vvm, and a stirrer speed of 300 rpm. A Rushton turbine with a diameter of 46 mm was chosen as stirrer.

5.2.5 Organic acid and carbohydrate analytics

For the malic acid quantification by HPLC, fermentation broth samples were pretreated and analyzed as described in Ochsenreither *et al*. (2014) with slight modifications. Malic acid was released from precipitated calcium malate by mixing 1 mL sample with 1 mL of 3 M H_2SO_4 and 3 mL of water incubating the homogenate at 80 °C for 20 min. 1 mL of the mixture was transferred to a 1.5 mL Eppendorf tube and centrifuged in a table top centrifuge for 5 min at 20,000×g. The supernatant was used for HPLC analysis. The analysis was performed with a standard HPLC device (Agilent 1100 Series, Agilent, Germany) equipped with a 15 cm reversed phase column (Synergi™4 µm Fusion-RP 80 Å, LC Column 150×4.6 mm, Phenomenex, Aschaffenburg, Germany) at 30 °C. Mobile phase solution A was methanol, and solution B was 20 mM KH_2PO_4, pH 2.5. The flow rate was 1 mL/min and a gradient was used for the separation of organic acids: 0 - 0.5 min 100% eluent B, 0.5 - 10 min linear increase of eluent A from 0 to 10%, 10 - 12 min decrease of eluent A back to 0%, and 12 - 14 min again 100% eluent B. The injection volume was 10 µL and the detection was performed with a UV detector at a wavelength of 220 nm. Standards were purchased from Sigma-Aldrich (Munich, Germany) and used for peak identification and calibration. Linear detection ranged from 0.1 to 5 g/L malic acid and 0.02 to 0.5 g/L fumaric acid.

For the carbohydrate quantification by HPLC, fermentation broth samples were pretreated and analyzed as described by Buchholz *et al.* (2013) with slight modifications described by Siebenhaller *et al.* (2017). A protocol for phosphate precipitation was applied before measurement. 45 µL 4 M NH_3 and 100 µL 1.2 M $MgSO_4$ were added to 1000 µL sample and subsequently centrifuged for 5 min at 20,000×g after 5 minutes of incubation. 500 µL supernatant was then mixed with 500 µL 0.1 M H_2SO_4 and incubated for 15 min. After the final centrifugation step

of 15 min at 20,000×g, the supernatant was used for HPLC analysis. The analysis was performed with a standard HPLC device (Agilent 1100 Series, Agilent, Germany) with a Rezex ROA organic acid H+ (8%) column (300 by 7.8 mm, 8 m; Phenomenex) and a Rezex ROA organic acid H+ (8%) guard column (50 by 7.8 mm). Separation was performed under isocratic conditions at 50 °C (column temperature) for 45 min with 5 mM H_2SO_4 as the mobile phase at a constant flow rate of 0.5 ml/min. Detection of carbohydrates was achieved via a refractive index detector (Agilent 1200 series, Agilent, Germany).

5.2.6 Data analysis

Carbon source consumption and malic acid production was fitted using a logistic equation with four parameters with a scientific data analysis and graphing software (Sigma Plot 9.0, Systat, San Jose, USA). The used equation was:

$$y(x) = y_0 + \frac{a}{1+\left(\frac{x}{x_0}\right)^b}$$

The four parameters are the following: y_0 indicates the minimum concentration of the carbon source/product; a indicates the maximum carbon source/product concentration; x_0 indicates the process time when half of the carbon source amount is consumed or half of the maximum product concentration is produced; b is a shape parameter and difficult to explain biologically (Erkmen and Alben 2002).

Consumption and production rates were calculated as the derivation of this equation.

5.3 Results

5.3.1 Pure sugar conversion experiments

The main challenge of using nonfood sugars in biotechnological applications is firstly the ability of the respective organism to metabolize different sugars in general, and secondly, especially for pretreated lignocellulosic material, the tolerance concerning degradation products formed during the pretreatment process.

Therefore, several mono- and disaccharides possibly contained in alternative carbon sources were tested for their suitability as substrates for malic acid production with *A. oryzae*. This includes the anhydrosugar levoglucosan, formed during flash pyrolysis, carbohydrates contained in lignocellulose like glucose, galactose, mannose, arabinose, xylose, ribose and cellobiose, as well as cheap and easy available sugars like fructose and maltose. The results are summarized in **Table 5-2**.

Table 5 2: Calculated parameters of tested carbon sources in shake flask cultivation of *A. oryzae*. Flasks were incubated at 32 °C for 168 hours.

	Carbon Source	g/L (carbon source)[a]	c (Malate) g/L[b]	$Y_{P/S}$ g/g[b]	Q_p max g/(L*h)[c]	time span of Q_p max h[c]	Q_s max g/(L*h)[c]	time of Q_s max h[c]	Q_p overall g/(L*h)[b]
(Anhydro-) Hexoses	Glucose	109	40.5 ± 3.7	0.65	0.41	64.13 - 79.31	0.61	0 - 1.13	0.24
	Fructose	109	24.8 ± 1.9	0.63	0.22	107.63 - 131.25	0.39	61.69 - 73.5	0.15
	Galactose	109	1.8 ± 0.5	0.06	0.015	164.72 - 168	0.25	0 - 1.97	0.01
	Mannose	109	32.8 ± 0.5	0.69	0.29	108.94 - 134.53	0.40	6.56 - 34.78	0.19
	Levoglucosan	98.2	17.2 ± 1.7	0.34	0.14	103.03 - 143.72	0.36	0 - 10.5	0.1
Pentoses	Arabinose	109	7.2 ± 2.9	0.22	0.06	108.94 - 140.44	0.42	0 - 1.97	0.04
	Ribose	109	20.7 ± 5.7	0.45	0.18	110.25 - 148.31	0.33	0 - 10.5	0.12
	Xylose	109	24.3 ± 3.3	0.49	0.20	97.78 - 123.38	0.31	139.78 - 168	0.14
Di-saccharides	Maltose	103.6	34.1 ± 10.8	0.34*	0.30	96.47 - 110.25	1.57*	56.44 - 60.38*	0.2
	Cellobiose	103.6	8.8 ± 1	0.14*	0.10	101.06 - 130.59	0.65*	0 - 0.66*	0.05
Mixed	75% Glucose, 25% Xylose	81.8 27.3	29.4 ± 1.9	0.38	0.29	47.81 - 67.50	1.02	0 - 0.56	0.2
	25% Glucose, 75% Xylose	27.3 81.8	31.9 ± 0.3	0.59	0.31	140.63 - 149.63	0.90	0 - 0.56	0.22

c (Malate) = Final Product concentration; $Y_{P/S}$ = substrate specific yield; Q_p max = maximal volumetric production rate; Q_s max = maximal volumetric consumption rate; *Based on disaccharide cleavage; [a]Weighed amount; [b]Measured values; [c]Calculated values

A. oryzae is able to convert every tested sugar to malate, albeit with different yields. The hexose fructose and the disaccharide maltose, not derived from lignocellulosic material, turned out to be a very promising substrate.

The highest malic acid titer was achieved with glucose (40.5 ± 3.7 g/L). This approach is also used as the control cultivation. Subsequently, maltose led to the second highest malic acid concentration of 34.1 ± 10.8 g/L. Cultivations with mannose and the two testes mixture of glucose and xylose resulted in final product concentrations around 30 g/L. Around 20 g/L could be achieved by using fructose (24.8 ± 1.9 g/L), levoglucosan (17.2 ± 1.7 g/L), ribose (20.7 ± 5.7 g/L) and xylose (24.3 ± 3.3 g/L) as sole carbon source (**Table 5-2**). Only three of the tested carbon sources led to a final malic acid concentration below 10 g/L, namely cellobiose, arabinose and galactose. Product yields correlate slightly with malic acid titers. The theoretical yields of (anhydro)hexoses are 2 mol organic acid per mol carbon source which is 1.49 g/g for malic acid and 1.29 g/g fumaric acid with hexoses and 1.65 g/g malic acid with levoglucosan. For disaccharides 4 mol organic acid per mol carbon source (1.57 g/g) and for pentoses 1.67 mol organic acid per mol carbon source (1.49 g/g) (Ochsenreither *et al.* 2014).

The highest yield was achieved with mannose with 0.69 g/g which corresponds to 46% of the maximum theoretical yield. The control approach with glucose resulted in a yield of 0.65 g/g (44%), the mixture of 25% glucose and 75% xylose in a yield of 0.59 g/g (40%) and with fructose in a yield of 0.63 g/g (42%). The yields for all of the other tested carbon sources were below 0.5 g/g. Cultivation with xylose (0.49 g/g; 33%), ribose (0.45 g/g, 30%), the mixture of 75% glucose and 25% xylose (0.38 g/g, 26%), levoglucosan (0.34 g/g; 21%), maltose (0.34 g/g; 22%) and arabinose (0.22 g/g; 18%) resulted in concentrations in the middle range. Lowest yields were achieved for cellobiose with 0.14 g/g (9%) and galactose with 0.06 (4%) g/g.

Production rates were calculated as derivation of malic acid concentration fit (sigmoidal, 4 parameters) during cultivation time. The malic acid concentration as well as the corresponding volumetric production rates during cultivation with glucose, mixture of 75% glucose and 25% xylose, cellobiose, fructose and levoglucosan are exemplary shown in **Figure 5-1**.

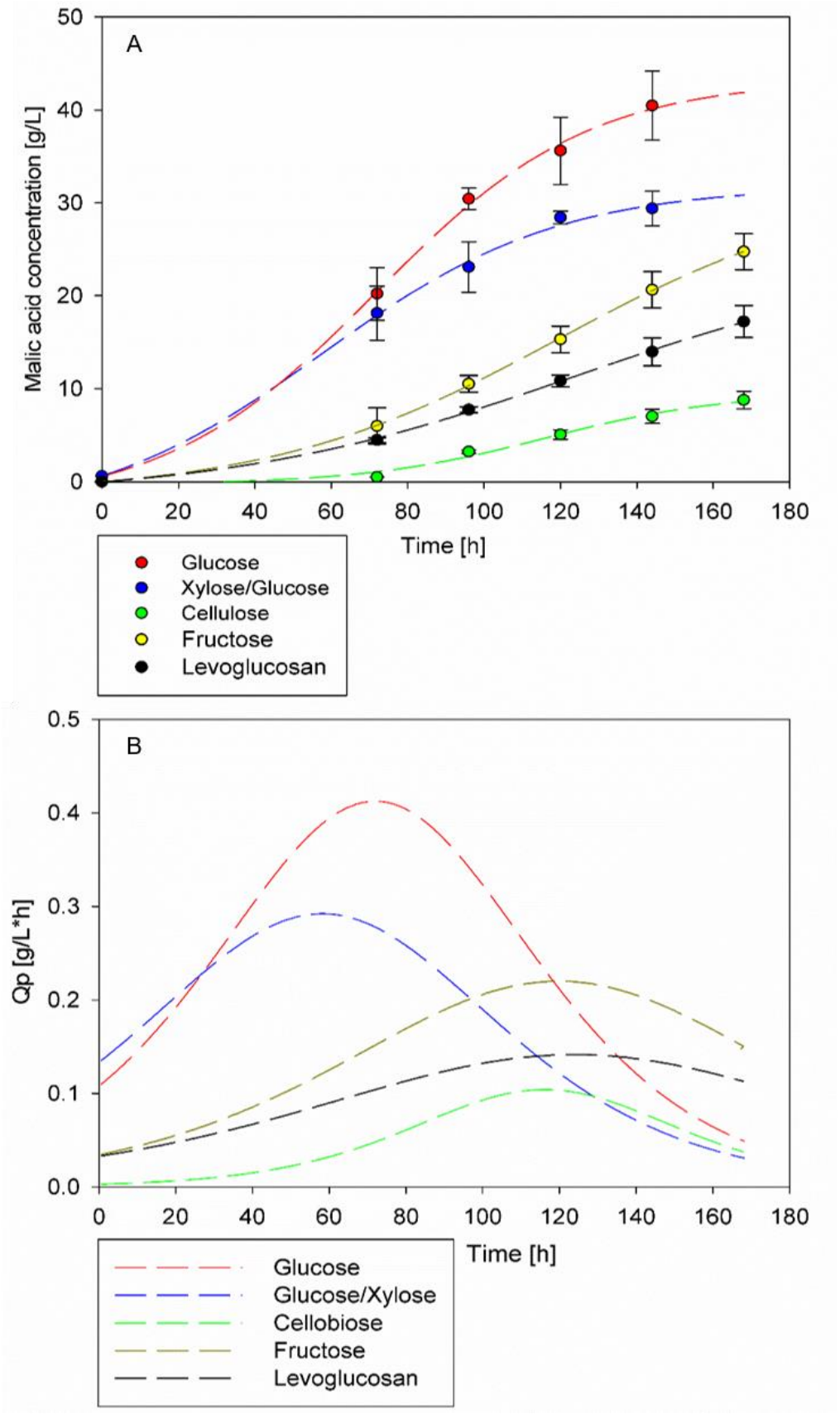

Figure 5-1: Examples of malic acid formation (A) and volumetric production rates (B) during cultivation of *A. oryzae* DSM 1863 by using different carbon sources. Flasks were incubated at 32 °C for 168 h. Q_p = volumetric production rate.

The volumetric production rates of malic acid differ widely between the different carbon sources. The production rate plotted against the cultivation time results in a parabolic curve. Their peak corresponds to the time point of the maximum production rate.
Highest maximal rate could be observed during cultivation with glucose (0.41 g/(L*h)) between 64.13 h and 79.31 h of cultivation. Production rates of both glucose and xylose mixtures, mannose and maltose were approximately 0.3 g/(L*h). Using fructose as carbon source resulted in a maximal production rate of 0.22 g/(L*h) and was observed during the cultivation period from 107.63 h to 131.25 h. For xylose, ribose, levoglucosan and cellobiose the highest volumetric production rate were 0.20 g/(L*h), 0.18 g/(L*h), 0.14 g/(L*h) and 0.10 g/(L*h). Lowest maximal production rates could be observed with arabinose (0.06 g/(L*h)) between cultivation hour 108.94 to 140.44 and with galactose (0.015 g/(L*h)).
Maximal volumetric consumption rates of carbon sources did not correlate with maximal production rates of malic acid. The highest consumption rate was detected for maltose (1.57 g/(L*h)) from. Second highest consumption rate could be observed with the mixture of 75% glucose and 25% xylose of 1.02 g/(L*h) followed by 25% glucose, 75% xylose of 0.90 g/(L*h). Cellobiose and the control approach glucose, showed the maximum of consumption rate in an early stage of cultivation (0.65 g/(L*h) and 0.61 g/(L*h)). By using arabinose, mannose and fructose, the maximal consumption rate of about 0.40 g/(L*h) was achieved. Ribose, xylose and finally galactose showed the lowest maximal consumption rates of about 0.30 g/(L*h).

The pure sugar conversion experiment showed the suitability of several sugars. By focusing on organosolv pretreated lignocellulose fractions, the most important sugars are cellobiose, glucose and xylose, whereas galactose, ribose, arabinose and mannose only occur in trace amounts. Beside the sugars, toxic compounds formed during organosolv process derived from sugars and lignin can be a major problem for many organisms. The limiting inhibiting concentrations for several typical impurities derived from lignin, as well as some impurities derived from sugars are described in earlier studies by our group (Dörsam *et al.* 2016) but does not include hydroxymethylfurfural (HMF), the most common impurity. To investigate the tolerance during malic acid production phase, various concentrations of HMF were added to main-culture medium and malic acid concentration was measured during cultivation period. The analyzed HMF contents were 0%, 0.1%, 0.15% and 0.2%. No inhibiting influence could be observed for all concentrations tested (data not shown).

5.3.2 Cultivation of *A. oryzae* with different lignocellulose-derived fractions

Because of the promising results from the pure sugar conversion experiments, fractions of the organosolv process were further assessed as carbon source for cultivation. For this study, fractions of two different plants were used (*Fagus sylvatica* and *Miscanthus giganteus*). For each plant, fibers were separated and pretreated as described in the materials section. Both cellulose fractions were saccharified and concentrated. For *F. sylvatica* cellulose hydrolysate, no HMF could be detected. 108.7 g of beechwood fraction was used for cultivation. *Miscanthus giganteus* hydrolysate solution was diluted to 100 g/L carbon source. During cultivation in shake flasks, carbon source concentration and product formation was determined. The curves are shown in **Figure 5-2**.

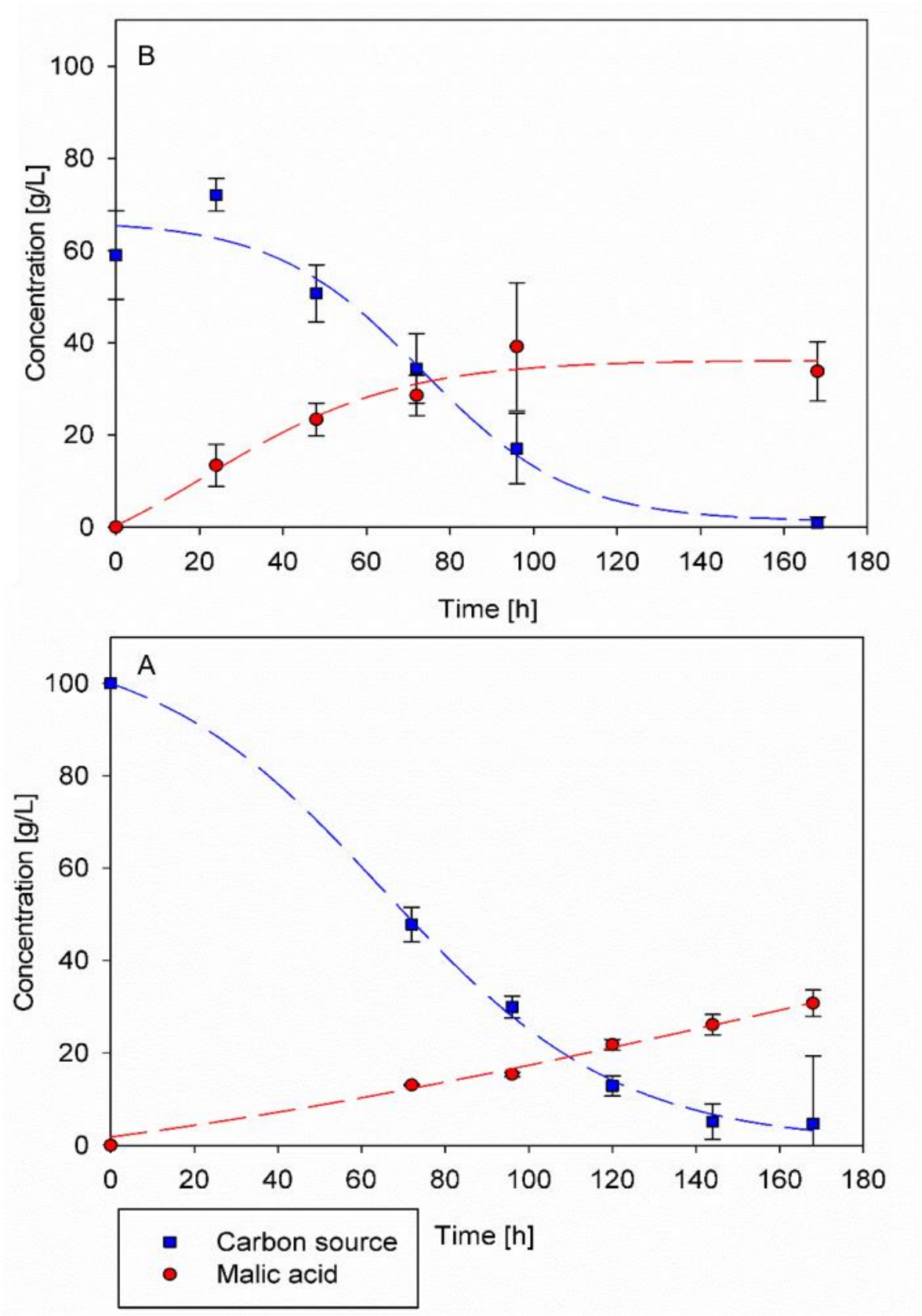

Figure 5-2: Carbon source and malic acid concentration during cultivation with *A. oryzae* DSM 1863 and cellulose/fiber hydrolysates from *F. sylvatica* (A) and *M. giganteus* (B). Flasks were incubated at 32 °C for 168 hours.

During cultivation with *F. sylvatica* fiber hydrolysate, the detectable carbon sources (glucose and xylose) decreased from 59 g/L to 0.9 g/L, whereas the malic acid concentration increased from 0 to 33.8 ± 6.4 g/L corresponds to an overall production rate of 0.2 g/(L*h). This results in a yield related to glucose and xylose of 0.58 g/g (39%). Shake flasks with *M. giganteus* cellulose hydrolysate showed a decrease of carbon source concentration (glucose and xylose) from 100 g/L to 4.7 g/L and a final malic acid titer of 30.8 ± 2.9 g/L which results in a yield of 0.32 g/g (22%) and an overall production rate of 0.18 g/(L*h). The highest volumetric production rate (0.54 g/(L*h)) was determined after 18.38 h and decreased after 24.28 h for beechwood cellulose hydrolysate. For *M. giganteus* cellulose hydrolysate, the maximal production rate of 0.21 g/(L*h) was reached after 156.84 h until the end of cultivation. Compared to this, maximal volumetric consumption rate of 0.92 g/(L*h) were calculated from cultivation hour 71.53 until hour 75.47 for beechwood and for *M. giganteus* from 60.38 h to 70.88 h (0.97 g/(L*h)).

In contrast to the fiber hydrolysate, the hemicellulose fraction of *F. sylvatica* was not saccharified. The fraction was used as the carbon source in the main culture in shake flask cultivations. The monosaccharide content is formed during the harsh organosolv process conditions. As the major impurity, 4.5 g/L furfural could be detected in this fraction. Further impurities derived from lignin and sugars were expected. HPLC measurements showed that they only occur in trace amounts. Beside furfural, acetic acid (15 g/L) must be seen as an impurity, but is also a possible carbon source for *A. oryzae* for malic acid production (Oswald *et al.* 2016). Because of the impact of impurities, three different amounts of *F. sylvatica* hemicellulose were used to observe possible inhibition effects. Malic acid concentrations during cultivation time were measured and curves are shown in **Figure 5-3**. Amount of beechwood hemicellulose fraction (BHF) was used correlated to the amount of carbon in the control approach with glucose: 99.5 g, 49.8 g and 24.9 g

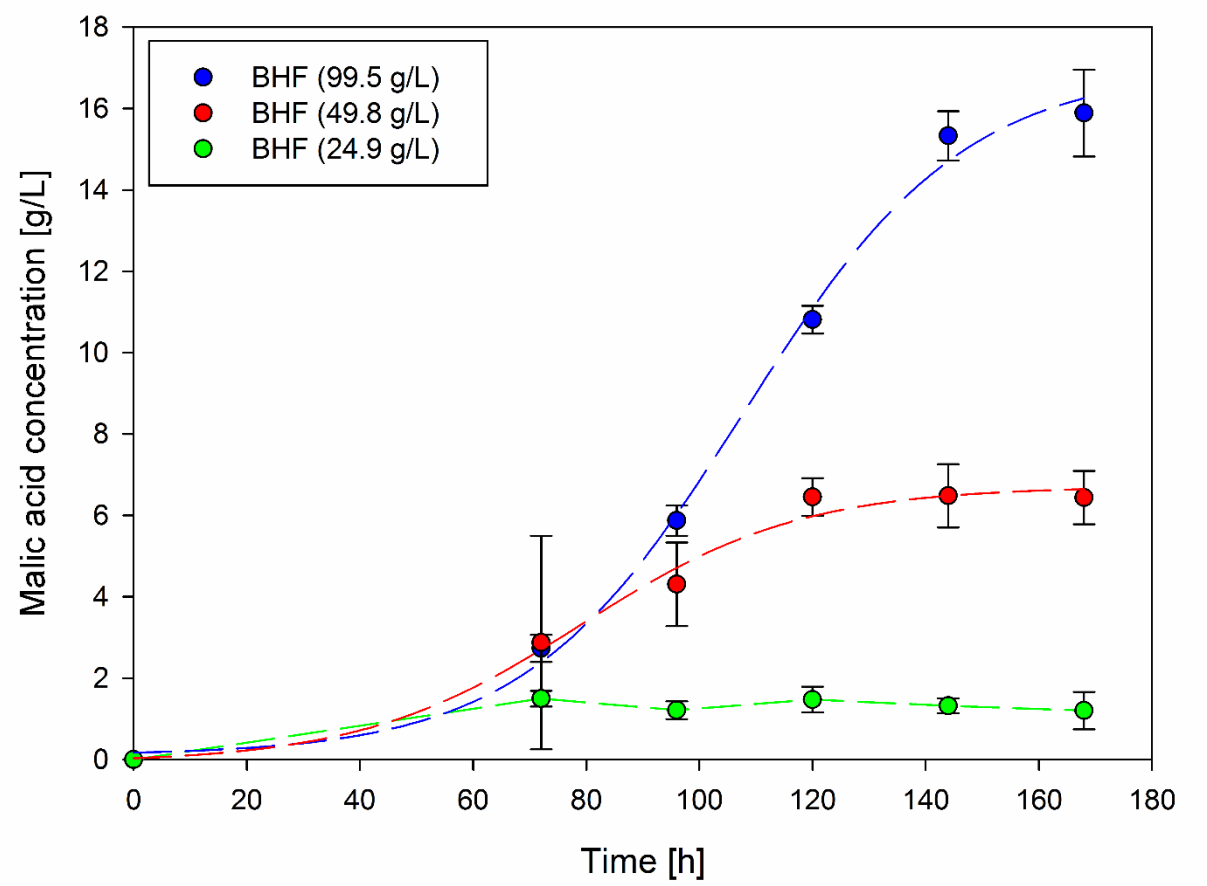

Figure 5-3: Malic acid concentration during cultivation of *A. oryzae* DSM 1863 with hemicellulose fraction from beechwood (BHF) in three different concentrations. Flasks were incubated at 32 °C for 168 hours.

With all concentrations a lag phase of about 48 h was observed before malic acid production started which did not occur in the other cultivations with refined sugars. Using 99.5 g/L beechwood hemicellulose fraction, a malic acid titer of 15.9 ± 1.1 g/L could be achieved. A concentration of 49.8 g/L resulted in a final product concentration of 6.4 ± 0.7 g/L and 24.9 g/L carbon source resulted in 1.2 ± 0.5 g/L malic acid after a cultivation time of 168 h. Maximal volumetric production rate (0.22 g/(L*h)) could be observed during 103.69 h and 112.22 h for 99.5 g/L and between 72.19 h and 85.97 h for 49.75 g/L (0.09 g/(L*h)). The amount of malic acid produced from a concentration of 24.9 g/L was low. The production rate between the first two samples can be calculated and is approximately 0.02 g/(L*h). Because of the complexity of the hemicellulose hydrolysate, carbohydrates could not be fully quantified during cultivation. Related to all known carbon sources (glucose, xylose, oligoxylose, rhamnose, arabinose and acetic acid) at the beginning, the yields were 0.42 g/g, 0.34 g/g and 0.13 g/g with decreasing amount of hemicellulose fraction. The overall production rates were 0.09 g/(L*h), 0.04 g/(L*h) and 0.007 g/(L*h).

5.3.3 Scale-up fermentation of organosolv fractionated lignocellulose with *A. oryzae*

Since promising results gained with the shake flask experiments with hemicellulose and cellulose from beechwood, batch fermentations in a small-scale bioreactor (vessel volume: 2.0 L) have been performed. The hemicellulose cultivation approaches revealed that the highest yield could be achieved using 99.5 g/L of hemicellulose fraction. Because of this, we used this approach for fermentation. The malic acid and carbon source concentration of beechwood cellulose fraction, as well as the malic acid concentration of beechwood hemicellulose fraction during fermentation are shown in **Figure 5-4**.

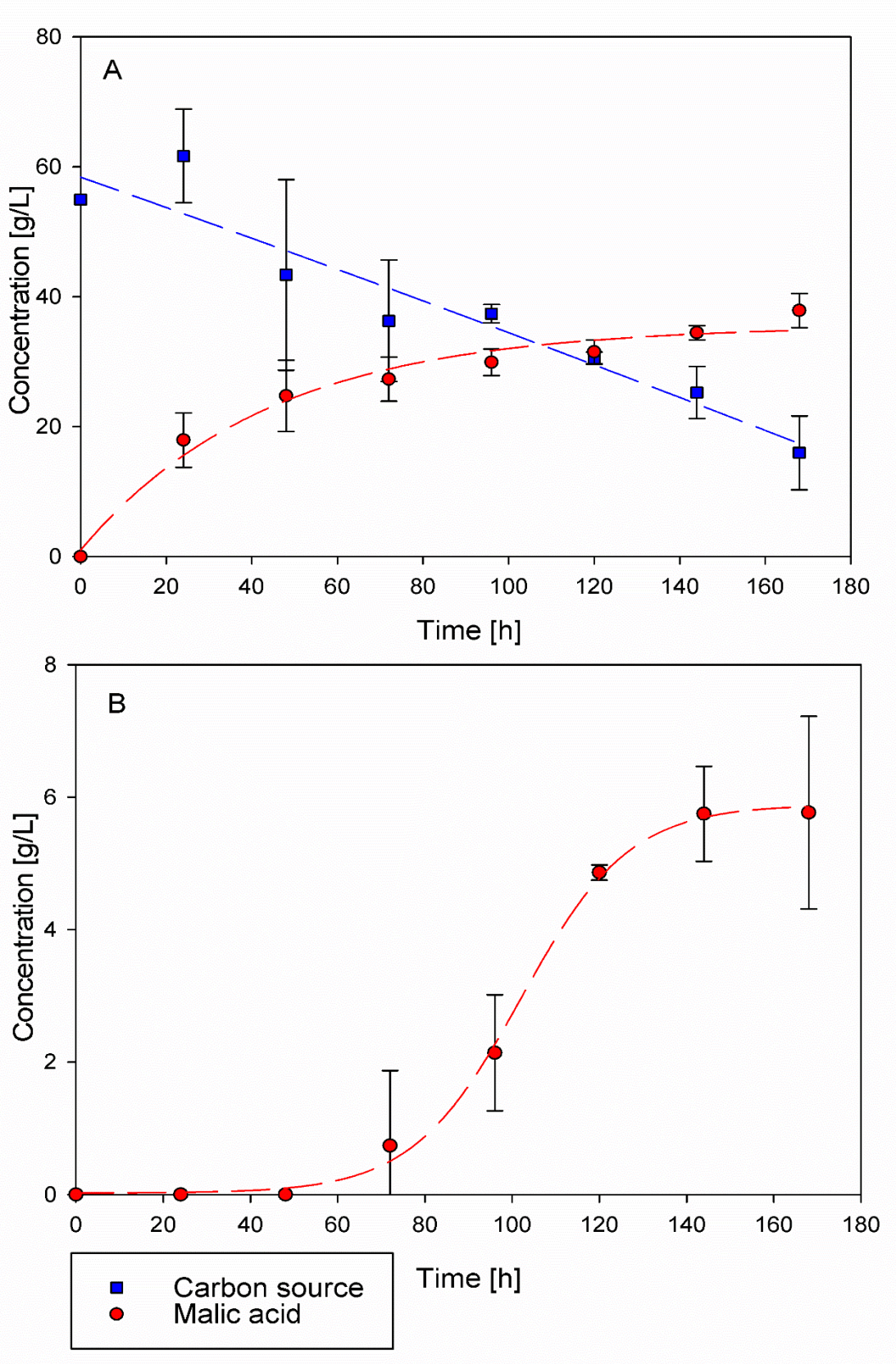

Figure 5-4: Carbon source and malic acid concentration by fermentation of *A. oryzae* DSM 1863 with cellulose/fiber hydrolysate (A) and hemicellulose fraction (B) from *F. sylvatica*. Batch fermentation was carried out in a small-scale bioreactor at 35 °C and 300 rpm for 168 hours.

During fermentation with beechwood cellulose/fiber hydrolysate, malic acid concentration increased to 37.9 ± 2.6 g/L. The carbon source concentration (glucose and xylose) simultaneously decreased from 55 g/L to 16 g/L. This results in a yield of 0.97 g/g (65%). The maximal production rate of 0.78 g/(L*h) was determined in the beginning of the fermentation (0 h – 0.66 h). Maximal volumetric consumption rate (0.26 g/(L*h)) lasted from 160.78 h until the end of cultivation. The production rate of the whole fermentation process was 0.23 g/(L*h).

During fermentation with beechwood hemicellulose fraction the extended lag phase of about 48 h observed at shake flask cultivation occurred again in bioreactor fermentation. The malic acid concentration increased to a final concentration of 5.8 ± 1.5 g/L resulting in an overall production rate of 0.03 g/(L*h). The maximal volumetric production rate of 0.12 g/(L*h) was from 97.78 h to 106.31 h of fermentation time.

5.3.4 Fermentation of organosolv fractionated lignocellulose with *R. delemar*

The results of fermentation with beechwood hydrolysates with *A. oryzae* showed the general suitability of this kind of carbon source for fermentation. To demonstrate the suitability of the beechwood carbohydrates as substrate for other fungi, a small-scale batch fermentation was subsequently repeated with the industrial relevant fumaric acid producer *Rhizopus delemar* DSM 905 with same amounts of organosolv fraction and same fermentation conditions. Toxicity tests of a selection of possible impurities were done in earlier studies of our group (Dörsam *et al.* 2016) with this fungi. Cultivation with beechwood hemicellulose fraction did not result in product formation. Using beechwood fiber hydrolysate, 16.2 ± 0.2 g/L fumaric acid could be produced. During fermentation, the carbon sources (glucose and xylose) decreased from 53.6 g/L to 12.1 g/L resulting in a yield of 0.39 g/g (30%) and an overall production rate of 0.1 g/(L*h). The results are shown in **Figure 5-5**.

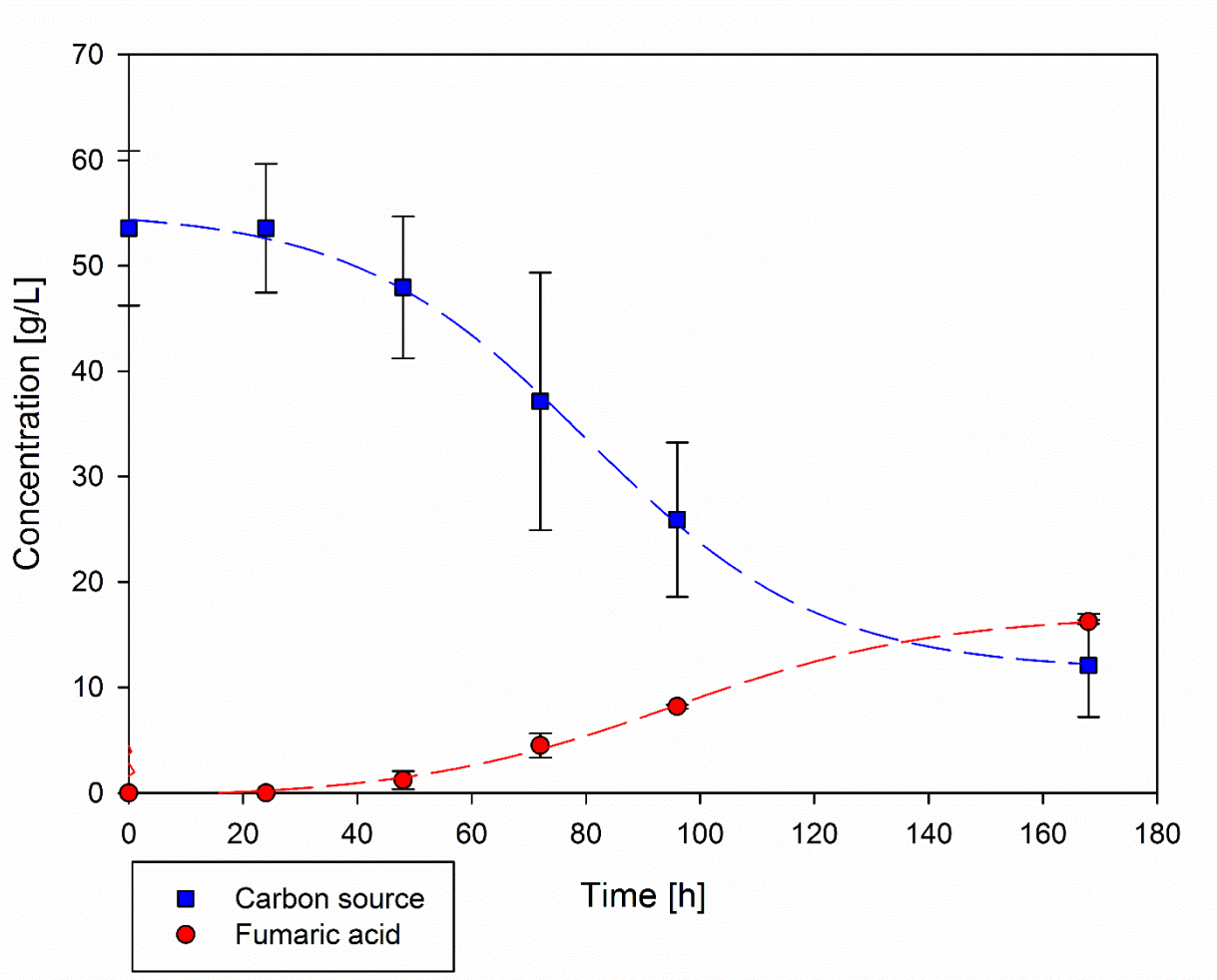

Figure 5-5: Carbon source and fumaric acid concentration by fermentation of *R. delemar* DSM 905 with cellulose hydrolysate from beechwood. Batch fermentation was done in a small-scale bioreactor at 35 °C and 300 rpm for 168 hours.

The maximal production rate of 0.19 g/(L*h) was reached after the first half of fermentation (89.91 h to 101.06 h). Maximal volumetric consumption rate (0.53 g/(L*h)) was observed from 76.13 h until 84.66 h.

5.4 Discussion

The number of possible carbon sources for malic acid production with *A. oryzae* is high. The observed flexibility makes this filamentous fungus still interesting for industrial application, although higher yields and titers could be achieved with other fungi like *Ustilago trichophora* (Zambanini *et al.* 2016). For industrial application the main focus is the price of the carbon source, the productivity and the yield of the process. Fructose and maltose, both commonly used in food industry, were proved to be very good sources for malic acid fermentation. Both sugars resulted in a good product yield; fructose even results in the highest yield of all tested sugars. The glucose dimer maltose, led to the second highest malic acid concentration of 34.2 g/L. Beside most other microorganism's, fungi are only able to consume simple molecules like monosaccharides and amino acids but they are able to secrete enzymes to cleave more complex structures externally. The rate-limiting step of the metabolization of disaccharides of fungi is the extracellular cleavage of the α/β -1→2-glycosidic bond. Because the disaccharide concentration does not reflect the concentration of metabolizable glucose concentration in the media, calculated yield seems too low and consumption rate too high. As all other hexoses can easily be converted to glucose-6-phosphate to enter the glycolysis, the metabolic pathway for galactose is more complex. Although *A. oryzae* expresses the enzyme galactose-1-phosphate uridylyltransferase, an important enzyme for galactose metabolism, and a decrease of galactose during cultivation could be observed, the conversion to malic acid seems not possible. Regarding the "food or fuel" dilemma, focus of this study was to identify suitable non-edible carbon sources. To achieve a high productivity of the process, a high volumetric production rate is striven. This high production rate, reached in an early stage of fermentation over a long term is the ideal case. Both parameters vary highly between the sugars investigated. As observed, the time of maximal consumption rate (mostly at the beginning of the fermentation) and the time of maximal production rate (mostly in the middle or end of fermentation) are not correlating with each other. Glucose as the control forms an exception. This is due to the adaptation of the fungus to the respective carbon source, which is not happening by cultivation on glucose because of the pre-culture cultivation on glucose. The longest phase of the highest production rate was observed in cultivations with levoglucosan. The ability of A. *oryzae* to metabolize and produce malic acid from this anhydrosugar was not described before, but it is shown for citric acid production with *A. niger* (Zhuang *et al.* 2001) and itaconic acid production with *A. terreus* (Nakagawa *et al.* 1984). It was demonstrated that *A. oryzae* can convert levoglucosan to malic acid with a yield of 0.34 g/g to a final titer of 17.2 g/L. Both are about half of the values obtained for glucose. Like glucose, levoglucosan will be converted to glucose-6-phosphate as the first

step of the metabolic pathway. Because of the higher K_m value of the levoglucosan kinase compared to the hexokinase, this difference can be explained by activity differences (Kitamura and Yasui 1991). Energetic differences in levoglucosan metabolization (like ATP consuming transport systems) are speculative and not known until now. Nevertheless, pyrolytic sugar is a possible future carbon source.

The other tested sugars are all contained in organosolv-pretreated and fractionated lignocellulose. They showed a very diverse suitability as carbon source. The suitability of xylose as carbon source for malic acid production has already been shown by Ochsenreither *et al*. (2014) and could be verified in this study (0.44 g/g yield). The main components in lignocellulose are by far glucose and xylose. Glucose is the established carbon source for this fermentation process, but its dimeric form in organosolv-derived pretreated cellulose, cellobiose is a challenge for the organism. The results in **Table 5-2** showed a general suitability but the resulting yield and titer is in a very low range (0.14 g/g and 8.8 g/L). As well as for maltose, the calculated yield and consumption rate is related to the disaccharide cleavage and does not reflect the real values adequately. However, it shows the better adaption of *A. oryzae* to a starch-containing substrate compared to a lignocellulosic substrate. In preparation for fermentation with enzymatic treated organosolv fractions, mixtures of glucose and xylose were tested. Product titer for both tested mixtures and the yields differ greatly (75% glucose: 29.4 ± 1.9 g/L, 0.38 g/g and 25% glucose: 31.9 ± 0.3 g/L, 0.59 g/g). As observed, *A. oryzae* prefers to metabolize glucose first, until the concentration decreases under a certain level (about 20 g/L). Below that threshold value xylose also gets metabolized. This double usage of glucose and xylose is described for other organisms like *Clostridium* sp. (Strain BOH3) (Xin *et al.* 2014) for butanol production and for the yeasts *T. cutaneum* (Hu *et al.* 2011) for lipid production. In the 75% glucose and 25% xylose approach, this adaption process has to be done in the middle of the fermentation. Compared to this, in the 25% glucose and 75% xylose approach it happened already during the adaption process of the fungus to the conditions in the main-culture medium. This double usage of sugars could be one of the reasons, why yield for the approach with 25% glucose and 75% xylose is much higher than the opposite around. Interestingly, this does not affect the malic acid production rate.

The logical next step was the cultivation on lignocellulosic carbon sources. In the past, fermentation approaches with pretreated lignocellulosic biomass were mostly done for ethanol production for example with bacteria like *E. coli* KO11/SL40 or *Zymomomas* CP4 (pZB5) summarized by Bothast *et al.* (1999) and yeasts like *S. cerevisiae* (Eliasson *et al.* 2000; Katahira *et al.* 2006; Jung *et al.* 2013), *S. passalidarum* (Long *et al.* 2012) and *P. stipitis* (Yücel and Aksu

2015). There is also an approach for the direct conversion of wheat straw without pre-treatment with the cellulolytic strain A. *oryzae* A-4 *A.* In this experiment a lipid yield of 62.87 mg/g dry substrate could be achieved (Lin *et al.* 2010). Approaches for the production of value-added substances are quite low. One of the challenges using organosolv fractions as carbon source are impurities formed during the process, as summarized by Jönsson *et al.* (2013) on the one hand. On the other hand, not focused in this study, the purification of the products is much more complicated using this complex carbon source.

For the majority of the fermentation processes, either an elaborate detoxification process is required or the organism has to be adapted through strain development (Larsson *et al.* 2001). Because of the high tolerance level against toxic impurities of *A. oryzae* (Dörsam *et al.* 2016), this organism is predestinated for this kind of carbon source.

Comparing the shake flask cultivation of *A. oryzae* with *F. sylvatica* fiber hydrolysate with *M. giganteus* fiber hydrolysate showed very different results. The starting concentration of glucose and xylose in both approaches differ greatly between 100 g/L and 60 g/L. The volumetric production rate for beechwood cellulose fraction reaches its maximum in an early stage of fermentation, and after most of the sugars are metabolized the malic acid concentration subsequently converges to a limit. In comparison, the *M. giganteus* cellulose hydrolysate cultivation shows a linear decrease of sugars, and the highest production rate at the end of cultivation, when sugar is nearly consumed. This indicates possibly non-detectable, but convertible carbon sources in this approach.

The lag phase of malic acid concentration of about 48 h by cultivation with hemicellulose fraction of beechwood can be explained with a necessary fungal adaptation to the media composition. By comparing the yield (0.42 g/g) of the 99.5 g approach to the yield with pure xylose (0.49 g/g) it is very similar. A detailed look on the composition of this fraction shows, that about one quarter of the carbohydrates are oligoxylose. *A. oryzae* is described as xylanolytic strain (Kimura *et al.* 1998), hence the adaption time can be explained with delayed gene regulation expressing enzymes capable of xylan degradation. The dilution approaches (49.8 g and 24.9 g) led to a decrease of the yield. On the one hand, it is known, that high amounts of carbon source support the malic acid formation (Ochsenreither *et al.* 2014) resulting in a lower yield for lower carbon source concentrations. On the other hand, the possible impurities in this fraction seem to be not above an inhibitory limit. Even the main impurity, furfural which is present in the fermentation medium with 0.45 g/L (0.045%), the inhibiting concentration of 0.7% was not reached (Dörsam *et al.* 2016).

The scale-up of the beechwood fiber fraction led to a similar malic acid concentration curve. Maximal volumetric production rate is with 0.78 g/(L*h) higher than in the shake flask experiments, but this value is only achieved in the very beginning of the fermentation process, and decreased after 40 min of fermentation. Carbon source consumption is significantly slower and a higher concentration of sugars is left at the end of the process. This resulted in a higher yield in the bioreactor approach and could be triggered by providing the optimal conditions, in case of oxygen supply and homogenous mixing of the cultivation broth. In all cultivations with beechwood cellulose fraction, an increase of carbon source from the beginning of the cultivation to first sample after 24 h, with a subsequent decrease. Most possible reason is the cleavage of oligosaccharides, because only mono- and disaccharides were measured. The scale-up of the *F. sylvatica* hemicellulose fraction differ even more from the shake flask cultivation. Less than the half amount of malic acid was produced in the bioreactor process. Conceivable is a reaction of impurities to more toxic compounds because of the better oxygen input. Compared to shake flask cultivation, greater amounts of foam was produced during fermentation and must be treated with antifoam. Further a negative impact of the bioreactor conditions for the oligoxylose digestion is possible, but not yet described and because of the xylose detection problems in this fraction not provable.

For fermentation of lignocellulose fractions with *R. delemar*, no pure sugar conversion experiments are necessary. As a well-known fumaric acid producer, a lot of studies were done with several alternative carbon sources, including different waste products and hydrolysates from lignocellulose (Maas *et al.* 2006; Millati *et al.* 2005;Bai *et al.* 2008; Zhang *et al.* 2007; Thongchul *et al.* 2010; Huang *et al.* 2005). *R. delemar* is able to convert xylose, and is also described as xylanolytic (Bakir *et al.* 2001). As this fungi is more sensitive towards inhibiting compounds than *A. oryzae* (Dörsam *et al.* 2016) lacking fumaric acid production with *F. sylvatica* hemicellulose fraction is a consequence of the possibly higher amounts of impurities. The beechwood cellulose hydrolysate fits well as carbon source for fumaric acid production. The achieved product concentration of *R. delemar* DSM 905 was even slightly higher with the hydrolysate than with refined glucose (13.1 ± 1.6 g/L, 0.26 g/g, 20%). As shown in further studies of our group, small amounts of phenols can support the organic acid production, which could be the reason for this (Dörsam *et al.* 2016).

5.5 Conclusion

In this study it was shown, that the range of convertible sugars for *A. oryzae* is even higher than known before. Beside glucose, fructose and maltose could be pointed out as a promising carbon source derived from first generation renewable resources. Regarding to the "food or fuel"-debate a biotechnological malic acid production based on alternative carbon sources not competing with food or feed production would be desirable. Lignocellulose, as an abundant renewable resource of the second generation, is easily available but its microbial accessibility is a challenge. The anhydrosugar levoglucosan, derived from cellulose during flash pyrolysis, could be figured out as a suitable carbon source. The suitability of fiber/cellulose hydrolysate of the plants *F. sylvatica* (beechwood) and *M. giganteus* obtained from the organosolv process as carbon source for *A. oryzae* in shake flasks as well as in a small-scale bioreactor was approved. Additionally, the more challenging hemicellulose fraction of *F. sylvatica* was also positively evaluated for malic acid production with *A. oryzae*. Both fractions of beechwood were also tested as carbon source for the fumaric acid producer *R. delemar*. Hemicellulose fraction of *F. sylvatica* was only suitable for *A. oryzae*.

5.6 References Chapter 5

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6 Summary & Conclusions

The evaluation of carbon sources for the fermentative organic acid production with fungi was successfully done for fractions from two different pretreatment methods of lignocellulose. During the flash pyrolysis of wheat straw, an organic and an aqueous condensate is formed, which can be further gasified to synthesis gas. The separation and subsequent enzymatic saccharification of cellulose and hemicellulose during the organosolv process of beechwood and *M. giganteus* biomass is leading to fractions mostly containing glucose or xylose, respectively.

During the evaluation of pyrolysis oil as carbon source, it was observed that *A. oryzae* and *R. delemar* tend to be more tolerant towards toxic compounds during the acid production phase than during the active growth phase. One compound was identified to be a main reason for the low tolerance level of pyrolysis oil: 2-cyclopenten-1-one, which is present in a concentration of 0.308%. However, even by testing single substances, the results give an idea of the complex nature of pyrolysis oil with many possible and different inhibition mechanisms of its compounds. Highly hydrophobic compounds are not present in the aqueous condensate. Furthermore 2-cyclopenten-1-one, which was discovered as toxic in chapter 2 is with 0.262% still present in a concentration which is problematic (0.308% in pyrolysis oil). The idea of the pretreatment of the aqueous condensate was to reduce the amount of solvents and organic substances as much as possible, so the most challenging substances are the one with similar physicochemical properties as water, regarding boiling point and hydrophilicity. None of the tested detoxification methods led to a usable fermentation substrate.

The possibility of the production of L-malate from syngas was successfully shown using only wild type strains of *C. ljungdahlii* and *A. oryzae* were used in a sequential coupled process. The advantage of this kind of biotechnological process is the extension of the product portfolio of anaerobic syngas fermentation. Because of the toxicity of oxygen to *Clostridia*, no further step is necessary than changing the sparging from syngas to air. Further, in this thesis it was shown, that the range of convertible sugars for *A. oryzae* is even higher than known before. The anhydrosugar levoglucosan, derived from cellulose during flash pyrolysis, could be figured out as a suitable carbon source. The suitability of fiber/cellulose hydrolysate of the plants *F. sylvatica* (beechwood) and *M. giganteus* obtained from the organosolv process as carbon source for *A. oryzae* in shake flasks as well as in a small-scale bioreactor was approved. Additionally, the more challenging hemicellulose fraction of *F. sylvatica* was also positively evaluated for malic acid production with *A. oryzae*. Hemicellulose fraction of *F. sylvatica* was only suitable for *A. oryzae*.

From a bioeconomical perspective, the different pretreatment methods have different advantages and disadvantages for biotechnological application. Although pyrolysis oil and the aqueous condensate used in this thesis were negatively evaluated as carbon sources for organic acid production, the process itself is promising. As shown, the pyrolytic sugar levoglucosan can be converted by *A. oryzae* to malic acid. Having the toxicity experiments in mind, tailor made pyrolysis oils with a higher sugar content, that can be further fractioned seem to be a possible sustainable carbon source. Beside a sugary fraction, used for fermentation, aromatic compounds derived from lignin decomposition can also be a valuable resource for the chemical industry. As the pyrolysis at the KIT is a self-propelled process with nearly no energy loss, using wheat straw as feedstock which is an agricultural by product, the sustainability is highly given. The high amount of different compounds in pyrolysis products gives the disadvantage of costly purification processes which lead to a loss of feedstock material as impurities in the different fractions. The further gasification to synthesis gas solves this problem by giving an almost completely conversion of the feedstock to usable gas. This process is energy consuming, the range of organisms able to convert syngas to value added substances is limited and the main products are mostly small C_2-molecules. Nevertheless the sequential mixed culture, established in chapter 4, shows the possibilities given by the wide range of carbon sources for fungi and the promising usage of syngas as feedstock for fermentation.

The disadvantage of the organosolv process is the energy and resource consumption during the separation of the lignocellulose compartments and the subsequent saccharification. The big advantage is the almost complete material use of lignocellulosic biomass. The precipitated lignin is because of its complexity and the preservation of functional groups much more valuable for chemical applications than the phenolic compounds gained from pyrolysis. The almost complete conversion of the carbohydrate part to sugar, especially for the cellulose fraction makes this process to the most promising pretreatment method for lignocellulose for biotechnological conversion. As the cellulose fraction resulted in a glucose rich solution, probably metabolizeable for many organisms, the hemicellulose fraction led to a xylose and oligoxylose rich solution, still mixed with lignin derived compounds. This fraction is until now a side stream product of the process. Many fungi are naturally able to metabolize a range of hexoses and pentoses without any genetically modification. As shown in chapter 5 the ability of *A. oryzae* to use this fraction as carbon source points out the suitability for fungi, especially for the important organic acid producers of the genus *Aspergillus*.

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Appendix

Appendix 1: Composition of pyrolysis oil, classified by kind of substance and concentration.

CAS-No.	Compound	wt.%
Non-aromatic compounds		
64-19-7	Acetic acid	5.004
79-09-4	Propionic acid	1.302
107-92-6	Butyric acid	0.164
-----	poss. Propanoid acid, ethenyl ester	
107-21-1	Ethylene glycol	1.258
141-46-8	Hydroxyacetaldehyde	0.314
116-09-6	Hydroxypropanone (Hydroxyacetone)	4.631
110-13-4	2,5-Hexandione (Acetonylacetone)	0.039
78-93-3	2-Butanone	0.038
5077-67-8	1-Hydroxy-2-Butanone	0.844
431-03-8	2,3-Butandione (Diacetyl)	0.036
513-86-0	3-Hydroxy-2-Butanone (Acetoin)	0.119
592-20-1	1-Acetyloxy-Propan-2-one	0.2018
120-92-3	Cyclopentanone	0.095
930-30-3	2-Cyclopenten-1-one	0.308
1121-05-7	2,3-Dimethyl-2-Cyclopenten-1-one	0.222
1120-73-6	2-Methyl-2-Cyclopenten-1-one	0.117
2758-18-1	3-Methyl-2-Cyclopenten-1-one	0.229
566-26-99	3-Ethyl-2-Cyclopenten-1-one	0.056
10493-98-8	2-Hydroxy-2-Cyclopenten-1-one	0.032
80-71-7	2-Hydroxy-1-methyl-1-Cyclopenten-1-one	0.939
21835-01-8	3-Ethyl-2-hydroxy-2-Cyclopenten-1-one	0.300
930-68-7	2-Cyclohexen-1-one	0.024
	poss: 2-Butenone	0.013
	Isomer of 3-Methyl-2-Cyclopenten-1-one	0.019
	Isomer of 3,4-Dimethyl-Cyclopentenone	0.034
	Dimethyl-Cyclopenten-one	0.026
	poss: 1-Acetyloxy-Butan-2-one	0.027
	Isomere of 2,3-Dimethyl-2-Cyclopenten-1-one	0.062

	Derivative of Dimethyl-Cyclopentenone	0.060
	2,3,4-Trimethyl-2-Cyclopenten-1-one	0.068
	Isomer of 3-Ethyl-2-hydroxy-Cyclopenten-1-one	0.094
	poss: 2-Cyclohexene-1,1-dione	0.028
	poss: 2,4-dimethyl-1,3-Cyclopenten-1-one	0.049
	Trimethyl-2-Cyclopenten-1-one	0.029
	poss: Trimethyl-2-Cyclopenten-1-one	0.035
	poss: 2-hydroxy-3-propyl-2-c-Cyclopenten-1-one	0.036
	2-Heptadecanone	0.108
	poss: 2-Pentadecanone or Isomere	0.029
Heterocyclic Compounds		
98-00-0	2-Furfuryl alcohol	0.124
2082-571-2	2(3H)-Furanone	0.065
497-23-4	2(5H)-Furanone	0.176
98-01-1	2-Furaldehyde	0.265
498-60-2	3-Furaldehyde	0.026
620-02-0	5-Methyl-2-Furaldehyd	0.032
1192-62-7	1-(2-Furanyl)-Ethanone	0.041
22122-36-7	(5H)-3-Methyl-Furan-2-one	0.094
	2,5-Dehydro-3,5-Dimethyl-Furan-2-one	0.127
96-48-0	γ-Butyrolactone	0.335
	poss: 5-Methyl-2(5H)-Furanone	0.042
	4-Methyl-(5H)-Furan-2-one	0.081
	poss: Isomere of 2,5-Dihydro-3,5-dimethyl-Furan-2-one	0.044
	Lactone derivative	0.037
Aromatic Compounds		
4265-25-2	2-Methyl-Benzofuran	0.016
83-33-0	2,3-Dihydro-1H-Inden-1-one	0.44
	Benzene	0.005
	2H-1-3,4-Dihydro-6-hydroxy-Benopyran-2-one	0.012
	poss: 3-Hydroxy-Benzaldehyde	0.048
98-86-2	Acetophenone	0.017
108-95-2	Phenol	0.384
95-48-7	o-Cresol	0.151
106-44-5	p-Cresol	0.134
108-39-4	m-Cresol	0.170

95-87-4	2,5-Dimethyl-Phenol	0.068
105-67-9	2,4-Dimethyl-Phenol	0.053
576-26-1	2,6-Dimethyl-Phenol	0.047
526-75-0	2,3-Dimethyl-Phenol	0.03
108-68-9	3,5-Dimethyl-Phenol	0.03
527-60-6	2,4,6-Timethyl-Phenol	0.012
90-00-6	2-Ethyl-Phenol	0.065
620-17-7	3-Ethyl-Phenol	0.067
123-07-9	4-Ethyl-Phenol	0.171
	4-Vinyl-Phenol	0.308
401-92-8	Trans-4-propenyl-Phenol	0.061
	Derivative of 2,3,4- or 2,4,5-Trimethyl-Phenol	0.034
	Ethyl-Methyl-Phenol	0.040
	Trimethyl-Phenol	0.030
90-05-1	Guaiacol	0.469
93-51-6	4-Methyl-Guaiacol	0.150
2785-89-9	4-Ethyl-Guaiacol	0.191
7786-61-0	4-Vinyl-Guaiacol	0.412
97-53-0	4-Allyl-Guaiacol (Eugenol)	0.085
2785-87-7	4-Propyl-Guaiacol	0.039
97-54-1	Cis-4-Propenyl-Guaiacol (Isoeugenol)	0.144
5932-68-3	Trans-4-Propenyl-Guaiacol (Isoeugenol)	0.524
121-33-5	Vanillin	0.200
498-02-2	4-Hydroxy-3-methoxy-Phenylethanone (Acetoguaja-cone)	0.099
2503-46-0	Guaiacylacetone	0.094
458-36-6	Coniferylaldehyde	0.023
91-10-1	Syringol	0.556
	4-Methyl-Syringol	0.162
	4-Ethyl-Syringol	0.094
	4-Vinyl-Syringol	0.218
6627-88-9	4-Allyl-Syringol	0.079
	4-Propyl-Syringol	0.048
627-88-9	Cis-4-(1-propenyl)-Syringol	0.077
	Trans-4-(1-propenyl)-Syringol	0.299
134-96-3	Syringaldehyde	0.177

2478-38-8	Acetosyringone	0.095
	Propiosyringone	0.059
	Syringylacetone	0.042
Carbohydrates		
7732-18-5	1,5-Anhydro-β-D-arabinofuranose	0.238
498-07-7	1,6-Anhydro-β-D-glucopyranose (Levoglucosan)	0.965
	1,4:3,6-Dianhydro-α-D-glucopyranose	0.431
	1,2-Ethanediol-monoacetate	0.048
	unknown compunds	0.251

Appendix 2: Composition of the aqueous condensate, classified by kind of substance and concentration

CAS-No.	Compound	wt. %
Non-aromatic Compounds		
64-19-7	Acetic acid	4.492
79-09-4	Propionic acid	0.404
	Pentanoic acid	0.055
	Hexanoic acid	0.015
	2-Butenoic acid	0.024
	Methanol	1.689
107-21-1	Ethylene glycol	0.437
	poss: 2-Methyl-2-Butenal	0.019
123-73-9	*trans*-Crotonaldehyde	0.142
116-09-6	Hydroxypropanone (Hydroxyacetone)	3.484
5077-67-8	1-Hydroxy-2-Butanone	0.490
431-03-8	2,3-Butandione (Diacetyl)	0.430
513-86-0	3-Hydroxy-2-Butanone (Acetoin)	0.095
592-20-1	1-Acetyloxy-Propan-2-one	0.125
	3-Methyl-3-Buten-2-one	0.026
	Poss: 3-Hexene-2-one	0.054
	3-Penten-2-one	0.152
	4-Hexene-3-one	0.009
	Poss: 3-Hexen-2-one	0.140
	1-Hydroxy-3-methyl-2-Butanone	0.023
120-92-3	Cyclopentanone	0.075
930-30-3	2-Cyclopenten-1-one	0.262
1121-05-7	2,3-Dimethyl-2-Cyclopenten-1-one	0.045
1120-73-6	2-Methyl-2-Cyclopenten-1-one	0.125
2758-18-1	3-Methyl-2-Cyclopenten-1-one	0.058
80-71-7	2-Hydroxy-1-methyl-1-Cyclopenten-3-one	0.093
21835-01-8	3-Ethyl-2-hydroxy-2-Cyclopenten-1-one	0.021
	2-methyl-Cyclopentanone	0.019
	3-methyl-Cyclopentanone	0.010
	Isomere of 3-methyl-Cyclopententen-1-one	0.020
	poss: 3,4-Dimethyl-2-Cyclopenten-1-one	0.021
	3,4-Dimethyl-2-Cyclopenten-1-one	0.020

	2,3,4-Trimethyl-2-Cyclopenten-1-one	0.010
	2-Cyclohexen-1,4-dione	0.008
	2-Hydroxy-3,4-dimethyl-2-Cyclopenten-1-on	0.023
Heterocyclic Compounds		
98-00-0	2-Furfuryl alcohol	0.020
497-23-4	2(5H)-Furanone	0.027
98-01-1	2-Furaldehyde	0.281
498-60-2	3-Furaldehyde	0.057
620-02-0	5-Methyl-2-Furaldehyd	0.016
1192-62-7	1-(2-Furanyl)-Ethanone	0.043
22122-36-7	(5H)-3-Methyl-Furan-2-one	0.024
96-48-0	γ-Butyrolactone	0.115
Aromatic Compounds		
98-86-2	Acetophenone	0.010
108-95-2	Phenol	0.041
95-48-7	o-Cresol	0.031
106-44-5	p-Cresol	0.015
108-39-4	m-Cresol	0.012
576-26-1	2,6-Dimethyl-Phenol	0.005
123-07-9	4-Ethyl-Phenol	0.008
90-05-1	Guaiacol	0.104
	3-Methyl-Guaiacol	0.005
93-51-6	4-Methyl-Guaiacol	0.150
2785-89-9	4-Ethyl-Guaiacol	0.009
7786-61-0	4-Vinyl-Guaiacol	0.003
91-10-1	Syringol	0.011
Other Organic Compounds		
542-59-6	Acetic acid 2-hydroxyethyl ester	0.128
110-86-1	Pyridine	0.020
	Acetonitrile	0.039
	2-Methyl-Pyridine	0.010
	unknown compunds	0.310