Municipal Green Waste as Basis to Produce Bio-Based Platform Chemicals

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Abstract

Considering the depletion of fossil fuels, the ongoing climate crisis, and geopolitical developments, it is evident that the current fossil-based industry is not sustainable. A key strategy for addressing these challenges is the decarbonization of industry, which involves transitioning to clean energy sources and alternative carbon feedstocks to reduce CO_2 emissions. Central to this transformation is the shift from a linear economy to a circular bioeconomy. In its context, materials previously considered waste are redefined as valuable resources. Given its composition and abundance, lignocellulosic biomass represents a promising alternative feedstock.

Municipal green waste encompasses all lignocellulosic biomass generated in urban areas. It offers the advantage of not competing with agricultural land use and currently lacks economically viable applications, making it an attractive feedstock for biotechnological processes. This work introduces the concept of municipal green waste and evaluates its potential. The necessity of pretreating lignocellulosic biomass is analyzed. It is demonstrated that both the press juice from herbaceous biomass and the enzymatic hydrolysate of hydrothermally pretreated biomass with a higher lignin content can serve as a fermentation medium or medium supplement for a range of microorganisms. *Saccharomyces cerevisiae*, *Lactobacillus delbrueckii* subsp. *lactis*, *Clostridium acetobutylicum*, and *Ustilago maydis* were successfully cultivated on media derived from municipal green waste to produce solvents and organic acids. Since pretreatment is often the most cost-intensive step, press juice from herbaceous biomass is identified as the most promising substrate due to the low pretreatment requirements and associated cost benefits. To enable industrial-scale implementation, a comprehensive understanding and optimization of the microbial cultivation processes are essential. Therefore, a protocol for a robust cultivation process of *C. acetobutylicum* was developed. Furthermore, the impact of oxygen supply – a critical parameter for scaling up – was investigated during the cultivation of *U. maydis*. Modulating the oxygen regime may provide a cost-effective tool to enhance productivity in this organism.

The present work demonstrates the viability of municipal green waste as a feedstock to produce platform chemicals and therefore contributes to developing a new carbon source for the chemical industry. In doing so, it supports both the decarbonization of industrial processes and the transition toward a more circular bioeconomy.

Kurzzusammenfassung

In Anbetracht endlicher fossiler Brennstoffe, der anhaltenden Klimakrise und geopolitischer Entwicklungen ist es offensichtlich, dass die derzeitige auf fossilen Brennstoffen basierende Industrie nicht nachhaltig ist. Eine Schlüsselstrategie zur Bewältigung dieser Herausforderungen ist die Dekarbonisierung der Industrie, das heißt der Übergang zu sauberen Energiequellen und alternativen Kohlenstoffrohstoffen, um die CO_2-Emissionen zu verringern. Von zentraler Bedeutung für diesen Wandel ist die Umstellung von einer linearen Wirtschaft auf eine zirkuläre Bioökonomie. In diesem Kontext werden Materialien, die bisher als Abfall galten, als wertvolle Ressourcen neu definiert. Lignozellulosehaltige Biomasse ist aufgrund ihrer Zusammensetzung und ihrer Häufigkeit ein vielversprechender alternativer Rohstoff.

Kommunaler Grünschnitt umfasst alle lignozellulosehaltige Biomasse, die in städtischen Gebieten anfällt. Er hat den Vorteil, dass er nicht mit landwirtschaftlicher Flächennutzung konkurrieren und derzeit nicht wirtschaftlich genutzt wird, was ihn zu einem attraktiven Ausgangsmaterial für biotechnologische Prozesse macht. In dieser Arbeit wird das Konzept des kommunalen Grünschnitts vorgestellt und sein Potenzial bewertet. Die Notwendigkeit der Vorbehandlung von lignozellulosehaltiger Biomasse wird analysiert. Es wird gezeigt, dass sowohl der Presssaft aus grasartiger Biomasse als auch das enzymatische Hydrolysat aus hydrothermisch vorbehandelter Biomasse mit höherem Ligningehalt als Fermentationsmedium oder Mediumszusatz für eine Reihe von Mikroorganismen dienen kann. *Saccharomyces cerevisiae*, *Lactobacillus delbrueckii* subsp. *lactis*, *Clostridium acetobutylicum* und *Ustilago maydis* wurden erfolgreich auf Medien kultiviert, die aus kommunalem Grünschnitt gewonnen wurden, um Lösungsmittel und organische Säuren zu produzieren. Da die Vorbehandlung häufig der kostenintensivste Schritt ist, wurde Presssaft aus grasartiger Biomasse aufgrund der geringen Vorbehandlungsanforderungen und der damit verbundenen Kostenvorteile als das vielversprechendste Substrat identifiziert. Um eine Umsetzung im industriellen Maßstab zu ermöglichen, sind ein umfassendes Verständnis und eine Optimierung der mikrobiellen Kultivierungsprozesse unerlässlich. Daher wurde ein Protokoll für den robusten Kultivierungsprozess von *C. acetobutylicum* entwickelt. Darüber hinaus wurde der Einfluss der Sauerstoffzufuhr – ein kritischer Parameter für das Scale-up – während der Kultivierung von *U. maydis* untersucht. Die Modulation des Sauerstoffregimes könnte ein kosteneffizientes Instrument zur Steigerung der Produktivität dieses Organismus darstellen. Die vorliegende Arbeit zeigt, dass kommunale Grünabfälle als Ausgangsmaterial für die Herstellung von Plattformchemikalien geeignet sind, und trägt somit zur Entwicklung einer neuen Kohlenstoffquelle für die chemische Industrie bei. Auf diese Weise unterstützt sie sowohl die Dekarbonisierung industrieller Prozesse als auch den Übergang zu einer stärker kreislauforientierten Bioökonomie.

List of Abbreviations, units, and symbols

5-HMF	5-hydroxymethylfurfural
ABE	acetone, butanol, ethanol
GHG	greenhouse gas
Gt	gigaton
HTC	hydrothermal carbonization
IEA	International Energy Agency
IINAS	Internationales Institut für Nachhaltigkeitsanalysen und -strategien, International Institute for Sustainability Analysis and Strategies
k_La	volumetric mass transfer coefficient
K_M	Michaelis Menten constant
Mt	megaton
OD_{600}	optical density at 600 nm
rpm	revolutions per minute
t	ton
TCA cycle	tricarboxylic acid cycle
v_{max}	maximum velocity achieved at the maximum substrate concentration

List of Publications

This list contains peer-reviewed publications that are part of this thesis. Further publications not within the scope of this thesis can be found on page 166.

Alexander Langsdorf*, Marianne Volkmar*, Dirk Holtmann, Roland Ulber; Material utilization of green waste: a review on potential valorization methods; Bioresources and Bioprocessing (2021) 8:19; https://doi.org/10.1186/s40643-021-00367-5

Ludovica Varriale*, Marianne Volkmar*, Jens Weiermüller, Roland Ulber; Effects of Pretreatment on the Biocatalysis of Renewable Resources; Chemie Ingenieur Technik (2022), 94(11), 1818-1826; https://doi.org/10.1002/cite.202200137

Katharina Oehlenschläger*, Marianne Volkmar*, Judith Stiefelmaier, Alexander Langsdorf, Dirk Holtmann, Nils Tippkötter, Roland Ulber; New insights into the influence of the pre-culture on the solvent production of *C. acetobutylicum*; Applied Microbiology and Biotechnology (2024) 108:143; https://doi.org/10.1007/s00253-023-12981-8

Marianne Volkmar, Anna-Lena Maus, Martin Weisbrodt, Jonathan Bohlender, Alexander Langsdorf, Dirk Holtmann, Roland Ulber; Municipal green waste as carbon source for the fermentative production of platform chemicals**;** Bioresources and Bioprocessing (2023) 10:43; https://doi.org/10.1186/s40643-023-00663-2

Marianne Volkmar*, Wolfgang Laudensack*, Felix Bartzack, Niklas Erdmann, Sonja Schönrock, Emely Fuderer, Dirk Holtmann, Lars M. Blank, Roland Ulber; Low oxygen oxygen availability increases itaconate production by *Ustilago maydis*; Biotechnology and Bioengineering (2025) 122:3007–3017; https://doi.org/10.1002/bit.70035

*both authors contributed equally

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I. Introduction

I.1 Challenges of the Current Economy

The reality that the current, fossil-based industry has no future is not new information. Projections from 2017 expect the crude oil reserves to last for 80 years, and the reserves of gas for 85 years [1]. At the same time, the current world population of 8 billion is projected to reach 9.7 billion in 2050 [2], leading to an increasing demand for energy and resources in general. Already documented deposits could extend the range for another 80 years for crude oil and a few hundred years for gas [1], but these resources are not yet economically exploitable and only postpone the problem for a short time. Even more critical than the finite nature of fossil resources is the impact of their use. The harmful consequences of greenhouse gases (GHG) such as carbon dioxide, methane, and nitrogen oxides produced during the combustion of fossil fuels are well documented [3]. Their increasing amount accounts for the anthropogenic climate change, the negative impact of which is undisputed among experts [4]. On top of that, the political developments of the last few years have painfully demonstrated the consequences of the dependency on fossil resources. The Russian invasion of Ukraine led to a rise in oil and gas prices, which hit German industry hard [5–7]. Rising nationalistic tendencies in elections in Europe and worldwide with discussions about tariffs and trade restrictions also emphasize the need to reduce resource dependencies and establish independent resource supplies [8]. A shift away from fossil fuels can also be helpful in this respect. Many EU states have already adapted their renewable energy policies as a reaction to the Russian invasion of Ukraine [9].

I.1.1 Circular versus Linear Economy

The challenges mentioned in the previous chapter underline the necessity of a profound change in the economic system. It used to be dominated by linear thinking, where resources are extracted, used in products, and then discarded. This can be summarized succinctly in the phrase “take, make, waste”. Even just from the perspective of finite resources it is obvious that this form of industry is not sustainable. As a contrast to "linear economy", the term "circular economy" appears increasingly in the scientific literature from 2015 onwards [10]. However, there was no unanimous definition of the concept. This might lead to uncertainties and also to the term being misused for greenwashing by assuming that biomass-based processes are environmentally superior to petrochemical processes. Nobre et al. sharpened the concept by finding a definition and separating it from the basic principles and their applications. According to Nobre et al., “Circular Economy is an economic system that targets zero waste and pollution throughout materials lifecycles, from environment extraction to industrial transformation, and to final consumers, applying to all involved ecosystems. Upon its lifetime end, materials return to either an industrial process or, in case of a treated organic residual, safely back to the environment as in a natural regenerating cycle. It operates creating value at the macro, meso and

micro levels and exploits to the fullest the sustainability nested concept. Used energy sources are clean and renewable. Resources use and consumption are efficient. Government agencies and responsible consumers play an active role ensuring correct system long-term operation.” [10]

The Ellen MacArthur Foundation, a charity collaborating with industry, academia, and governments, has been working since 2010 on the promotion of the concept of a circular economy. According to them, a successful stock management of existing resources can be summarized by the keywords “reduce, reuse, recycle”. Wherever a reduction of resource use cannot be realized, the useful life should be extended, for example also through shared use. In case this is no longer possible, all material should be recycled. For the management of renewables, cascade use of biomass is envisaged. Organic or biochemical components are extracted from feedstocks occurring in between other steps in the lifetime circle. An important factor is the simultaneous regeneration of the biosphere, for example by recirculating nutrients. The key notion is that nothing is ever considered to be waste, but always the starting material for something new [11].

The model from the Ellen MacArthur Foundation is idealized. Practically speaking, not all materials can be recycled indefinitely, and losses occur during the transformation processes. Additionally, they require energy, which needs to come exclusively from sustainable sources to ensure that there is no further pollution. Although it is unrealistic to completely fulfill the model in the medium term, it is a suitable guideline for inventions for the transformation of the industry.

The need to move away from a linear economy has long since reached politics. In 2012, the EU published its first "European bioeconomy strategy", which was updated in 2018. Same as for the term “circular economy”, there is no unanimous definition of “bioeconomy”. The EU defines it as “all sectors and systems that rely on biological resources (animals, plants, micro-organisms and derived biomass, organic waste), their functions and principles” [12]. It links terrestrial and maritime ecosystems and their services provided with all primary production sectors and with economic and industrial sectors using these resources. Therefore, bioeconomy includes agriculture, aquaculture, and forestry as well as the energy industry and the consumer [13]. Since then, many bioeconomy strategy papers on different organizational levels followed, for example, the "Nationale Bioökonomiestrategie” in Germany from 2020 to combine ecology and economy [14].

I.1.2 Alternative Carbon Sources

The term bioeconomy is strongly connected to decarbonization, which describes the process of lowering the amount of CO_2 released per unit of activity. Possible adaptations are switching to clean, renewable energy sources as well as improving both energy and process efficiency across all industrial operations [15,16]. These modifications are equally applicable to all branches of industries, although this is especially challenging for sectors with a generally high energy requirement such as the chemical

industry, which depends heavily on thermal processes. Therefore, establishing methods for the decarbonization of heating systems has a high priority [16]. However, the decarbonization of the chemical industry poses an additional challenge, since carbon is an essential structural element of organic chemicals and materials, and many chemical products inherently require carbon as a core building block [17]. Consequently, it is necessary to identify and develop alternative carbon sources beside fossil fuels and to establish efficient pathways to make them accessible for a sustainable industry [18].

One approach involves the utilization of CO_2 as a resource. For example, through the intermediate methylammonium, the nitrogen-based fertilizer urea can be synthesized from ammonia and CO_2 [19]. Furthermore, catalytic hydrogenation of CO_2 offers a route to produce so-called green organic fuels, such as methane or methanol, which serve as renewable energy carriers and chemical precursors [20]. In addition to chemical conversion, biological utilization of CO_2 is also possible, for example via direct fixation by plants in greenhouses or by microalgae to produce biofuels and other valuable compounds [21]. Besides the obvious benefit of serving as an alternative feedstock, the utilization of CO_2 could also contribute to mitigating climate change by reducing the amount of atmospheric CO_2 through carbon capture and utilization processes. However, these procedures are far from being technologically mature and established yet, and significant research and innovation efforts are required to reach industrial-scale deployment [22,23].

Other possible alternatives to fossil fuels are substrates such as renewable biomass and organic waste. This refers to any organic, biodegradable material that originates from biological systems and can be broken down by microbial activity. Examples include dedicated energy crops, food waste or agricultural residues, animal manure, as well as paper and cardboard. The major difficulty in using such alternative instead of fossil carbon sources lies in the accessibility and chemical availability of carbon in these complex matrices. Therefore, the main technological challenge remains to develop efficient conversion and processing methods that make the carbon content of these materials usable for industrial applications.

I.2 Lignocellulosic Biomass

The central alternative resource is currently lignocellulosic biomass. Forming roughly half of all biomasses, lignocellulose is the most abundant organic molecule on earth [24].

I.2.1 Structure of Lignocellulosic Biomass

Lignocellulosic biomass is the building block of plant cell walls and is depicted in Figure 1. The complex polymeric structure comprises the three main components lignin, cellulose, and hemicellulose. These

components are intricately linked through chemical bonds, making the fractionation and utilization of lignocellulosic biomass challenging.

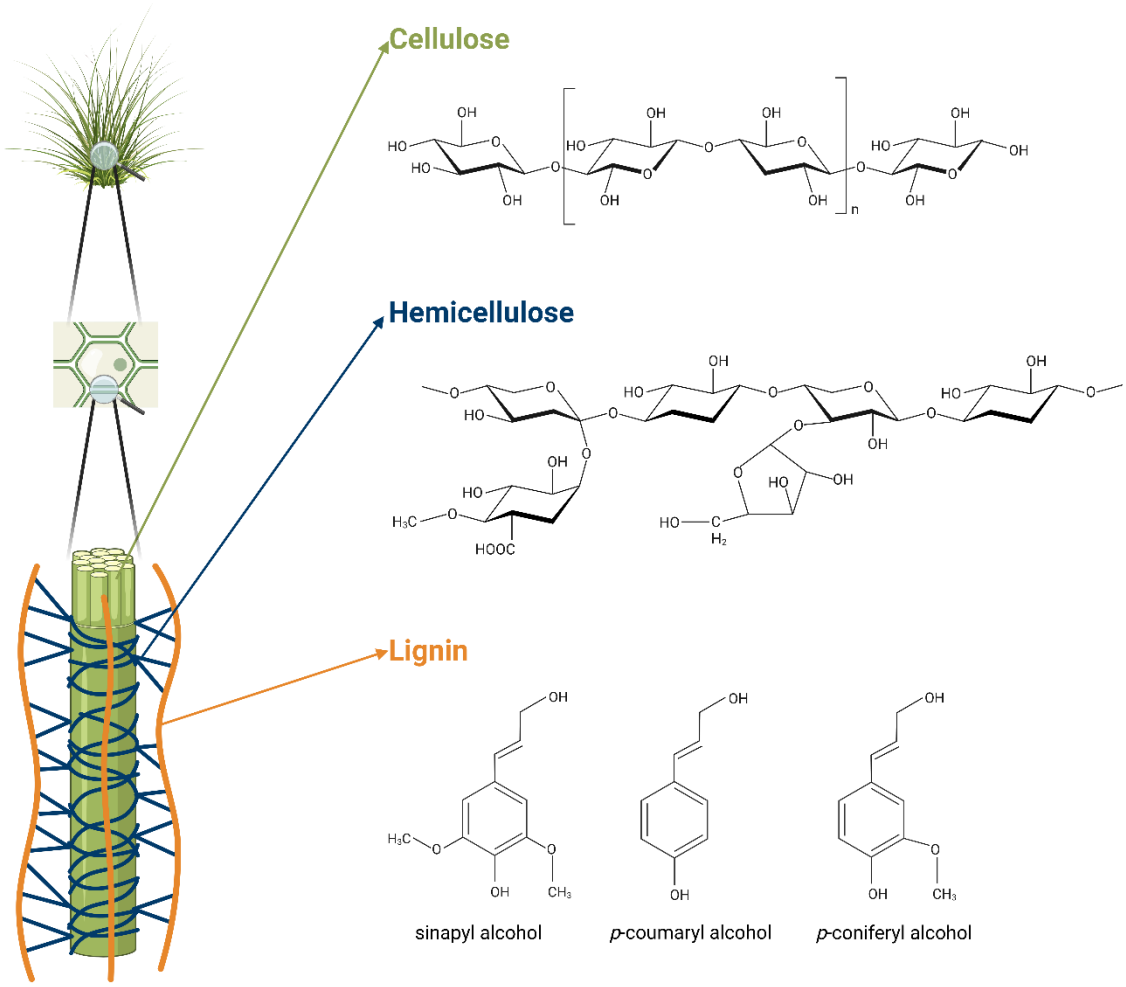

Figure 1: Composition of lignocellulosic biomass. Plant cell walls are mainly made of lignocellulose, consisting of cellulose, hemicellulose, and lignin. Cellulose fibrils (green) are formed by β-1,4-glycosidically linked β-D-glucose molecules. Hemicellulose (blue) consists of different pentoses and hexoses, and the main components of lignin (orange) are the phenols sinapyl alcohol, p-coumaryl alcohol, and p-coniferyl alcohol. Especially due to the branched structure of hemicellulose, the three components are strongly intertwined, making lignocellulose a recalcitrant material. Figure created in BioRender.com.

The heterophenolic polymer lignin consists of the phenols coniferylalcohol, coumarylalcohol, and sinapylalcohol. In biomass, lignin is responsible for maintaining the structural integrity of the cells, especially by providing compressive strength in the lower parts of the plants such as the stem, where mechanical stress is highest [25]. The structure and chemical diversity of lignin makes the molecule an interesting precursor for various phenolic compounds. Among the potential industrially relevant products derived from lignin are benzene, quinones, cyclohexane, and lignosulfonates, which are of interest to the chemical, pharmaceutical, and materials industry [26]. Additionally, lignin finds use in various material applications. It can serve as a binder, as a matrix component for composite materials,

as filler in thermoplastics and biocomposites, and as a base for producing hydrogels and nanostructured material such as nanofibers [25,27,28]. Han et al. summarize the possibilities of using lignin in biobased functional barrier materials with UV-blocking and even flame-retardant features [29]. Another possible application is the production of anodes for energy storage devices via hard carbon materials derived from lignin, which has gained attention in the context of sustainable battery technologies [30]. However, lignin is currently predominantly used for energy production in the form of combustion. The only product derived from lignin produced on an industrial scale so far is vanillin [31,32].

Cellulose is composed exclusively of β-1,4-glycosidically linked β-D-glucose units, forming highly ordered, linear crystalline fibrils. These fibrils are tightly packed and aligned, which is the reason for the mechanical anisotropy of lignocellulosic material [33]. Cellulose has a variety of applications, ranging from traditional uses in textiles and paper to modern applications in building and insulation material, as well as in the pharmaceutical industry where it serves as a filling agent. Fibrillated cellulose may be used as bioplastic alternative, and can also be employed as a porous membrane, for example for water filtration, or formulated into soft gels suitable for wound dressing applications [34]. Due to the highly ordered crystalline structure, cellulose can also be used for the targeted manipulation of light and is therefore a candidate for optical applications such as photonic crystals [35]. Another possible implementation is the use in electrochemical energy storage systems, for example as electrode material, current collectors, or separators for supercapacitors [36].

Hemicellulose, in contrast, is a heterogeneous polysaccharide formed from various pentoses and hexoses. Common monosaccharides include xylose, glucose, and mannose, but the exact composition depends on the species and is influenced by a range of factors such as the organism's developmental stage and environmental conditions like climate, soil nutrients, and geographical location [37]. The resulting heterogeneous structure leads to a highly branched molecule, which enables hemicellulose to act as a connecting agent within the plant cell wall matrix that links cellulose, lignin, pectin, and cell wall proteins, contributing to cell wall cohesion [25]. One notable application of hemicellulose lies in the chemical valorization of pentoses, especially xylose, which serves as a precursor for furfural. Furfural is an important platform molecule in the chemical industry, acting as key intermediate for the synthesis of organic acids such as maleic, succinic, levulinic and furoic acid, furfurylamine, furfural alcohol, aromatics, and cyclopentanone [38]. Furfural therefore serves either directly or via intermediates as a starting point for the production of solvents, resins, and biofuels [39]. Additionally, furans derived from hexoses, especially 5-hydroxymethylfurfural (5-HMF), are a versatile heteroaromatic building block. For example, dantrolene, a muscle relaxant, is based on 5-HMF. Furthermore, 2,5-furandicarboxylic acid, also derived from 5-HMF, could serve as a monomer for

polyethylene furanoate, a biobased plastic alternative, and is also being investigated as a building block for metal-organic frameworks, which could be used in gas capture and separation [40].

Last but not least, carbohydrates obtained both from cellulose and hemicellulose hydrolysis can serve as carbon source for a variety of microorganisms, which opens up the potential of a broad range of chemicals produced via biocatalysis [41]. This is also the main focus of this thesis.

Lignin, cellulose and hemicellulose combine to form an extremely robust unit that provides mechanical strength and rigidity to plant biomass. Individual cellulose strands are grouped in microfibrils, which are internally stabilized via hydrogen bonds. The high degree of order within these cellulose strands results in tightly packed crystalline regions which are resistant to both solvents and enzymatic digestion, posing a significant barrier to efficient biomass processing. The microfibrils are connected to the surrounding hemicellulose and lignin via hydrogen bonds [42,43]. In turn, hemicellulose and lignin form covalent bonds with one another, further reinforcing the cell wall's composite structure [25]. Subsequently, multiple microfibrils associate to form larger microfibril bundles [25]. This results in a very robust structure with a recalcitrant nature, which impedes both chemical and enzymatic degradation. To harness the valuable building blocks lignin, cellulose, and hemicellulose for industrial purposes, an effective pretreatment process is necessary to break up the lignocellulose structure and separate the three components. The initial step involves physical fragmentation into smaller particles to enlarge the surface, making the subsequent processes more efficient. Thermal processing steps are primarily employed to disrupt hydrogen bonds, resulting in the dissolution of the three main components. In contrast, chemical pretreatments such as acid or alkaline hydrolysis, aim to dissolve the crystalline cellulose structure [44]. All this enables saccharolytic enzymes to attack the cellulose and hemicellulose structures in a subsequent biological process step. The necessity of pretreatment and the practical implementation thereof are further discussed in the chapters II and III.

I.2.2 Availability of Lignocellulosic Biomass

The global net primary production of biomass is 104.9 petagrams of carbon per year [45]. This includes all organic matter that is produced by primary producers, usually plants, from atmospheric or aquatic CO_2, minus the carbon that is necessary to maintain their metabolism. 54 % of the production is terrestrial, while 46 % is aquatic. Although almost half of the biomass is produced in oceans, only 0.2 % of the available material is found there [45,46] as the algae immediately enter the food chain. Onshore, the majority of biomass is produced in temperate forests. Of the almost 105 billion tons of carbon that are incorporated in biomass every year, a big share is needed to maintain the respective biospheres. Only 11 t arise on cultivated lands, most of which are food crops.

Data from 2024 show that 24,790 km^2 of acreage is used to grow renewable resources in Germany. While 2,790 km^2 is accounted for by industrial plants, for example, to obtain starch, sugar, oil, or fibers, the rest of 22,000 km^2 is occupied by energy crops to produce biogas, biodiesel or bioethanol [47]. The international institute for sustainability analysis and strategies IINAS (Internationales Institut für Nachhaltigkeitsanalysen und -strategien) states in their final report “sustainable bioeconomy” for the nature conservation association NABU, that 86 million tons of biomass, excluding food or feed, could be produced annually in Germany in a sustainable way [13]. Currently, 150 million tons are produced, significantly more that can be sustained long-term [48]. This underlines the fact that arable land, too, is a finite resource.

The ”food or fuel" problem describes the question of whether farmland should be used to grow food crops or biomass as a resource, for example, to produce fuel. According to Thompson et al., publications concerning the food or fuel problem either emphasize the high impact on food prices assuming an inelastic feedstock supply, or focus on the impact on GHG emissions due to increased land use when assuming an elastic feedstock supply [49]. This publication bias aside, both conclusions point to the legitimate considerations concerning land use. Following the principles of a circular bioeconomy, only biomass that serves no other important purpose should be considered as feedstock. Prime examples therefore are agricultural, forestry, or food wastes. This way, conflicts with food production as well as with increased land use can be avoided.

Agricultural waste includes all non-edible parts of cultivated plants such as cereal straw or leaves of root vegetables. Forestry waste describes residues from forest management and wood production, and food waste comprises all residues occurring during food preparation, including rotten material and indigestible parts like peels or pits. Bedoić et al. gathered the ratios of usable parts and waste material for the fruit, cereal, vegetable, and animal sector during cultivation, harvest, processing or slaughtering, and consumption [50]. For Germany, Leverez et al. determined an annual amount of food waste of 11.9±2.4 million tons, of which around one million tons is attributable to agricultural waste. Improving for example supply chain management could significantly reduce the amount of food waste. But according to Leverez et al., at least 14 % of food waste is unavoidable as it consists of inherently indigestible parts [51].

Another lignocellulosic biomass resource with hitherto untapped potential is municipal green waste. There is no uniform definition of it in literature. As discussed in chapter II, it comprises of lignocellulosic material that occurs in urban areas such as private and public gardens and parks as well as roadside greenery. This includes hedge pruning and tree cuttings as well as leaves and grass cuttings, making municipal green waste a quite heterogeneous material stream. The vast variety of plants as well as their respective locations with different soil conditions lead to an inhomogeneous lignocellulose

feedstock with differing ratios of the three lignocellulose monomers. Due to seasonal changes in the fauna, the composition on the macroscopic level also changes throughout the year [52].

Medick et al. determined the technical biomass potential of Berlin to be 263.990 tons of fresh matter per year. The term technical potential considers all theoretically available biomass, without taking economic aspects such as the collecting process into account [53]. The actual amount collected in this region in the year of the study was 12.700 tons [54]. The gap between the two numbers is a strong indicator of the future challenges when municipal green waste as renewable feedstock. To harness the full potential of this biomass, collecting processes and logistic procedures need to be established. The federal environmental agency reports 5.2 million tons of biodegradable garden and park waste collected in 2022. Since 2010, this amount remained constant between 5.0 and 6.1 million tons [55]. This shows that the biomass currently collected is also considerable, and the trend underlines the reliability of the biomass as a feedstock.

Municipal green waste has two main advantages as a lignocellulosic feedstock. Firstly, the area where it grows does not compete with agricultural purposes. Secondly, most of the green waste is disposed of by city authorities. While it is partly composted or used to produce biogas [53,56], the associated costs currently exceed the profit [57,58]. Municipal green waste is therefore a promising potential lignocellulosic feedstock for further valorization in a circular bioeconomy.

I.2.3 Application of Lignocellulosic Biomass as Feedstock

Lignocellulosic biomass has already successfully been applied as renewable feedstock. The principle of a biorefinery is based on the sustainable and integrated processing of biomass into a spectrum of marketable products and energy [59]. The European Commission currently lists a total of 2362 biorefineries in Europe, of which 2185 operate on a commercial scale, indicating a well-established infrastructure for biomass conversion. In Germany, the predominant feedstock utilized in these facilities is derived from forestry, followed by agriculture, and subsequently by grass or short-rotice coppice. 35 % of German biorefineries are primarily focused on producing biomethane, while 19 % produce chemicals. Other products derived from lignocellulosic biomass include pulp and paper, timber, liquid biofuels such as bioethanol and biodiesel, as well as starch and sugar. Value-added materials such as composites and fibers are produced as well [60]. As mentioned above, the main technical challenge when using lignocellulosic biomass as a resource lies in making the carbon it contains accessible.

I.3 Aim of the Thesis

This thesis aims to establish municipal green waste as an alternative feedstock to produce chemicals and thereby contributes to developing a new carbon source for the chemical industry. In doing so, it

supports the decarbonization of industrial processes and the transition toward a more circular bioeconomy. Chapter II introduces the concept of municipal green waste and explores its potential in the context of sustainable biorefinery systems. It provides a detailed overview of the composition of herbaceous and other low-lignin biomass types commonly found in urban environments, analyzing these materials at both the macroscopic and the molecular level. Seasonal changes, botanical diversity, and differences in collection or harvesting sites strongly influence the composition of municipal green waste. This heterogeneity poses a major challenge for its consistent use as a biotechnological feedstock. To harness the potential of lignocellulosic biomass, pretreatment processes are essential. Chapter II presents the most commonly employed pretreatment methods, compares their efficacy, and introduces a standardized tool for evaluating and contrasting different approaches. Additionally, it highlights products that can be derived from municipal green waste with minimal processing. For example, pressing fresh or ensiled biomass yields a nutrient-rich press juice that can serve as a fermentation medium. Moreover, it is possible to recover valuable compounds such as proteins, lipids, acids, and lignin. Chapter II further discusses the bioconversion of municipal green waste into promising bio-based platform chemicals such as levulinic and succinic acid, furfural, HMF, and other value-added compounds. The concept of consolidated bioprocessing is introduced as innovative approach that integrates enzyme production, biomass hydrolysis, and product formation into a single step. Lastly, this chapter describes the carbonization of lignocellulosic biomass to produce biochar and even electrode materials for application in electro-biotechnological systems, thus demonstrating how green waste can be transformed into ecological functional materials.

Chapter III focuses on the pretreatment of different municipal green waste fractions for the purpose of enhancing biocatalytic sugar release. The necessity and intensity of pretreatment processes are shown to depend strongly on the lignin content of the biomass. In this context, grass clippings and a mixture of beech and pine wood chips are analyzed as representative examples of low- and high-lignin biomass, respectively. These substrates undergo various mechanical and hydrothermal pretreatments, and the effect on both composition and cellulose to glucose conversion during enzymatic hydrolysis are evaluated. A kinetic analysis of the biocatalytic process is performed to assess the rate and extent of sugar release over time.

The goal of the biomass pretreatment is to generate sugar monomers, which serve as a carbon source for fermentation-based production of relevant platform chemicals. Among the microbial candidates, clostridia species are particularly known for their capacity to produce important organic solvents. The bacterium *Clostridium acetobutylicum* has long been employed in the acetone-butanol-ethanol (ABE) fermentation process. Reliable solvent production, however, requires a stable and reproducible fermentation system. The cultivation of *C. acetobutylicum* is often challenging, partly due to a lack of

understanding of the metabolic processes such as the onset of the solvent production. Furthermore, literature often lacks detailed cultivation protocols, despite the well-established influence of pre-culture conditions on microbial performance. Chapter IV addresses this knowledge gap by investigating the impact of pre-culture parameters on solvent production by *C. acetobutylicum*, aiming to develop a more robust and reproducible cultivation protocol.

In chapter V, the previously gathered knowledge is integrated and applied. Different fractions of municipal green waste are pretreated to obtain fermentation media or media supplements. According to its moisture and lignin content, the biomass is either pressed or processed hydrothermally and enzymatically, resulting in a press juice or a hydrolysate. Both components are then successfully applied as source of nutrients for the cultivation of a variety of microorganisms. The successful use of these substrates for the fermentative production of ethanol, lactic acid, itaconic acid, and ABE solvents underlines the suitability of municipal green waste as feedstock for integrated biotechnological processes within a sustainable bioeconomy.

For the transfer of lab-scale fermentation results to industrial-scale applications, an effective scale up strategy for the processes is essential. Economically worthwhile processes are hardly possible without increasing the production volume. However, scale ups are intricate procedures, as the scale of a setup affects physical, chemical, and biological factors in cultivation systems. Chapter VI addresses the scale up of itaconic acid production using *Ustilago maydis*. Following initial screening experiments in a 0.4 L scale to determine optimal cultivation parameters, scale ups to a 30 L scale using different criteria are performed. Hereby, the influence of oxygen on productivity was investigated. Surprisingly, low oxygen availability correlates with increased itaconic acid yield. This observation applies to the experiments in the lab as well as on the technical scale. As oxygen limitation is likely to occur in industrial fermenters, this could be a new process control strategy to improve itaconic acid production.

I.4 References

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II. Material Utilization of Green Waste: A Review on Potential Valorization Methods

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Authorship contribution

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M. Volkmar	conceptualization, data curation, formal analysis, investigation, writing – original draft preparation, writing – review and editing
D. Holtmann	conceptualization, funding acquisition, project administration, resources, supervision, writing – review and editing
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Keywords: Green waste, Biomass pretreatment, Biomass conversion, Valorization, Carbonization

II.1 Abstract

Considering global developments like climate change and the depletion of fossil resources, the use of new and sustainable feedstocks such as lignocellulosic biomass becomes inevitable. Green waste comprises heterogeneous lignocellulosic biomass with low lignin content, which does not stem from agricultural processes or purposeful cultivation and therefore mainly arises in urban areas. So far, the majority of green waste is being composted or serves as feedstock for energy production. Here, the hitherto untapped potential of green waste for material utilization instead of conventional recycling is reviewed. Green waste is a promising starting material for the direct extraction of valuable compounds, the chemical and fermentative conversion into basic chemicals as well as the manufacturing of functional materials like electrodes for electro-biotechnological applications through carbonization. This review serves as a solid foundation for further work on the valorization of green waste.

II.2 Introduction

Especially in urban areas, various waste material streams occur, which are recycled economically unprofitable until now. Concerning the increasing interest in circular economy as well as bioeconomy, it is desirable that all waste is recycled as profitably and sustainably as possible. This is particularly important considering the intensified climate change as well as an increasing world population and urbanization. According to the updated bioeconomy strategy from the European Commission, EU cities should become a major hub for circular bioeconomy to exploit urban bio-waste (European Commission, 2018). Due to the depletion of fossil resources and pressing environmental issues, lignocellulosic biomass is increasing in attractiveness as a feedstock, not only for the energy sector but also for the chemical industry. In 2015, the manufacture of bio-based chemicals, excluding biofuels, recorded the highest value-added annual growth with +26 % (European Commission, 2018). One of the major biomass waste streams occurring in urban areas, which is not utilized economically worthwhile, is green waste. For example, in the Berlin (Germany) city districts, over 120,000 t a^{-1} of herbaceous fresh matter accumulate, comprising 72,000 t a^{-1} of leaves from yards and roads and 48,000 t a^{-1} of grass cuttings from lawns, roadside greenery, and biotope areas (Medick et al., 2017). With increasing urbanization, the number of urban green spaces increases. Subsequently, the amount of green waste is growing, affecting city development and the environment (Zhang et al., 2013).

Within the current literature, there is no uniform definition for the term green waste. Typically, green waste includes biodegradable garden waste and public park waste (Eades et al., 2020). Garden or park waste is generated by the maintenance of private gardens or public parks and can consist of organic materials like grass clippings, hedge cuttings, pruning, leaves, and wood as well as inorganic materials like stones and soil (Boldrin and Christensen, 2010). According to the European Waste Catalogue, this

garden and park waste also includes cemetery waste (German Federal Statistical Office, 2020). Green waste is also defined as the biodegradable fraction of municipal solid waste (MSW) (Zhang and Sun, 2016). Most of the time, the components of green waste are described as grass, leaves, and branch cuttings (Inghels et al., 2016; Zhang et al., 2013; Zhang and Sun, 2016, 2014). Leaves and grass cuttings, without woody materials, are also referred to as herbaceous green waste (Medick et al., 2017). Herein, green waste is defined as grass and leaves collected from public parks, private gardens as well as cuttings from roadside greenery including a small amount of branches or other woody materials, resulting in a mainly non-woody or low-lignin and herbaceous green material.

Nowadays, composting and the subsequent application as fertilizer in agriculture is the method of choice for the majority of current green waste management in and around the largest cities in Germany (e. g. Berlin) (Medick et al., 2017). Another uprising method is the production of biogas. According to the German Federal Statistical Office (German Federal Statistical Office, 2020), about 5.6 million tonnes of biodegradable garden and park waste were produced in 2018 within Germany. Almost the entire amount (5.5 million tonnes) was recycled. In 2017, there were 631 green waste composting plants for exclusively green waste and 297 fermentation plants (biogas plants including combined fermentation and composting plants) throughout Germany (German Federal Ministry for the Environment, Nature Conservation and Nuclear Safety, 2019). These plants produced 4.2 million tonnes of compost, almost 3.4 million tonnes of digestate, and 626 million m^3 of biogas in 2017 (German Federal Environment Agency, 2020). The fermentation residues of biogas plants also represent a valuable fertilizer and humus supplier for application in agriculture. A lot of lignocellulosic material around the world is also disposed of by burning. However, the burning of waste biomass releases particulate matter, polycyclic aromatic hydrocarbons, dioxins, and toxic air pollutants, which can affect air quality negatively especially in urban areas (Sivertsen, 2006). On top of that, its high amount of moisture, oxygen, and alkaline earth metals, as well as the production of flue gas emissions, make green waste unattractive for incineration (Shao et al., 2019). Besides the mentioned common methods, the production of ethanol is also an interesting and one of the most economically feasible methods for recycling of lignocellulosic biomass (Pérez et al., 2002). There is a large number of publications on bioethanol production from grass and even more from biomass in general. Due to the structural features of lignin, plants with low lignin and high cellulose/hemicellulose content are increasingly interesting for biofuel production. For example, Mohapatra et al. reviewed different methods of bioethanol production from herbaceous biomasses like *Miscanthus*, switchgrass, Napier grass, or Coastal Bermuda grass (Mohapatra et al., 2017). Nevertheless, composting is generally the method of choice for green waste utilization based on economic and environmental aspects (Zhang et al., 2013; Zhang and Sun, 2014). Altogether the recycling of green waste still costs considerably more than thereby is generated.

Nowadays, there are plenty of publications regarding the utilization of woody biomass with low moisture content and/or high lignin content as well as agricultural residues for the generation of higher value, while the utilization of herbaceous material has been researched comparatively less. Regarding herbaceous or green biomass, energy crops like *Miscanthus*, switchgrass, *Arundo donax*, *Populus nigra*, or *Eucalyptus camaldulensis* (Ventorino et al., 2017) have been a focus of research, since the production of biofuels and bioenergy is currently a popular research topic. However, such energy crops hold the disadvantage of competing with food for bio-productive land (Johansson and Azar, 2007). With an increasing world population, the food-fuel competition will become a more pressing issue. A great advantage of green waste compared to energy crops is that it does not require any arable land but arises anyway. Therefore, the feedstock production costs are zero, if you do not consider the cultivation, the care of plants and lawns, or the collection and pretreatment of green waste. The greater difficulty in processing green waste lies in the collection, since it does not accumulate in certain fields, but is distributed over large areas. In metropolitan regions, green waste is typically collected and disposed of by public waste management companies, park departments, and private landscaping/gardening companies, while in rural areas green waste is collected and disposed of by municipal green waste collection points (Medick et al., 2017). Furthermore, the processing of green waste cannot be optimized by genetic or molecular modifications of the feedstock. The probably largest problem with the utilization of green waste is its heterogeneity. The heterogeneity of green waste from a single meadow, lawn, park, or garden is manageable. When green waste is collected from a large number of sources, there is a huge incline in heterogeneity. In addition, green waste composition varies depending on several other factors, which are explained more in detail hereinafter. Unfortunately, the publications investigating the composition and utilization of green waste in its entirety are limited. Therefore, in this review, especially low-lignin or non-woody herbaceous materials that might appear in green waste, predominantly grasses, but also leaves or low-lignin branches, should be considered for the generation of higher value. Agricultural residues like wheat straw or corn residues are left out since there are already plenty of publications investigating these bio-wastes. Furthermore, the potential energetic use of biomass, including methane and ethanol production, will not be considered in this review for the same reason. Instead, the current state of science for the material utilization of herbaceous materials and green waste is presented to lay a foundation for future work on green waste valorization.

II.3 Green waste composition

The heterogeneity of green waste is caused by various factors. First of all, the type of plants arising in green waste varies depending on the place in which the green waste was collected. In Singapore for example, abundant green waste materials are dead eucalyptus leaves (Liu et al., 2013), which do hardly

ever occur in European cities. Zhao et al. investigated the plant species composition in built-up areas of Beijing City and determined a total of 618 plant species within different green spaces (Zhao et al., 2010), which underlines the possible variety of plants in green cuttings, even in dense urban areas. Differences in composition and physical shapes, also through seasonal variations, result in challenges in transportation and storage (Liu et al., 2013). A pretreatment at the place of collection of green waste might be advantageous for further logistics. While public park waste is relatively easy to collect separately by authorities, garden waste from private households is rarely separated from other bio-waste (Eades et al., 2020). Since garden and kitchen waste are often combined into organic waste, it is difficult to determine exact numbers for the green waste of private households. Nevertheless, some studies can be found on the composition of MSW from individual cities, regions, or countries. The amount of garden-related grass and wood waste from MSW varies mainly due to residential buildings being equipped or lacking private gardens (Gu et al., 2015). Hanc et al. described the differences of bio-waste from an urban settlement (multi-story apartment buildings) and individual family houses with small gardens in Prague, Czech Republic during different seasons (Hanc et al., 2011). While urban bio-waste varies hardly, bio-waste from family houses is influenced by the seasonal garden activities, since over 75 % of the average yearly bio-waste from family houses consists of grass, leaves, wood, and plants (Hanc et al., 2011). The strong seasonal variations of green waste have also been shown by Boldrin and Christensen (Boldrin and Christensen, 2010). They showed that the amount of garden waste in Aarhus, Denmark varies from 19.4 kg person^{-1} month^{-1} in the summer to 2.5 kg person^{-1} month^{-1} in winter. In the study, garden waste from the summer consisted of more than 90 % of “small stuff” like grass clippings, flowers, hedge cuttings, or soil, while the winter was dominated by woody materials (Boldrin and Christensen, 2010).

Besides the regional and seasonal variation in green waste composition, the physicochemical characteristics of the individual plants vary depending on several factors. The composition of herbaceous materials includes carbohydrate polymers like cellulose and hemicellulose, phenolic polymers (lignin), and other components like proteins, acids, salts, and minerals (Mohapatra et al., 2017). The chemical composition of a plant differs depending on the species, its developmental stage (maturity), and the environmental conditions in which it was grown (Mohapatra et al., 2017). Herrmann et al. showed the differences in dry matter, crude fat, crude protein, crude fiber, and sugar of freshly harvested samples from three typical grassland biotopes from north Germany depending on the type of grassland and date of harvest (Herrmann et al., 2014). Differences in the concentrations of cellulose, hemicellulose, lignin, fibers, and crude proteins depending on the harvest date have also been demonstrated by Mashingo et al. (Mashingo et al., 2008). Kim et al. displayed the effect of growing conditions on prairie cordgrass (*Spartina pectinata L.*), resulting in significant differences in the composition of the plant between two years (Kim et al., 2015). Typical protein contents of

herbaceous material range from 1.5 to 4.5 % for grassland (Oregon, USA) (Juneja et al., 2011), 5 to 6 % in elephant grass (Menegol et al., 2016; Scholl et al., 2015), 12.3 % in timothy (Alvo and Belkacemi, 1997) to 18.5 % in alfalfa (Alvo and Belkacemi, 1997). The amount of extractives can differ similarly as shown for e. g. *Miscanthus* (6.9 %) and switchgrass (13.6 %) (Reza et al., 2013). The concentration of fats/lipids was shown to be around 2.5 % of dry weight for yard waste as well as grass and leaves (Komilis and Ham, 2003).

Probably one of the most important parameters of biomass resources is their composition of cellulose, hemicellulose, and lignin. Together, those three polymers form lignocellulose, which is the major component of plant biomass (Pérez et al., 2002). While cellulose and hemicellulose are macromolecules composed of sugars, lignin is an aromatic polymer, which is synthesized from phenylpropanoid precursors (Pérez et al., 2002). Cellulose is a linear polymer composed of subunits of *D*-glucose, which are linked by ß-1,4-glycosidic bonds (Pérez et al., 2002; Peterson et al., 2008). The so-called cellobiose molecules form fibrils, which are linked together by hydrogen bonds as well as van der Waals forces (Pérez et al., 2002). Due to these bonds, the monomers are crystalline as well as resistant to swelling in water and enzymatic attacks (Peterson et al., 2008). But if water with high temperature and pressure is applied, it can break the hydrogen-bound crystalline structure and hydrolyze the ß-1,4-glycosidic bond, which results in glucose monomers (Peterson et al., 2008). Hemicellulose, on the other hand, does not contain as many repeating ß-1,4-glycosidic bonds and has a more random structure, resulting in a less crystalline and less resistant structure than cellulose (Peterson et al., 2008). Hemicellulose is a polysaccharide comprised of *D*-xylose, *D*-mannose, *D*-galactose, *D*-glucose, *L*-arabinose, 4-O-methyl-glucuronic acid, *D*-galacturonic acid, and *D*-glucuronic acid, which are not only linked by ß-1,4-glycosidic bonds but also by ß-1,3-glycosidic bonds (Pérez et al., 2002). Therefore, hemicellulose contains branches and is more vulnerable to hydrothermal extraction or hydrolysis than cellulose (Pérez et al., 2002; Peterson et al., 2008). Lignin is an amorphous heteropolymer consisting of phenylpropane units, which are linked by multiple different bonds (Pérez et al., 2002). It consists of the monomers coniferyl alcohol, sinapyl alcohol, and *p*-coumaryl alcohol, while the relative composition varies strongly depending on the source. Lignin from grass comprises mainly guaiacyl and syringyl units and additionally *p*-coumaric acid and ferulic acid (Lin, 1992). Within the cell wall, lignin acts as structural support and a permeability barrier as well as resistance against microbial attacks and oxidative stress (Pérez et al., 2002). The structures of cellulose, typical grass hemicellulose, and lignin monomers are shown in figure 1.

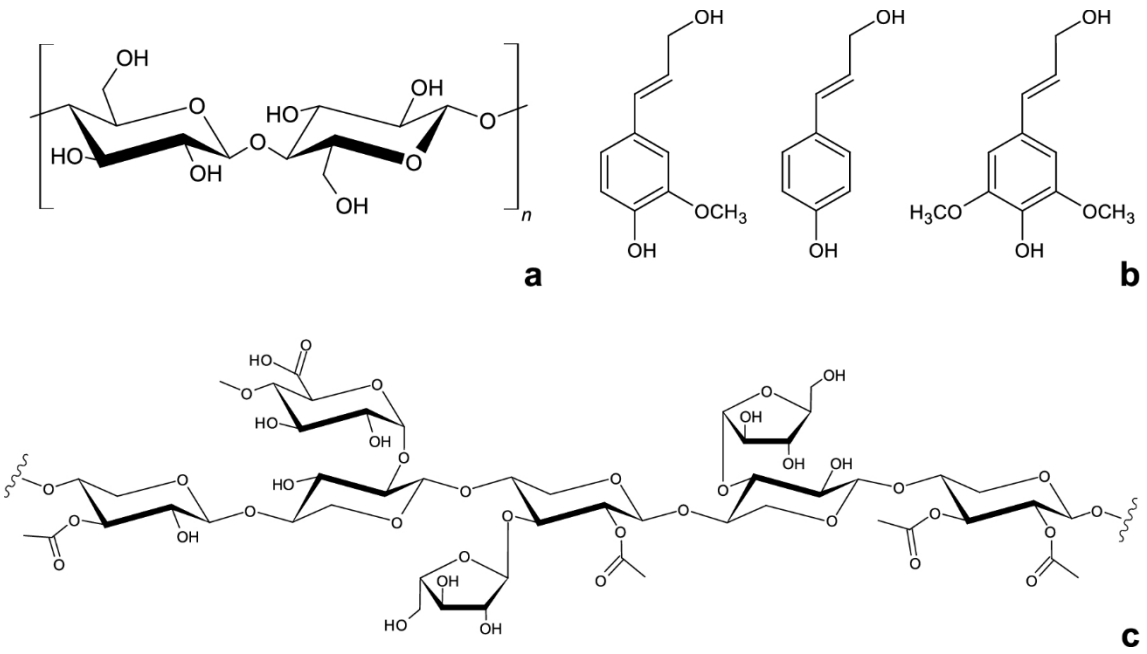

Figure 1: Components of lignocellulose. Chemical structures of cellulose (a), the lignin building blocks coniferyl alcohol, p-coumaryl alcohol, and sinapyl alcohol (b) as well as hemicellulose from grass (c) adapted from Escobar et al. (Escobar et al., 2020)

The composition of the lignocellulose components varies considerably between different plants. The cellulose/lignin ratio of herbaceous materials like grass is much higher than the ratio of woody materials like branches (Komilis and Ham, 2003). Non-woody biomass has a lignin content of around 5 to 25 % (Bagby et al., 1971; Barakat et al., 2013). The amount differs even within species and depends among others on the maturity of the plant (Sawatdeenarunat et al., 2015). Lawn grass for example contains high cellulose (41.7 %), high hemicellulose (35.8 %), and low lignin content (8.0 %) (Guo et al., 2015). Cellulose, hemicellulose, and lignin content of grassland from Oregon, USA ranged from 28.8 to 36.0 %, 18.4 to 24.7 %, and 13.4 to 17.4 %, respectively (Juneja et al., 2011). Premjet et al. showed the composition of 77 indigenous weed species from Thailand varying from 16.1 to 56.0 % in cellulose, from 3.3 to 34.2 % in hemicellulose, and from 4.6 to 20.4 % in lignin (Premjet et al., 2012). Leaves often contain a lower amount of cellulose and hemicellulose and a higher concentration of lignin compared to grassy material (Komilis and Ham, 2003). Jung et al. compared the content of cellulose, hemicellulose, and lignin of stems and leaves from *Miscanthus*, switchgrass, reed, and sorghum (Jung et al., 2015). The results showed that the woodier reed (*Phragmites australis*) contains significantly more lignin than *Miscanthus*, switchgrass, or sorghum (Jung et al., 2015). Typically, stalks contain higher concentrations of lignin, but also higher concentrations of cellulose and hemicellulose, than leaves (Jung et al., 2015). At the same time, leaves contain a higher amount of extractives (Jung et al., 2015). There are also leaves from e. g. bamboo (27.7 %) or the Java plum (27.4 %), which can contain more than 25 % lignin (Das et al., 2013). However, pruning from the olive tree has been shown to contain 39.4 % cellulose, 16.1 % hemicellulose, and only 16.8 % lignin (Raspolli Galletti et al., 2012). In

table 1, an overview of the composition of various low-lignin plants with lignin contents less than 25 % is shown. This overview demonstrates the variability in the composition of lignocellulose depending on the species and the origin of the plant. However, these differences are additionally related to the mentioned parameters cultivation conditions and maturity during harvest. Besides individual plants or grassland, there have also been some examinations of the lignocellulosic composition of green waste. Zhang and Sun determined a cellulose and hemicellulose concentration of green waste of 25.3 and 46.3 %, respectively (Zhang and Sun, 2014). According to Komilis and Ham, yard waste contains around 27 % cellulose, 11 % hemicellulose, and 24 % lignin (Komilis and Ham, 2003). The high amount of lignin, in this case, arises due to a high amount of woody branches in the yard waste.

Table 1: Cellulose, hemicellulose, and lignin content from various low-lignin plants

Plant	**Origin**	**Cellulose [%]**	**Hemicellulose [%]**	**Lignin [%]**	**References**
Alfalfa (*Medicago sativa*)	Québec, Canada	27.4	11.7	4.8	(Alvo and Belkacemi, 1997)
Asoka leaves (*Saraca indica*)	India	26.6	30.1	21.8	(Das et al., 2013)
Dwarf Napier grass (*Pennisetum purpureum cv. Mahasarakham*)	Thailand	35.6	34.2	3.7	(Wongwatana paiboon et al., 2012)
Eucalyptus leaves (*Eucalyptus marginata*)	India	35.7	47.4	16.9	(Das et al., 2013)
Finger flatsedge (*Cyperus digitatus*)	Malaysia	44.8	42.8	11.8	(Emmclan et al., 2018)
Greater club rush (*Scirpus grossus*)	Malaysia	36.2	49.9	13.4	(Bidin et al., 2015)
	Malaysia	42.1	45.6	11.4	(Emmclan et al., 2018)
King Napier grass (*Pennisetum Purpureum x P. Americanum*)	Thailand	32.0	31.1	3.1	(Wongwatana paiboon et al., 2012)
Lesser bulrush (*Typha angustifolia*)	Malaysia	44.1	54.8	20.0	(Bidin et al., 2015)
Mango leaves (*Mangifera indica*)	India	27.2	54.0	18.9	(Das et al., 2013)
Miscanthus sacchariflorus	Korea	31.7 – 36.1	22.3 – 28.9	14.1 – 18.1	(Jung et al., 2015)

Miscanthus sinensis	India	32.4	21.3	19.0	(Jung et al., 2015)
	Korea	30.5 – 33.4	24.6 – 29.9	14.7 – 24.7	(Jung et al., 2015)
Miscanthus × *giganteus*	Illinois, USA	33.2	23.3	17.2	(Jung et al., 2015)
Mission grass (*Pennisetum polystachion*)	Thailand	40.0	23.3	6.2	(Premjet et al., 2012)
	Thailand	39.8	29.2	14.6	(Tatijarern et al., 2013)
Napier grass/elephant grass (*Pennisetum purpureum*)	Brazil	36	22	20	(Menegol et al., 2016)
	Thailand	32.9	36.5	3.6	(Wongwatana paiboon et al., 2012)
Neem leaves (*Azadirachta indica*)	India	20.6	50.8	18.5	(Das et al., 2013)
Pangola grass (*Digitaria eriantha*)	Thailand	33.1	35.5	4.5	(Wongwatana paiboon et al., 2012)
Poplar leaves (*Populus nigra*)	India	29.4	48.8	21.8	(Das et al., 2013)
Purple guinea grass (*Panicum maximum*)	Thailand	33.4	31.3	4.0	(Wongwatana paiboon et al., 2012)
Purple nutsedge (*Cyperus rotundus*)	Malaysia	42.6	45.6	9.5	(Bidin et al., 2015)
Rice flatsedge (*Cyperus iria*)	Malaysia	44.6	43.4	10.6	(Emmclan et al., 2018)
	Thailand	33.4	31.0	6.3	(Premjet et al., 2012)
Ruzi grass (*Brachiaria ruziziensis*)	Thailand	33.6	34.0	4.6	(Wongwatana paiboon et al., 2012)
Sorghum (*Sorghum bicolor* (L.) Moench)	Nigeria	31.4	23.4	17.9	(Jung et al., 2015)
	Sudan	35.3	23.6	20.7	(Jung et al., 2015)
Sorghum (*Sorghum halepense*)	Thailand	44.4	25.8	6.6	(Premjet et al., 2012)
Switchgrass (*Panicum virgatum*)	Illinois, USA	29.5 – 37.8	21.5 – 27.4	13.9 – 21.1	(Jung et al., 2015)
	USA	35.3	33.7	8.4	(Reza et al., 2013)

Tall fescue grass (*Festuca arundinacea Schreb*)	Oregon, USA	31.0	20.2	14.4	(Kumar and Murthy, 2011)
Timothy (*Phleum pratense*)	Québec, Canada	28.8	27.2	4.6	(Alvo and Belkacemi, 1997)
Ubon paspalum (*Paspalum atratum*)	Thailand	34.9	32.6	5.6	(Wongwatana paiboon et al., 2012)
Vetiver grasses (*Chrysopogon zizanioides*)	Thailand	31.9 – 38.5	37.9 – 42.6	3.7 – 5.1	(Wongwatana paiboon et al., 2012)
Water hyacinth (*Eichhornia crassipes*)	Indonesia	43.0	29.1	6.9	(Asrofi et al., 2018)
	Thailand	57.0	25.6	4.1	(Tanpichai et al., 2019)
Wild grass (*Achnatherum hymenoides*)	India	51.2	30.1	18.7	(Das et al., 2013)

II.4 Pretreatment methods of lignocellulosic biomass

As described above, lignocellulosic biomass contains valuable components, which makes it an interesting feedstock for industrial applications. Constituting up to 60 % of total biomass (Álvarez et al., 2016), one of the main components of interest are carbohydrates, which can serve as a carbon source for fermentation. The conversion of biomass usually starts with size reduction, followed by one or several pretreatment steps. Subsequently, monomeric sugars like glucose and xylose are obtained via hydrolysis, which can then be converted by various microorganisms into desired products like bulk and fine chemicals. An exemplary pretreatment process of green waste for fermentative utilization is given in figure 2. Factors impacting the digestibility of biomass include the chemical composition, notably the lignin, ash, acetate, hemicellulose, and cellulose content as well as the particle size, surface area, pore volume, and the crystallinity of the cellulose (Chang and Holtzapple, 2000). Therefore, the pretreatment is crucial for further conversion of the biomass. The main goals of the pretreatment are delignification and structural conversion of cellulose and hemicellulose, making them more accessible for the ensuing hydrolysis into monomeric sugars by enzymes. Yang and Wyman summarized the main requirements for a successful pretreatment procedure of lignocellulosic biomass. The process should result in a liquid hydrolysate with a high concentration of fermentable sugars compatible with the fermenting microorganisms. Occurring by-products should be recovered for further use as far as possible. To reduce costs, the pretreatment method should require as little heat and power as possible, expensive and possible hazardous chemicals should also be avoided (Yang and Wyman, 2008). The

impact of a successful pretreatment method is emphasized for example by the findings of Rezende et al. By optimizing the pretreatment strategy the sugar release from elephant grass leaves was increased fivefold compared to a sample without pretreatment, resulting in a yield of 205 mg of reducing sugars per gram substrate via acid-alkali pretreatment (Rezende et al., 2018).

Because of the great importance of pretreating lignocellulosic biomass prior to fermentation, there exists a broad variety of different methods. Hendriks and Zeeman identified the increase of the accessible surface area as the critical factor, which is present in all pretreatment methods (Hendriks and Zeeman, 2009). According to their underlying principle, the methods usually are subdivided into physical, chemical, physicochemical, and biological methods, although most procedures combine several principles. In this chapter, a short overview of different methods and their underlying principles is provided. Focussing on the pretreatment of green waste, insights stemming from the pretreatment of other lignocellulosic biomass feedstocks like agricultural waste are mentioned in case the principle is applicable to low-lignin green waste biomass. Table 2 summarizes the mentioned methods together with their respective effects. For more detailed and in-depth reviews with a broad range of substrates see for example the publications of Kucharska et al. (Kucharska et al., 2018), Mohapatra et al. (Mohapatra et al., 2017), Hendriks and Zeeman (Hendriks and Zeeman, 2009), Yang and Wyman (Yang and Wyman, 2008), and Taherzadeh and Karimi (Taherzadeh and Karimi, 2008).

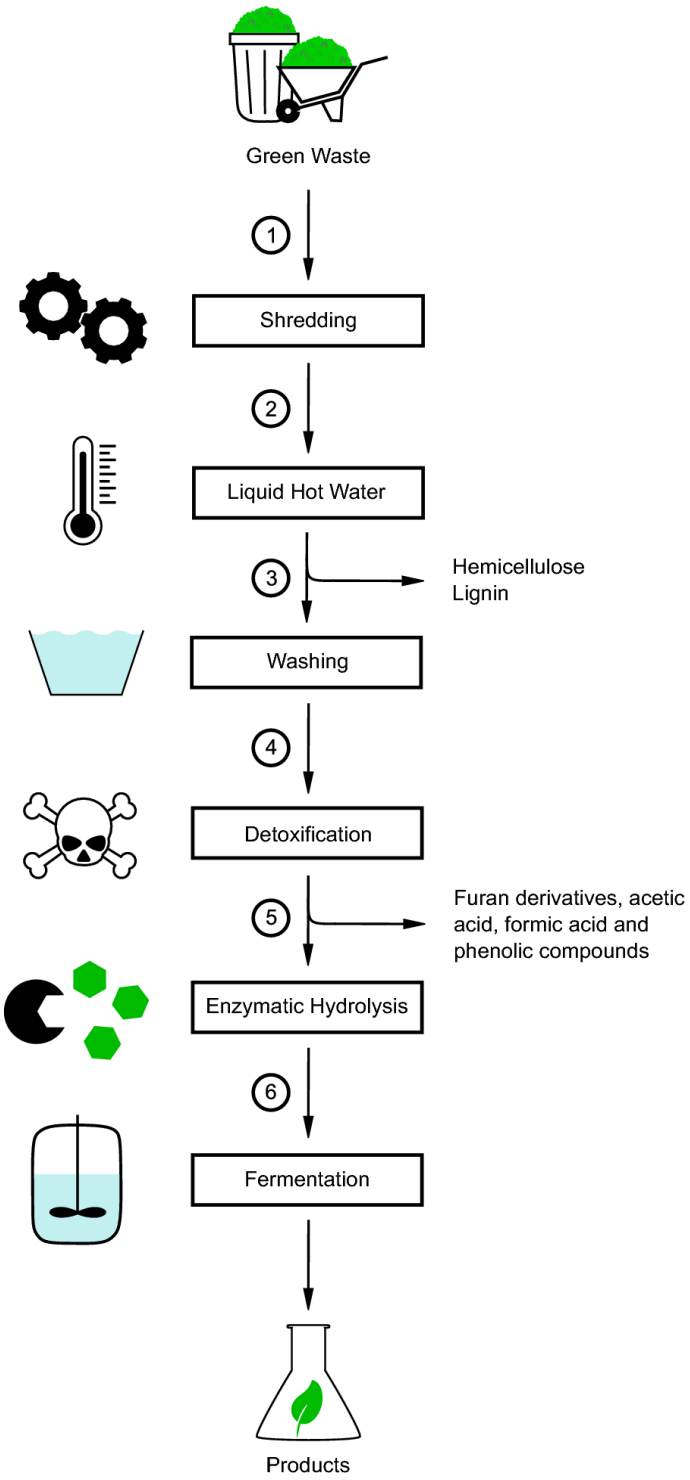

Figure 2: Exemplary pretreatment process of green waste for fermentative utilization. Green waste is shredded (1) and pretreated using elevated temperature and pressure, removing hemicellulose and lignin (2). The resulting hydrolysate is washed (3) and detoxified, e.g. using activated carbon, to eliminate furan derivatives, acids, and phenolic compounds (4). The process is followed by an enzymatic hydrolysis to obtain monosaccharides (5), which are then used in microbial fermentations (6).

II.4.1 Physical pretreatment methods

Physical pretreatment mainly focuses on the size reduction of biomass. Because of the resulting increase in the specific surface, enzymatic hydrolysis is more effective (Dasari and Berson, 2007).

Therefore, fragmentation methods like cutting, shredding, grinding, and milling are employed. Licari et al. compared different milling methods considering their efficiency for later enzymatic hydrolysis as well as their energy consumption. Vibro-ball milled bagasse samples displayed a cellulose conversion rate of 95 % in subsequent enzymatic hydrolysis, while ball and centrifugal mills only achieved rates of 76 and 54 %, respectively (Licari et al., 2016). As no additional compounds are needed, this approach has a smaller environmental impact than chemical pretreatment methods. A severe disadvantage however is the high energy consumption compared to most chemical pretreatment methods (Holtzapple et al., 1991; Licari et al., 2016).

Other physical pretreatment methods aim at reducing the crystallinity of cellulose to improve accessibility for further conversion. One example is the irradiation with electron beams with doses of up to 1000 kGy, resulting in a 14 % reduction of the crystallinity of microcrystalline cellulose (Jusri et al., 2018). Further types of radiation are employed to treat lignocellulosic biomass prior to fermentation. As they are usually coupled with chemical methods, they are grouped into the physicochemical section below.

II.4.2 Chemical pretreatment methods

Chemical pretreatment methods are based on chemical reactions taking place in aqueous solution between lignocellulosic biomass and various chemical compounds. Most common are treatments with acid and alkaline solutions.

Acid pretreatment aims at breaking up the lignin structure as well as dissolving hemicellulose and depolymerizing cellulose. It takes place at acid concentrations of 10 – 30 %, elevated pressures and temperatures, and reaches hemicellulose degradation rates of up to 90 % (Kucharska et al., 2018). Here, a balance needs to be found between effective biomass pretreatment and the limitation of inhibitor formation under harsher conditions. The separation in a first stage under mild conditions to dissolve hemicellulose and a second step in a harsher environment to break down cellulose into monomers helps to reduce the occurrence of interfering degradation products. Another approach of acid pretreatment is the dilute acid method. By applying acid concentrations between 1 and 2 %, Karapatsia et al. achieved hemicellulose conversion rates of up to 81 % from the perennial grass *Phalaris aquatica* (Karapatsia et al., 2017).

Alkaline pretreatment is based on the saponification process. The presence of usually ammonia, as well as sodium or calcium hydroxides (Rabemanolontsoa and Saka, 2016), causes swelling of the biomass. Thereby, the reduction of cellulose crystallinity results in an increase in the specific surface. Xu et al. achieved a 3.8-fold increase in total reducing sugars from switchgrass by applying mild conditions of 50 °C and 1 % NaOH for 12 h, resulting in a yield of total reducing sugars of 45 % and a

delignification rate of 78 %. Harsher conditions resulted in a higher degree of delignification of up to 86 % but did not lead to an increased yield of fermentable sugars (Xu et al., 2010).

A variety of organic solvents are used to pretreat lignocellulosic biomass as well. Here, the high degree of delignification and hydrolysis of hemicellulose is the result of the hydrolysis of microfibrillar structures by breaking internal lignin and hemicellulose bonds as well as the bonds between the two structures (Kucharska et al., 2018). Schmetz et al. describe an increase in delignification of the tall fescue grass *Festuca arundinacea* Schreb. from 23 to 87 % using a butanol pretreatment compared to dilute acid. While the effect on the recovery of xylose and glucose is marginal, the use of butanol during pretreatment results in 74 % conversion during subsequent enzymatic hydrolysis, compared to 20 % following dilute acid pretreatment (Schmetz et al., 2019). A major disadvantage of solvent pretreatment is the necessity to remove the solvent prior to microbial fermentation.

Ionic liquids, salts with a melting point below 100 °C, are applied to dissolve cellulose by reacting with carbohydrate hydroxyl protons and thereby facilitating the hydrolysis into fermentable sugars (Remsing et al., 2006). A variation is the use of deep eutectic solvents (DES). Although having similar properties, DES are different from ionic liquids as they are composed of a eutectic mixture of Lewis or Brønsted acids and bases, while ionic liquids contain only one type of discrete anion and cation (Smith et al., 2014). Yu et al. applied a mixture of water and chloroform in a Liquid Hot Water experiment (described below), proposing the formation of an *in situ* poly-hydrogen bonding DES. This is supposed to selectively delignify biomass without degrading carbohydrate structures. For *Roystonea regia* leaf sheaths delignification rates of up to 59.6 % were achieved while glucan and xylan removal rates were 4.8 and 10.8 %, respectively (Yu et al., 2019).

M'Arimi et al. give an extensive overview of a variety of advanced oxidation processes applied for biomass pretreatment, for example, ozonation, Fenton oxidation, cavitation methods causing the formation of hydroxyl ions, and photocatalysis (M'Arimi et al., 2020). The application of peroxides effects electrophilic substitution, shifts of side chains, and the breaking of aryl-alkyl bonds, which lead to the dissolution of lignin, hemicellulose, and amorphous cellulose (Hendriks and Zeeman, 2009; Kucharska et al., 2018).

II.4.3 Physicochemical pretreatment methods

Physicochemical pretreatment methods combine the usage of chemicals with the application of physical forces. The basis of hydrothermal methods like Liquid Hot Water, steam explosion, and supercritical water is the simultaneous application of high temperature and pressure in aqueous solution. The latter method is reviewed extensively by Peterson et al. (Peterson et al., 2008). The fractionation of biomass is caused by ionic parts of the liquid, where water as well as carbon dioxide is

used (Akalın et al., 2017; Kucharska et al., 2018). In the autocatalytic process of steam explosion, acetyl residues are released from the biomass, whereby the produced acetic acid acts as a catalyst for further hydrolysis. The sudden release of pressure is the key element of this method. Although it is cost-effective, the incomplete lignin removal and the formation of inhibitors are major drawbacks (Kim, 2018). A similar process is the Ammonia Fiber Expansion (AFEX), where an anhydrous ammonia suspension serves as solvent. It effects a decrease in cellulose crystallinity and an increased accessible surface area (Holtzapple et al., 1991). In the related Ammonia Recovery Process (ARP), ammonia aqueous solution is repeatedly pumped through lignocellulosic biomass at temperatures between 150 and 190 °C, effecting delignification as well as solubilization of hemicellulose (Yoon et al., 1991). Mes-Hartree et al. compared the methods steam explosion and AFEX on several substrates, but could not find a superior method suitable for all biomasses (Mes-Hartree et al., 1988). This emphasizes the diversity of combinations of substrates and pretreatment methods. Rollin et al. compared the digestibility of switchgrass after cellulose solvent- and organic solvent-based lignocellulose fractionation (COSLIF) and soaking in aqueous ammonia (SAA). They observed a 16-fold increase in cellulose accessibility to cellulase after COSLIF pretreatment, which primarily breaks lignocellulosic bonds, compared to a 1.4-fold cellulose accessibility increase after SAA pretreatment, mainly eliminating lignin. Therefore, they concluded the accessibility of cellulose to cellulases to be more important than the removal of lignin (Rollin et al., 2011).

During Liquid Hot Water treatment, elevated temperature and pressure cause the hydrolysis of hemicellulose and, at temperatures above 180 °C, lignin, leaving cellulose behind (Kucharska et al., 2018; Kumar et al., 2009). By applying 180 °C for up to 60 min, Yu et al. obtained a total xylose yield of 83.6 % of the theoretical maximum from switchgrass. The pretreatment enhanced subsequent enzymatic hydrolysis by 113 % and thereby nearly achieved the total theoretical digestibility (Yu et al., 2016).

Microwave irradiation is a way of heating the biomass, resulting in the disruption of lignocellulosic structures. It is usually coupled with another, often chemical, method (Chaturvedi and Verma, 2013). There is no explicit proof of a higher saccharification rate in subsequent hydrolysis compared to other thermal pretreatments (Li et al., 2016). The mechano-acoustic effect of ultrasound radiation is shown to cause a reduction in lignin condensation at lower frequencies than 40 kHz and an increase in the solubilization of carbohydrates at a higher frequency than 995 kHz (Bussemaker et al., 2013).

II.4.4 Biological pretreatment methods

Another approach to treat lignocellulosic biomass are biological methods through the application of microorganisms and isolated enzymes. According to their purpose, these methods can be divided into delignification and saccharification. In addition, lactic acid bacteria can be used to pretreat especially

herbaceous biomass through ensiling. While the process requires at least two weeks, the produced organic acids preserve the feedstock, circumventing the problem of only seasonally available biomass. Rabemanolontsoa and Saka summarize various microorganisms including a variety of bacteria and fungi applied for biological pretreatment (Rabemanolontsoa and Saka, 2016).

Biological delignification is mainly based on fungi and fungal enzymes. Wood-rot fungi, more specific white-, brown-, and soft-rot fungi, are known to degrade cellulose and lignin (Kerem et al., 1992; Sun and Cheng, 2002). Akin et al. examined the delignification of Bermuda grass stems by white-rot fungi *Ceriporiopsis subvermispora* and *Cyathus stercoreus*. They showed removal of up to 41 % of total aromatic compounds, mainly *p*-coumaric and ferulic acid, while significantly improving the biodegradability through ruminal microorganisms by up to 77 % (Akin et al., 1995). Dionisi et al. showed a lignin degradation rate of up to 83 % by mixed cultures. This applied especially for biomass with a low initial lignin content of 6 – 10 % (Dionisi et al., 2015). One major disadvantage of biological delignification is the processing time. Dionisi et al. calculated lignin degradation rates for the microbial delignification process and compared them with common, non-biological approaches as described in Wyman et al. (Wyman et al., 2011). Biomass like cotton stalk had delignification rates of up to 0.1 g L^{-1} h^{-1}. Compared to up to 23 g L^{-1} h^{-1} for Liquid Hot Water pretreatment, the biological method cannot compete (Dionisi et al., 2015).

The application of lignin-degrading enzymes promises faster results than using whole organisms. One example is the multicopper oxidase laccase (EC 1.10.3.2). It oxidizes substituted phenols and therefore degrades lignin by modifying its three phenolic subunits *p*-coumaryl alcohol, coniferyl alcohol, and sinapyl alcohol. Gutiérrez et al. demonstrated delignification rates of elephant grass and eucalypt of up to 35 and 58 % by using laccase in combination with 1-hydroxybenzotriazole as a mediator. The subsequent hydrolysis rate of total reducing sugars increased by 10 and 63 %, respectively (Gutiérrez et al., 2012).

One of the best-studied group of organisms for biological saccharification are clostridia. Especially *Clostridium thermocellum* is promising because of its tolerance to high temperatures. To degrade polysaccharides, clostridia possess an intricate extracellular complex of enzymes called cellulosome. It consists of cellulases, xylanases, exo- and endo-glucanases, hemicellulases, chitinases, pectate lyases, and lichenases (Bayer et al., 2004). In contrast, other bacteria and fungi produce free cellulases. A challenging aspect of using microorganisms instead of enzymes for saccharification is to prevent further metabolization of generated glucose to other, unwanted products (Rabemanolontsoa and Saka, 2016). An issue concerning the use of enzymes is product inhibition. Prawitwong et al. avoided the inhibition of *C. thermocellum* with cellobiose through the addition of a thermostable β-glucosidase.

In combination with an alkali pretreatment saccharification of 72 % of glucan was achieved (Prawitwong et al., 2013).

Usually, no compounds produced during biological pretreatment procedures are inhibitory to further microbial or enzymatic hydrolysis processes (Sindhu et al., 2016). This poses a major advantage over most physicochemical pretreatment methods as there is no detoxification or purification step necessary. Even more, Du et al. observed a positive effect of by-products occurring during pretreatment with the fungus *Irpex lacteus* on the subsequent enzymatic hydrolysis. When the by-products were removed by rinsing the biomass after the biological pretreatment, the maximum reducing sugar yield in subsequent enzymatic hydrolysis decreased by 39 %. Additionally, enzymatic hydrolysis of enzymatically pretreated biomass could be enhanced by 32 % through the addition of the water extract rich in by-products of biological pretreatment (Du et al., 2011).

Table 2: Overview of pretreatment methods for lignocellulosic biomass

	Size reduction	Fractionation	Reduction of cellulose crystallinity	Dissolution of lignin	Dissolution/hydrolysis of hemicellulose	Dissolution/hydrolysis of cellulose	Delignification	Removal of aromatic compounds	References
Physical									
Milling etc.	+	+							(Holtzapple et al., 1991; Licari et al., 2016)
Irradiation			+						(Jusri et al., 2018)
Chemical									
Acidic pretreatment				+	+	+			(Kucharska et al., 2018),
Alkaline pretreatment			+						(Rabemanolontsoa and Saka, 2016; Xu et al., 2010)
Organic solvents					+	+	+		(Kucharska et al., 2018; Schmetz et al., 2019)
Ionic liquids, DES						+	+		(Remsing et al., 2006; Yu et al., 2019)
Oxidation processes				+	+	+			(Hendriks and Zeeman, 2009; M'Arimi et al., 2020)

Physico-chemical								
Liquid Hot Water				+				(Kumar et al., 2009; Yu et al., 2016)
Steam explosion	+		+	+	+			(Kim, 2018)
Supercritical water	+							(Akalın et al., 2017; Peterson et al., 2008)
AFEX		+						(Holtzapple et al., 1991; Mes-Hartree et al., 1988)
ARP				+		+		(Mes-Hartree et al., 1988; Yoon et al., 1991)
Ultrasound			+	+	+			(Bussemaker et al., 2013)
Biological								
Wood-rot fungi						+	+	(Akin et al., 1995; Dionisi et al., 2015; Kerem et al., 1992; Sun and Cheng, 2002)
Enzymes				+	+	+	+	(Bayer et al., 2004; Gutiérrez et al., 2012; Prawitwong et al., 2013; Rabemanolontsoa and Saka, 2016)

II.4.5 Inhibition and detoxification

Hydrolysis of lignocellulosic biomass does not exclusively result in monomeric sugars. Other components are formed as well, unfortunately, many of which inhibit the subsequent microbial fermentation. Depending on their origin, phenolic compounds, furan derivatives, and weak acids are differentiated (Palmqvist and Hahn-Hägerdal, 2000a). Degradation of lignin leads to the formation of phenolic compounds. Due to their hydrophobic structure, they can intercalate into biological membranes, thus destroying their functionality as selective barriers. The furan derivative furfural is formed from the pentose xylose, while 5-hydroxymethylfurfural (HMF) is a degradation product from the hexoses mannose, galactose, and glucose. Furfural has a negative impact on cell growth (Palmqvist and Hahn-Hägerdal, 2000a), with high concentration leading to cell death (Palmqvist et al., 1999). Both

furfural and HMF can react further to formic acid, while levulinic acid is only formed from HMF. Acetic acid results from the degradation of hemicellulose to monomeric sugars. Those weak acids inhibit cell growth by lowering the intracellular pH, which in turn is answered by the cell by pumping protons out of the cell via the plasma membrane ATPase, using ATP (Palmqvist and Hahn-Hägerdal, 2000a).

To alleviate the effects of inhibitory substances there exist a variety of biological, physical, and chemical detoxification methods (Jönsson et al., 2013; Palmqvist and Hahn-Hägerdal, 2000b). As described above, the enzyme laccase modifies phenolic compounds. Therefore, it can not only be used for delignification in a pretreatment step but also in a downstream process to remove inhibiting phenolic compounds (Fillat et al., 2017). The same applies to lignin peroxidase. Obtained from the fungus *Trametes versicolor*, Jönsson et al. showed an increase in glucose consumption and ethanol production during fermentation with *S. cerevisiae* of up to three times compared to an untreated control for both enzymes (Jönsson et al., 1998). Direct treatment of hydrolysate with the fungus *Trichoderma reesei* also resulted in a tripling of ethanol productivity (Palmqvist et al., 1997). One possibility to eliminate inhibitory acids, furfural and vanillin is evaporation and subsequent dilution of the hydrolysate. Satisfactory results were achieved as well with ethyl acetate or diethyl ether extraction (Palmqvist and Hahn-Hägerdal, 2000b; Wilson et al., 1989). Due to its adsorptive properties, activated carbon is also successfully employed for detoxification of hydrolysates. Lee et al. achieved removal rates of 42 % formic acid, 14 % acetic acid, 96 % HMF, and 93 % furfural with 2.5 % activated carbon in one hour. Prolonged reaction time and higher activated carbon loadings resulted in complete removal of HMF and furfural as well as 48 % removal of acetic acid and 84 % removal of formic acid (Lee et al., 2011). In the so-called overliming process the pH is augmented to over 9 through the addition of $Ca(OH)_2$ and subsequently decreased to 5.5. This leads to the destruction of inhibitors in the alkali milieu as well as their precipitation (Martinez et al., 2000; Palmqvist and Hahn-Hägerdal, 2000b). The procedure removes mainly HMF and furfural, while the effect on organic acids and phenolic compounds is less pronounced (Martinez et al., 2000). Zhang and Ezeji showed that *Clostridium beijerinckii* is able to reduce the inhibitors furfural, HMF, 4-hydroxybenzaldehyde, and *p*-coumaric acid during fermentation of Liquid Hot Water pretreated *Miscanthus* hydrolysate (Zhang and Ezeji, 2014).

II.4.6 Comparability of pretreatment methods

As demonstrated above, there exists a multitude of different pretreatment methods. But as factors like carbohydrate and lignin content, which strongly impact the pretreatment behavior, varies even within one biomass species, there is no single, optimal procedure. The implications of each pretreatment method need to be considered for every substrate and process anew.

In 2000, the Biomass Refining Consortium for Applied Fundamentals and Innovation (CAFI) made an effort to acquire a comparable database covering various pretreatment methods to obtain sugar from corn stover. The tested methods cover AFEX, ammonia recycle percolation, dilute sulfuric acid, flowthrough of hot water or dilute acid, lime, controlled pH hot water, and sulfur dioxide steam explosion pretreatment. The methods achieved total sugar yields between 86.6 % for lime and 96.6 % for flowthrough pretreatment of maximum possible values. The monomeric sugar yield however ranged from 60.6 % for partial flow to 91.5 % for dilute acid pretreatment. In general, the results of the different pretreatment methods are in the same range for corn stover (Elander et al., 2009). The project was expanded in 2004 to include poplar wood and switchgrass, covering the same pretreatment methods. The examination of switchgrass resulted in total sugar yields of 67.3 % for SAA (replacing the water and energy-consuming ARP) to up to 90.9 % for lime pretreatment. The authors emphasize to not select or exclude one method according to their data, as the experimental conditions were not optimized for the substrates (Wyman, 2013).

One approach to prevent excessive laboratory testing for pretreatment methods is the design of predictive models. Chang and Holtzapple establish a link between lignin content as well as cellulose crystallinity and digestibility of biomass (Chang and Holtzapple, 2000). Payne and Wolfrum built a model to predict not only the composition of biomass feedstocks but more importantly the yield of soluble carbohydrates resulting from a dilute acid pretreatment followed by enzymatic saccharification. The prediction is based on near-infrared spectral data and partial least squares multivariate data analysis. In contrast to other models, it uses 279 samples from six different feedstock groups instead of a single feedstock (Payne and Wolfrum, 2015). Bai et al. developed a predictive model for the release of sugar from a feedstock mixture of garden waste of the leguminous tree *Bauhinia blakeana Dunn*, rice straw, and sugarcane bagasse during a microwave-assisted hot water pretreatment process. It allows identifying an optimal mixing ratio of the three feedstocks for a xylose yield of 67.8 % (Bai et al., 2020). Hoover et al. designed a model to grade different herbaceous biomass feedstocks according to their performance in bioconversions. As defining properties, they identified the content of structural glucan, hemicellulose carbohydrates, acid-soluble, and acid-insoluble lignin as well as ash (Hoover et al., 2019).

Although countless articles are published regarding various pretreatment methods with diverse substrates, it is difficult to compare the different results. There is no standardized evaluation protocol to assess the effectiveness of a pretreatment method. One approach is to state the decrease in hemicelluloses (Rollin et al., 2011), another to measure the amount of total reducing sugars before and after the treatment (Xu et al., 2010). A popular method to rate the effectiveness of the pretreatment is comparing the results of a subsequent enzymatic saccharification. Here, the reported

information varies from sugar conversion rates to end product yields. An important aspect due to which the results of different studies are not easy to compare is the fact that the enzymatic methods are rarely optimized to achieve maximum yields, but are merely tools to rank different parameter combinations within one study.

One difficulty concerning the rating of the effectiveness of delignification is the formation of so-called pseudo-lignin (Sannigrahi et al., 2011). During acid and hydrothermal pretreatment, the development of aromatic structures is observed. As they display lignin-like properties, they add to the Klason lignin value without emerging from actual lignin, therefore distorting the results. Pseudo-lignin originates from furfural- and HMF-derived substances for example 3,8-dihydroxy-2-methylchromone and 1,2,4-benzenetriol. That is why it occurs primarily when harsher pretreatment conditions are applied, which leads to increased formation of inhibitors (Sannigrahi et al., 2011; Wan et al., 2019).

Since the 1970s there were efforts to describe the effects of different pretreatment conditions in a comparable way (Chornet and Overend, 2017). Overend et al. suggested an expression to combine the pretreatment factors time *t* in minutes and temperature *T* in °C (Overend and Chornet, 1987). This severity factor R_0 is shown in equation 1. While a higher severity factor indicates harsher pretreatment conditions, all correlations between the factor and pretreatment results should be considered purely empirical (Chen et al., 2007; Kim et al., 2014).

$$R_0 = t\ exp\left\{\frac{(T-100)}{\omega}\right\} \qquad \text{Eq. 1}$$

The fitted parameter ω is inversely proportional to the activation energy (Chen et al., 2007) and usually determined to be 14.75. However, Kim et al. showed that the influence of the temperature is not adequately expressed by this value for the pretreatment of hardwood chips with the Liquid Hot Water method and adapted the severity factor accordingly (Kim et al., 2014).

An extension of the severity factor was developed by Abatzoglu et al. (Abatzoglou et al., 1992) and Chum et al. (Chum et al., 1990) as combined severity factor *CS* to include the acid concentration of the pretreatment method as well. Ruiz et al. (Ruiz et al., 2017) linked the severity factor R_0 and the combined severity factor *CS* to the expression in equation 2.

$$\log CS = \log R_0 - pH \quad \text{Eq. 2}$$

Ideally, a prediction model would suggest the ideal pretreatment method for a feedstock based on its composition. Due to the low content of lignin, green waste generally requires less harsh pretreatment conditions than other lignocellulosic material (Wolfrum et al., 2013; Xu et al., 2010). Therefore, the use of green waste for biomass conversion is advantageous regarding the consumption of energy and chemicals. But there is the need for a universal comparison of the impact of different pretreatment

methods. This includes the degradation rate of lignin, the conversion rate of cellulose and hemicellulose into monosaccharides as well as the formation of substances inhibitory to subsequent enzymatic reactions. The severity factor R_0 enables a classification of the harshness of pretreatment protocols but offers no indication of the product building rates. Up to now, the best comparative methods are studies like the CAFI project, using uniform experimental conditions. However, there is only limited comparability between different studies, among others due to the lack of a consistent reporting format.

II.5 Products obtained by pressing and extraction

A common use of lignocellulosic biomass is the fractionation in lignin, hemicellulose, and cellulose with the subsequent conversion into fermentable sugars. However, green waste contains further valuable compounds. The main products hereof are proteins, organic acids, lipids, and phenolic compounds. Their recovery and utilization are described hereinafter. Figure 3 gives an overview of processes and products from green waste obtained by extraction and pressing.

II.5.1 Pressing of lignocellulosic biomass

One commonly applied technique for biomass with low lignin content, especially herbaceous material, is its separation into a solid press cake and a liquid press juice by pressing. The press cake usually contains more carbohydrates than the press juice as the cellulosic fibers are less extractable. The press juice however contains a higher amount of crude protein and organic acids. Boakye-Boaten et al. obtained a portion of 64 % of dry weight cellulose in the press cake of *Miscanthus* versus only 39 % in the press juice, while the latter having a protein content of 5 % versus only 2.5 % in the press cake. Furthermore, 64 % of lignin remained in the solid fraction (Boakye-Boaten et al., 2016). As the juice also contains a vast number of organic acids and various nutrients (for a detailed composition of *Miscanthus* press juice see tables 1 and 2 in Boakye-Boaten et al., 2016), it can serve as a cultivation medium for microorganisms. Boakye-Boaten et al. showed improved growth of *Saccharomyces cerevisiae* in 90 % *Miscanthus* press juice compared to the commonly used YM medium (Boakye-Boaten et al., 2016).

Andersen et al. demonstrated the suitability of press juice from Italian ryegrass (*Lolium multiflorum*), white clover (*Trifolium repens*), and alfalfa (*Medicago sativa*) for the use as a fermentation medium. When glucose was added to reach the same amount as in MRS broth, the growth of lactobacilli in brown juice, press juice depleted of proteins, was higher than in the conventional cultivation medium. It is shown that lactic acid fermented alfalfa juice can replace peptone, yeast, or hydrolyzed soy protein in cultivation media for bacteria while improving growth (Andersen and Kiel, 2000). By lactic acid fermentation, it is also possible to preserve the juice for further use, for example, the fermentative production of amino acids or organic acids (Andersen and Kiel, 2000).

Leiß et al. produced *L*-lysine-*L*-lactate, a precursor for polylactic acid, with alfalfa press juice as a medium for lactobacilli. After the amount of sugar was adjusted to the same level as in the MRS medium, the lactate production rates of 6.4 g L^{-1} h^{-1} for the MRS medium and 6.8 g L^{-1} h^{-1} for press juice were comparable. The deproteinization of the press juice had no significant effect on the lactate production rate, allowing to gain surplus value from the extracted proteins for example as functional food or a foaming agent (Leiß et al., 2010). The suitability of alfalfa press juice for lactic acid production by *Bacillus coagulans* was confirmed by Papendiek and Venus. Again, the supplementation of glucose is necessary (Papendiek and Venus, 2014). For an integrated biorefinery process, the additional glucose necessary to use press juice as a fermentation medium could be obtained from the separated press cake via enzymatic saccharification. We already described this process for brewers' spent grain (Akermann et al., 2019).

II.5.2 Fermentation of silage press juice

A common approach for the processing of herbaceous biomass is ensiling. As shown above, biomass press juices can serve as cultivation media for fermentation procedures. Several studies examine the suitability of silage press juice for the same application. We obtained a press juice containing 20 g L^{-1} glucose, 17 g L^{-1} xylose/galactose, 44 g L^{-1} fructose/arabinose, and 44 g L^{-1} lactic acid from grass silage based on perennial ryegrass (Sieker et al., 2011). As the acid has a strong inhibitory effect on *S. cerevisiae*, the press juice is not suitable to use as a fermentation medium for the production of ethanol. The usage of hydrolyzed silage press cake also resulted in unsatisfactory yields as nutrients required for fermentation are washed out from the solid fraction. However, they can be replaced by adding up to 10 % of the press juice, resulting in a significant increase of productivity compared to the use of a mixture of salts, vitamins, and trace elements and a total ethanol yield of 91 g kg^{-1} (Sieker et al., 2011).

Cerrone et al. demonstrated the production of polyhydroxyalkanoates (PHA) by *Burkholderia sacchari* and *Pseudomonas chlororaphis* using press juice from ensiled perennial ryegrass as a carbon source. The juice contained 54 g L^{-1} of total sugars, 20 g L^{-1} of proteins, 15 g L^{-1} of *L*-lactic acid, and 5.4 g L^{-1} of *D*-lactic acid. The acid content is significantly lower than described by Sieker et al. (Cerrone et al., 2015). For storage, the press juice was concentrated by rotary evaporation to a content of 300 g L^{-1} total sugar and fermentation was conducted at 20 g L^{-1}. With *Burkholderia sacchari*, 44.5 g L^{-1} cell dry weight was achieved, with 33 % being polyhydroxybutyrate (PHB), while 37 g L^{-1} cell dry weight of *Pseudomonas chlororaphis* contained 10 % PHA (Cerrone et al., 2015). Koller et al. examined the addition of green grass juice and silage juice to fermentation media for the production of PHA by *Cupravidus necator*. Supplementing the minimal medium with 5 % (v/v) green grass juice resulted in a productivity of 0.28 g L^{-1} h^{-1} of PHB, which is no improvement compared to the productivity in minimal medium.

Supplemented with 5 % (v/v) silage juice, however, 0.65 g L^{-1} h^{-1} were achieved, exceeding the result obtained with the common addition of casamino acids by 5 % (Koller et al., 2005).

II.5.3 Extraction of proteins

The extraction of proteins from green waste biomass with low lignin content is studied since 1942 (Pirie, 1942). It is proposed to pulp the biomass with added water and then press the material. To precipitate the protein, salt or acid is added to the press juice. On a larger scale, heating the fluid is a more convenient process. Up to 60 – 70 % of the press cakes' dry matter is protein (Pirie, 1969). Dotsenko and Lange examined the recovery of protein from white clover and ryegrass press cake. Using aqueous extraction, 40 % of the crude Kjeldahl protein content, which is 10 % for white clover and 16 % for ryegrass, could be recovered. Treatment with proteases increased the recovery rate to 80 % and previous addition of carbohydrases improved the rate further to 95 % (Dotsenko and Lange, 2017). LaCour et al. increased the amount of precipitated protein in press juices from ryegrass, red clover, a grass-clover mixture, and spinach by adding lignosulfonates from 34 – 46 % to 41 – 55 % (La Cour et al., 2019).

Another method to obtain protein from biomass is the single-cell protein method. Hereby, the biomass serves as a substrate for microorganisms with high protein content. Pihlajaniemi et al. use pretreated ensiled grass hydrolysates as media for the fungus *Paecilomyces variotii*, which is known to have a protein content of about 50 %. Thereby, the overall protein yield does not only stem from the direct extraction of the biomass, but rather from the nutrients metabolized by the fungus. By using an ammonia-based treatment, part of the residual nitrogen can also be incorporated in the produced proteins (Pihlajaniemi et al., 2020).

II.5.4 Extraction of lipids and acids

Other value-added products obtainable from green waste biomass as feedstock are lipids or acids. However, there are only a few publications on their extraction. For example, Attard et al. extracted various lipophilic compounds from *Miscanthus* pretreated with supercritical carbon dioxide. From the leaves a yield of approximately 2 % wax was obtained, containing long-chain hydrocarbons, fatty acids, *n*-policosanols, aldehydes, wax esters, sterols, and steroid ketones (Attard et al., 2016).

Bichot et al. pretreated *Miscanthus* with microwaves and extracted 0.6 % ferulic acid and 3.9 % coumaric acid (Bichot et al., 2019). According to Karlen et al., extraction titers of at least 50 g kg^{-1} biomass are necessary to make the extraction of hydroxycinnamic acids economically feasible (Karlen et al., 2020).

II.5.5 Extraction of lignin

Although the lignin share of lignocellulosic biomass is problematic for the production of fermentable carbohydrates, it is also a valuable component of interest itself. Due to its abundance, it is an attractive feedstock to obtain various aromatic compounds. Common industrial extraction methods are Kraft and sulfite processes, both of which apply harsh conditions. This results in the formation of various stable polymers from intermediates, which complicate further use of the lignin components such as Kraft lignin and lignosulfonates (Bertella and Luterbacher, 2020). Although the organosolv process is a gentler extraction method, the hereby obtained lignin is highly polymerized as well. Generally, the degree of polymerization can be correlated with the severity of the pretreatment conditions (Dababi et al., 2016). Schwarz et al. demonstrated a lignin yield of 41 % from organosolv treatment of grass silage (Schwarz et al., 2016).

A possible use of lignin is as a component in bio-polymers (Bertella and Luterbacher, 2020). Other conceivable applications are nano-composites, bio-surfactants, and phenolic resins (Alzagameem et al., 2018). Due to its antioxidative and bioactive properties, lignin could also be used as an antimicrobial agent (Alzagameem et al., 2019). Huang et al. show further potential target chemicals derived from lignin such as vanillin, polyhydroxyalkanoates, muconic acid, cyclohexanes, and phenols (Huang et al., 2020). Microorganisms with the ability to secrete ligninolytic enzymes and to take up aromatic degradation products of lignin are a promising starting point for a consolidated bioprocess based on lignin (Salvachúa et al., 2015).

The direct extraction of lipophilic compounds such as fatty acids, long-chained carbohydrates and aldehydes from green biomass is not profitable due to the low yields received. The extraction of lignin, often as a by-product during pretreating green waste to obtain fermentable sugars, is more promising. But although there are several interesting approaches to utilize lignin, an industrial process for its valorization is still lacking. However, obtaining products via pressing green waste is seminal. Proteins are successfully extracted as feed supplement. Acidic precipitation of proteins can be conducted chemically by adding for example propionic acid (Brugger et al., 2016) or by fermenting the press juice using lactic acid bacteria (Santamaría-Fernández et al., 2020), reaching crude protein yields of up to 430 g kg^{-1} dry matter. Therefore, green waste is able to compete with usually employed soybean matter (Brugger et al., 2016). The press juice contains a range of nutrients suitable to replace or supplement fermentation media, even after depletion of proteins. The contained sugars are a valuable carbon source for fermentative processes, e.g. the production of PHA. Summarizing, green waste press juice constitutes an alternative for expensive fermentation media compounds.

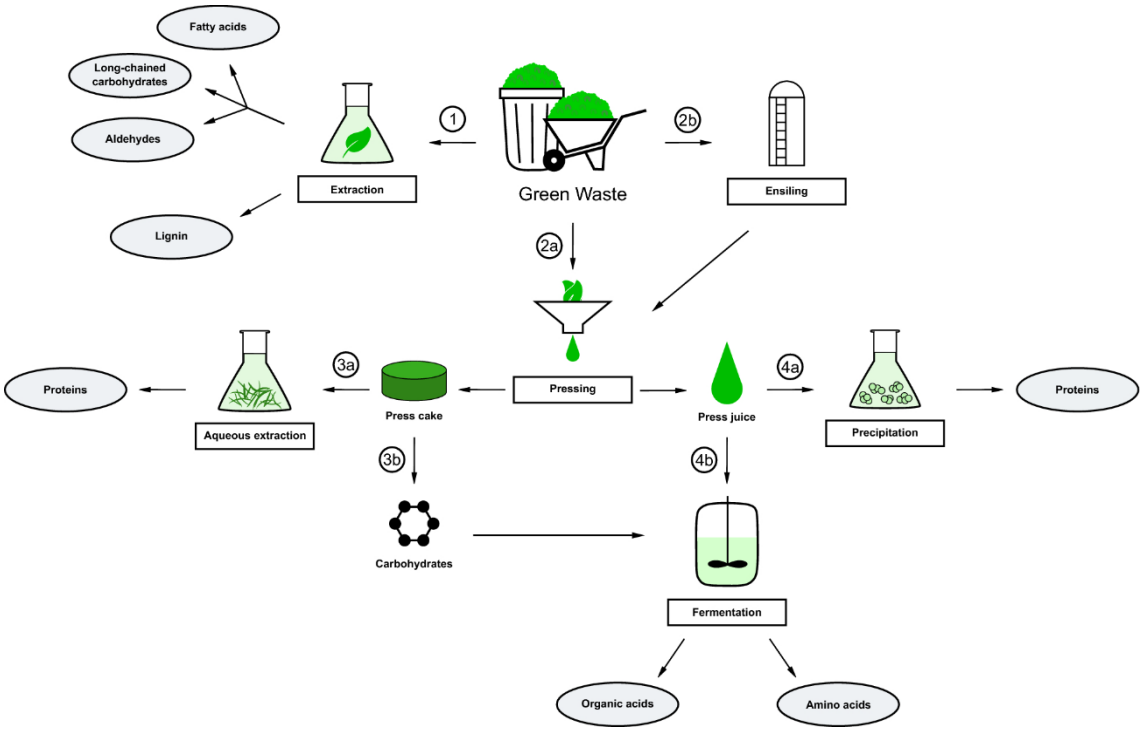

Figure 3: Processes for the production of extracts from green waste. Products are obtained from green waste directly by extraction (1) or via pressing (2a), possibly after ensiling the biomass (2b). After pressing, proteins are obtained from aqueous extraction of the press cake (3a) and precipitation of the press juice (4a). Carbohydrates gained from the press cake (3b) are added to the press juice for fermentation (4b).

II.6 Chemical and biotechnological conversion of green waste

The possibility of chemical or biotechnological conversion of biomass components to chemicals has already been shown in large quantities, while the conversion of green waste has not been investigated extensively yet. A relatively simple conversion of green waste, in particular, the included cellulose, hemicellulose, or lignin, to bulk and fine chemicals is a promising way to generate higher value. It is important that green waste is not processed too extensively as the recycling should be financially worthwhile. The U.S. Department of Energy identified 12 possible sugar-derived value-added building block chemicals from biomass (Werpy and Petersen, 2004). Succinic acid, levulinic acid, and xylitol are three of these building blocks, which have already been shown to be produced from various herbaceous green materials. Other frequently produced chemicals are furfural and HMF, which have already been described as inhibitors of fermentation but are also valuable basic chemicals. The production of these promising substances as well as other potential candidates by chemical and biotechnological conversion of green waste or herbaceous biomass is described hereinafter. The most promising substances are shown in figure 4.

II.6.1 Production of levulinic and succinic acid

Especially levulinic and succinic acid are popular building block chemicals produced from herbaceous biomass. Levulinic acid is obtained via an acid-catalyzed reaction. Girisuta et al. demonstrated the

optimized hydrolysis of water hyacinth to levulinic acid catalyzed by sulphuric acid (Girisuta et al., 2008). The washed, chopped and dried leaves of the water hyacinth were incubated with an aqueous solution of sulphuric acid at constant temperature and various reaction times. The reaction parameters temperature, sulphuric acid concentration, and water hyacinth intake have been investigated. High acid concentrations > 0.5 M yielded levulinic acid as the major organic acid with a maximum yield of 53 mol% based on the amount of C6-sugars. Raspolli Galletti et al. showed the hydrothermal conversion of olive tree pruning to levulinic acid in the presence of homogeneous acid catalysts (Raspolli Galletti et al., 2012). The powdered biomass was incubated with niobium phosphate and water with HCl in an autoclave under an inert atmosphere. At 200 °C, the reaction resulted in up to 66 % levulinic acid of the theoretical yield. Furthermore, Raspolli Galletti et al. demonstrated the hydrothermal conversion of giant reed (*Arundo donax* L.) to levulinic acid in the presence of dilute HCl (Raspolli Galletti et al., 2013). The reaction yielded up to 23.3 % of levulinic acid based on dry biomass, corresponding to a maximum theoretical yield of 82.7 %. A similar process was also shown by Antonetti et al. (Antonetti et al., 2015). They investigated the production of levulinic acid from giant reed in the presence of HCl and were able to achieve a theoretical yield of up to 90 %. Dussan et al. demonstrated the acidic hydrolysis of *Miscanthus* × *giganteus* cellulose and hemicellulose with H_2SO_4 in a two-stage process into levulinic acid (58 – 72 mol%) and furfural (27 mol%), respectively (Dussan et al., 2013).

Succinic acid is usually produced through the fermentation of herbaceous biomass. Dąbkowska et al. showed the fermentative production of succinic acid from *Miscanthus* × *giganteus* (Dąbkowska et al., 2019). The biomass was subjected to a glycerol-based pretreatment and enzymatically hydrolyzed. The hydrolysate was fermented with *Actinobacillus succinogenes* 130Z yielding up to 82 % succinic acid. Similarly, Kuglarz and Rom showed the succinic acid production from *Miscanthus* × *giganteus* hydrolysate by *A. succinogenes* 130Z resulting in a yield of up to 76 % (Kuglarz and Rom, 2019). Ventorino et al. demonstrated the succinic acid production from *Arundo donax* hydrolysate with *Basfia succiniciproducens BPP7* (Ventorino et al., 2017). They were able to produce 6.1 g L^{-1} of succinic acid via separate hydrolysis and fermentation in small-scale shake flask experiments. Through optimization of growth conditions and medium composition, a maximal titer of 9.4 g L^{-1} of succinic acid could be obtained with the *A. donax* hydrolysate as well as yeast extract as the main carbon and nitrogen sources in a 2.5 L batch experiment after 24 h. This corresponds to 0.072 kg of succinic acid from 1 kg of pretreated *A. donax* biomass. The highest succinic acid production and yield were achieved with just the liquid fraction. It was assumed that this is due to toxic compounds in the solid fraction of the biomass hydrolysate like e. g. furfural, HMF, *p*-hydroxybenzoic aldehyde, or vanillin. Previously, Ventorino et al. already demonstrated the production of the organic acids lactic acid, succinic acid, and acetic acid from an *A. donax* hydrolysate via the strain *Cosenzaea myxofaciens* BPM1, which was isolated from bovine rumen (Ventorino et al., 2016). Gunnarsson et al. demonstrated the conversion

of industrial hemp to succinic acid by *Actinobacillus succinogenes* (Gunnarsson et al., 2015). The most effective pretreatment conditions determined were thermochemical treatment with 3 % H_2O_2 at 121 °C prior to enzymatic hydrolysis, which resulted in a maximum sugar yield of 73.5 %. The fermentation with the hemp hydrolysate yielded a maximum of 83 % (21.9 g L^{-1}) of succinic acid.

II.6.2 Production of furfural and HMF

The aldehydes furfural and HMF are again produced by chemical processing. Rivas et al. developed a biorefinery strategy for the production of furfural and HMF from *Miscanthus* × *giganteus* (Rivas et al., 2019). *Miscanthus* was pretreated hydrothermally resulting in a soluble hemicellulose-rich and a solid cellulose- and lignin-rich fraction. Acidic processing of the soluble fraction yielded 78 % furfural, while enzymatic and subsequent chemical processing of the solid phase resulted in a HMF yield of 49 %. Mandalika and Runge demonstrated the sulfuric acid-based dehydration of hot water hydrolyzed *Miscanthus* and switchgrass for the production of furfural with yields of over 90 % for both substrates based on the total pentose (Mandalika and Runge, 2012). Yang et al. demonstrated the synthesis of furfural and HMF from switchgrass with $AlCl_3 \cdot 6H_2O$ as a catalyst in a biphasic medium of water/tetrahydrofuran (Yang et al., 2012). The reaction system yielded 66 % furfural based on the pentose content and 23 % HMF based on the hexose content. Zhang et al. demonstrated the dehydration of switchgrass to furfural catalyzed by polymer-bound sulfonic acid in ionic liquid yielding 22 % of furfural (Zhang et al., 2014). A conceptual design for the production of furfural and HMF as well as dimethyl furfural from switchgrass has been developed by Martín and Grossmann (Martín and Grossmann, 2016). Imteyaz Alam et al. demonstrated the conversion of various weed species (waste plant materials) from India into HMF as well as 5-ethoxymethyl-2-furfural, a promising next-generation biofuel, with a solid acid and ionic liquid catalysts (Imteyaz Alam et al., 2012). Using different Brønsted acidic ionic liquid catalysts resulted in HMF yields ranging from 11 to 58 wt% and 26 to 52 wt% for different weeds, respectively. The direct conversion of the weeds into HMF with a solid acid catalyst yielded 8 – 32 wt% of HMF. The stated maximum yields were each achieved for foxtail weed. Antonetti et al. showed the hydrothermal conversion of giant reed to furfural in the presence of an acid catalyst as described above. They were able to produce up to 70 % of the theoretical yield (Antonetti et al., 2015).

II.6.3 Production of other valuable substances

The biotechnological production of xylitol from grassy biomass has been shown for certain yeasts, which are able to produce xylitol from the xylose in hydrolyzed biomass. West demonstrated the production of xylitol with the yeast *Candida* on medium containing a hydrolysate of North American perennial prairie grass big bluestem (West, 2009). The grass hydrolysate was produced by a combination of dilute acid hydrolysis (1 % sulfuric acid) and enzymatic treatment with xylanase (West,

2009). Neeru et al. showed the xylitol production from acidic hydrolyzed switchgrass with *Pichia stipitis CBS 5773* (Neeru et al., 2013). They were able to yield 48 % xylitol based on the initial xylose.

The fermentative production of polyesters like polyhydroxyalkanoates from grassy biomass is also possible as shown by Davis et al. and Zhang et al. Davis et al. investigated the production of medium-chain length PHA from perennial ryegrass by different *Pseudomonas* strains (Davis et al., 2013). To evaluate different pretreatment methods prior to enzymatic hydrolysis, the grass was pretreated either with 2 % NaOH at 120 °C or water alone and with or without a subsequent sodium chlorite/acetic acid treatment for additional lignin removal. The best results were achieved for NaOH and sodium chlorite/acetic acid treatment, resulting in a predominantly glucose-containing hydrolysate. Using the glucose-rich hydrolysates (74 – 77 %) as a sole carbon source resulted in an accumulation of 20 – 34 % PHA of the cell dry mass by the *Pseudomonas* strains, which is comparable to growth and yields with conventional sugars. Zhang et al. demonstrated the production of PHB from alligator weed hydrolysate with *C. necator* (Zhang et al., 2020). The Alligator weed hydrolysate was prepared via acid or enzyme treatment. While the addition of enzymatic hydrolysate as a sole carbon source resulted in an increased PHB production, the acid hydrolysate produced less PHB due to inhibitors affecting microbial growth. After 72 h of fermentation with the enzymatic hydrolysate at optimized conditions, they were able to produce 4.8 g L^{-1} of PHB at a final cell dry weight of 8.5 g L^{-1}.

Another example of the production of esters is shown by Cao et al. They investigated the chemical cellulose acetate production directly from green landscaping waste after pretreatment with dilute phosphoric acid for separation of hemicellulose (Cao et al., 2018). The pretreated residues were treated with acetic anhydride and sulfuric acid to produce cellulose acetate. The mean yield of cellulose acetate from all green landscaping waste samples was approximately 35 %. Xylose and arabinose were obtained as value-added by-products from the pretreatment with dilute phosphoric acid.

The production of fats has also been shown. Employing a mixed culture of the microalga *Chlorella pyrenidosa* and the yeast *Yarrowia lipolytica* for the fermentation of garden wastes, Yu et al. obtained lipid contents of up to 0.8 g L^{-1} (Yu et al., 2019). Mast et al. achieved 0.93 g L^{-1} of mainly long-chain fatty acids by fermenting *Miscanthus* hydrolysates using the red-yeast *Rhodotorula glutinis* over 96 h (Mast et al., 2014).

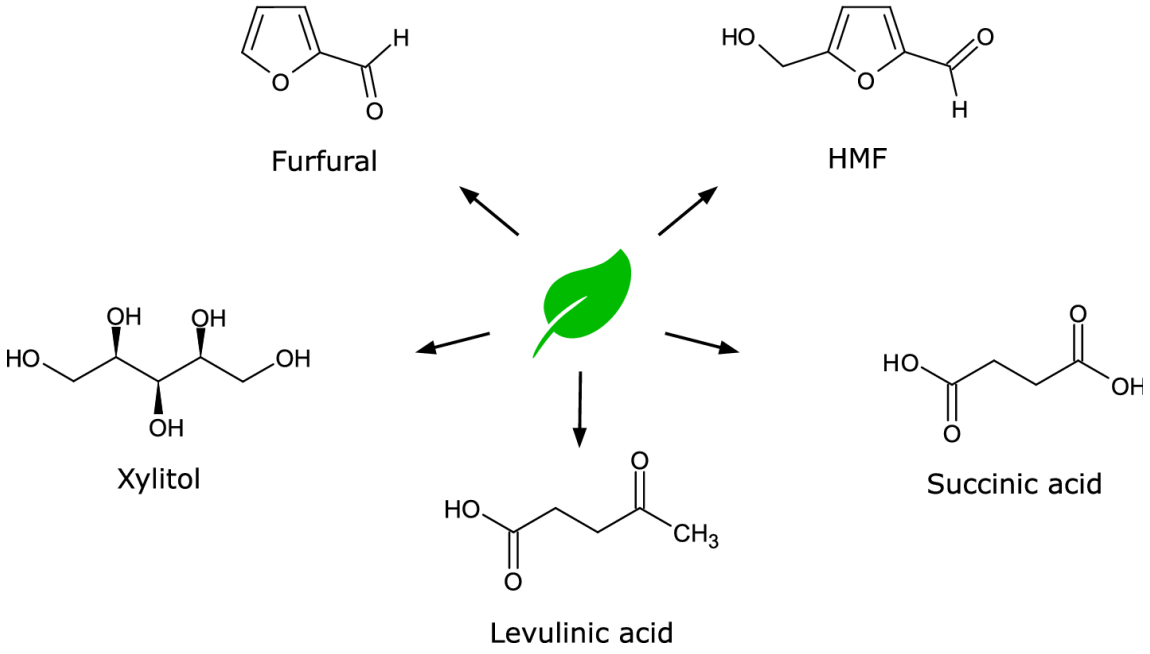

Figure 4: Potential chemicals from herbaceous lignocellulosic materials

II.6.4 Consolidated bioprocessing

When considering the fermentative production of chemicals from green waste, pretreatment e. g. through enzymes and product synthesis by microorganisms are usually separated processes. The combination of enzyme generation, biomass hydrolysis, and the subsequent conversion to final products in a single stage is known as consolidated bioprocessing, which can be achieved by engineering biomass-degrading as well as product synthesis capabilities into one organism (Bokinsky et al., 2011). A dedicated process of enzyme generation for pretreatment of lignocellulose represents a major cost factor, which could be avoided by this approach. Either a synthesis pathway, a biomass degradation pathway or both need to be integrated into the strain without overburdening the organism. For example, Steen et al. were able to engineer a pathway for the production of structurally tailored fatty esters (biodiesel), fatty alcohols, and waxes as well as the expression of hemicellulases into *E. coli* for a possible production of biodiesel from hemicellulose (Steen et al., 2010). A great example of consolidated bioprocessing of green waste has been shown by Bokinsky et al., who engineered biomass-degrading *E. coli*, which are able to grow on cellulose and hemicellulose fractions of plant biomass pretreated with ionic liquids (Bokinsky et al., 2011). They introduced three different biofuel synthesis pathways to produce fuel substitutes or precursors directly from ionic liquid-treated switchgrass. The fermentation on ionic liquid-treated switchgrass as the main carbon source resulted in the production of 71±43 mg L^{-1} fatty acid ethyl esters, 28±5 mg L^{-1} butanol, and 1.7±0.6 mg L^{-1} pinene. Bokinsky et al. furthermore showed that their engineered *E. coli* strains were not only able to grow on pretreated switchgrass but also as mono- and co-culture on pretreated *Eucalyptus globulus* and yard waste. The interesting point in the approach of Bokinsky et al. with regard to green waste

processing is that the engineered microorganisms appear to be applicable to a variety of pretreated green biomasses. To fully utilize plant biomass, the combination of microorganisms with both cellulose and hemicellulose degrading abilities could potentially be a promising approach for green waste conversion. Building on that, the desired product synthesis route has to be integrated without overloading the organism to produce value-added chemicals within a consolidated bioprocessing scheme.

II.7 Electrode materials from green waste

Besides the extraction of valuable compounds and the conversion into basic and fine chemicals, green waste might be utilized profitably as a feedstock for cheap and ecological functional materials. A promising approach to green waste functionalization is its carbonization and subsequent use as electrode material. Carbon-based materials have been proven to be suitable electrodes for electro-biotechnological applications, such as microbial fuel cells or bioelectrosynthesis. Bioelectrochemical systems can contribute to a greener chemistry and bioeconomy. We demonstrated several examples such as bioelectromethanogenesis, allowing conversion of electricity into methane through electroactive methanogens (Enzmann et al., 2019a). The production is influenced among other things by the carbon electrodes used. Besides cathodic working electrodes, we demonstrated that the anodic counter electrode plays an important role in the cathodic bioelectromethanogenesis process as well (Enzmann et al., 2019b). Another example of bioelectrochemical systems are electro-fermentations, which utilize electrical energy to control a reaction in the desired direction as shown for the carbon-efficient production of biobutanol with *Clostridium acetobutylicum* (Engel et al., 2019). High costs due to expensive electrode materials are a major bottleneck of hindering the commercialization of such technologies until now. We already reviewed different types of electrodes, including carbonized materials, for their application in bioelectrochemical systems and highlighted the importance of cost-effective designs (Krieg et al., 2014). Therefore, cheap biomass-derived electrodes could pave the way for commercialization of these novel technologies. The path from green waste to “green” chemicals or energy by functionalization of green waste as electrode material is summarized in figure 5.

The carbonization of herbaceous lignocellulosic biomass has already been investigated for the production of coal products as an energy source (Kambo and Dutta, 2015, 2014). Especially foliage, which is not suitable for fermentation due to its low content of anaerobically accessible substances, is a promising substrate for carbonization. Coal products from biomass are attractive fuel sources in comparison to raw biomass due to their higher carbon content and calorific value. Additionally, raw biomass causes ignition and combustion problems in combustors due to its high moisture and ash contents (Sadaka et al., 2014). Carbonized biomass is also easy to transport and to store. Biomass-

derived carbon materials have already been investigated pretty extensively as electrodes for supercapacitors (Gao et al., 2017; Lu and Zhao, 2017).

While biochar production was developed and investigated mainly for woody materials, during the last decades, it has also been studied extensively for herbaceous materials. Carbonized herbaceous biomass is mainly manufactured by hydrothermal carbonization (HTC), producing a solid, so-called biochar, biocoal, or hydrochar (Guo et al., 2015; Medick et al., 2017). During HTC, biomass is carbonized via hot compressed water. While reaction temperatures typically range from 200 to 275 °C, the pressure lies above the saturation pressure of water to ensure its liquidity (Reza et al., 2013). After cooling of the reactor following HTC, the hydrochar is separated by filtration and is dried at 105 °C for 24 h (Eibisch et al., 2013; Guo et al., 2015; Liu et al., 2013; Reza et al., 2013). The structural transformation of cellulose, hemicellulose, and lignin and therefore the characteristics of the biochar depends mainly on temperature and duration as well as the reaction environment (Sadaka et al., 2014). The main products of hydrothermal treatment can be distinguished in solid products (biochar), liquids, and aqueous as well as gaseous (mainly CO_2), while the properties and distribution of these products depend on the reaction conditions (Liu et al., 2013). Therefore, an important characteristic of HTC is the solid mass yield (Guo et al., 2015). Typically, the HTC of biomass leaves concentrated and dissolved sugars and organic acids in water (Reza et al., 2012). In the sense of a biorefinery concept, it should be considered, whether these dissolved molecules can be further used, although possible unwanted components like HMF are present in the liquid (Yan et al., 2010).

Due to the increasing interest in carbonized materials, there are several publications describing the carbonization of low-lignin materials like grasses, leaves, and even green waste. Reza et al. manufactured and characterized biochar from *Miscanthus* and switchgrass (Reza et al., 2013). The biomass was crushed and dried prior to HTC at 200, 230, or 260 °C with a liquid-to-solid-ratio (LTSR) of 1:5 for 5 min. The highest mass yields were achieved at 200 °C resulting in 79 and 87 % for *Miscanthus* and switchgrass, respectively. Another carbonization process of switchgrass has been shown by Sadaka et al. (Sadaka et al., 2014). They carbonized ground switchgrass at 300, 350, or 400 °C for 1, 2, or 3 h, but without the addition of water. As described above, biochar mass yield also decreased with increasing temperature and time from 82.6 % to 35.2 % due to loss of moisture and depolymerization of cellulose, hemicellulose, and lignin. Guo et al. investigated the HTC of lawn grass and the characteristics of the hydrochar (Guo et al., 2015). Lawn grass was dried, shredded, and carbonized at 200 or 240 °C for 30 to 180 min (LTSR 1:30). Solid mass yield ranged from 31 to 50 %. A prolonged residence time was shown to be favorable to the formation of hydrochar from lawn grass. Eibisch et al. compared the physicochemical properties of HTC products from various raw materials (Eibisch et al., 2013). The raw materials (woodchips and grass cuttings amongst others) were shredded and

hydrochars were produced at 180 °C and 20 bar over 8 h (LTSR 1:10). The hydrochars from grass showed a high amount of hydrophilic functional groups. The higher hydrophilicity of carbonized grass in comparison to hydrochar from woody biomass is advantageous for the use as electrode material since carbonized biomass is already more hydrophobic than the wet feedstock (Acharjee et al., 2011). Liu et al. investigated the HTC of dead eucalyptus leaves under different carbonization conditions (Liu et al., 2013). HTC was performed at 150 – 375 °C for 30 min (LTSR 1:10). Biochar yield decreased rapidly with increasing temperature from a yield of 90 % at 150 °C to 30 % at 375 °C. Zeymer et al. conducted a technical, economic, and environmental assessment of HTC of green waste for its conversion into coal as an energy source (Zeymer et al., 2017). Prior to the HTC, the green waste was chopped, sieved, and washed. The HTC yielded 76 % mass of hydrochar from the green waste. Unfortunately, they did not describe the HTC method further. However, Shao et al. investigated the microwave HTC of green waste consisting of fallen leaves and deadwood (Shao et al., 2019). The green waste was pretreated by removing dirt, milling, and drying at 105 °C for 24 h. They varied the parameters temperature (130 – 190 °C), holding time (0.5 – 2 h), and liquid-to-solid ratio (7:1 – 10:1) with regard to the calorific value of the hydrochar. The yield of hydrochar from green waste ranged from 50.4 % to 76.8 %.

It has been shown that the biochar mass yield decreases significantly with increasing temperature (Guo et al., 2015; Liu et al., 2013; Reza et al., 2013). Furthermore, carbon content increases with increasing temperature, while oxygen content decreases (Eibisch et al., 2013; Liu et al., 2013). Therefore, the temperature is a major parameter as it influences yield and carbon content. At 200 °C, hemicellulose is eliminated predominantly (Reza et al., 2013) and is almost entirely removed after 30 min (Guo et al., 2015). At reaction temperatures above 200 °C, hemicellulose degrades to the full extent (Reza et al., 2013). The amount of cellulose is also reduced with increasing temperature during HTC (Reza et al., 2013). Cellulose components slowly degrade at 240 °C with increasing residence time to form hydrochar, which is shown by a decrease in the crystallinity index (Guo et al., 2015). While most of the hemicellulose and cellulose already decompose at temperatures below 250 °C, lignin degradation only takes place at higher temperatures around 300 °C (Liu et al., 2013). Due to the loss of cellulose and hemicellulose, lignin percentage increases with reaction temperature (Reza et al., 2013). Reza et al. give a great overview of the composition of hydrochar from grass in comparison to the raw material (Reza et al., 2014). An overview of typically applied process parameters (Temperature, LTSR, pressure, and time) for the HTC of the raw materials described above as well as the resulting biochar mass yield is given in table 3.

Table 3: Investigated parameters for the HTC of herbaceous materials and green waste including hydrochar mass yields

Raw material	Temp. [°C]	LTSR[a] (w/w)	Pressure [bar]	Time	Mass yield [%]	References
Miscanthus, switchgrass	200, 230, 260	1:5	10 - 50	5 min	57 - 87	(Reza et al., 2013)
Lawn grass	200, 240	1:30	N/A	30 - 180 min	31 - 50	(Guo et al., 2015)
Grass cuttings	180	1:10	20	8 h	N/A	(Eibisch et al., 2013)
Eucalyptus leaves	150 - 375	1:10	N/A	30 min	30 - 90	(Liu et al., 2013)
Green waste	130 - 190	7:1 - 10:1	N/A	0.5 h - 2 h	50 - 77	(Shao et al., 2019)

[a]LTSR = Liquid-to-solid-ratio

Although there are several publications on the carbonization of herbaceous materials or green waste, publications addressing the utilization of hydrochars as electrode materials are only sparsely available. However, the usage of other biomass materials as electrodes has been investigated extensively in the last decade. Just recently, Yang and Chen summarized the potential of biomass-derived electrodes for microbial fuel cells (Yang and Chen, 2020). They show examples of electrodes from e. g. fruit residues, wood, mushrooms, nutshells, and sludge. The potential of novel electrode materials depends on various parameters like stability, structure, surface area, conductivity, biocompatibility, etc. The pore structure of the electrodes is particularly important for electro-biotechnological applications since the pores have a decisive influence on the bacterial growth on the internal electrode surface as well as substrate and ion transport within the electrode (Yang and Chen, 2020). The amount of extractives of biochars from grassy material in comparison to woody material is somewhat higher and therefore indicates a more porous structure than biochar from woody material (Reza et al., 2013). Pore sizes of hundreds of micrometers to millimeters allow bacteria to grow unhindered within the pores, while for smaller pore sizes the biofilm thickness decreases due to limited mass transport within the electrode (Yang and Chen, 2020). In addition to the pore size, colonization by bacteria is dependent on surface properties, temperature, and substrate concentration (Yang and Chen, 2020). As mentioned previously, hydrochar from grassy material exhibits higher hydrophilicity in comparison to hydrochar from woody biomass (Eibisch et al., 2013), which is advantageous for the growth of microorganisms. Some general advantages of biomass-derived electrodes naturally occurring within biological material are an increased surface area for microbial growth, pores for mass transport of ions and oxygen, high electrical conductivity for fast electron transport, and low resistance as well as the low production

costs (Yang and Chen, 2020). These characteristics are important prerequisites for their use in commercial and large-scale applications.

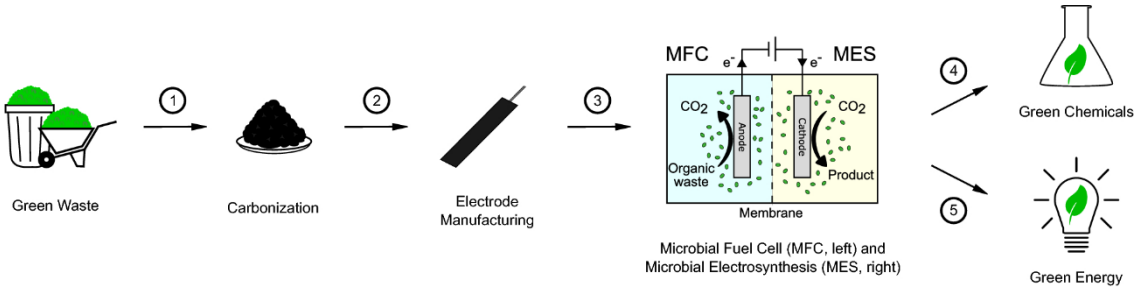

Figure 5: Schematic presentation of electrode manufacturing from green waste and application in bioelectrochemical systems for the production of "green" chemicals or energy. Potential utilization of green waste for electrodes in electro-biotechnological applications: Green waste is carbonized and activated through hydrothermal carbonization and/or pyrolysis (1), formed into suitable electrodes (2) and used in electro-biotechnological applications (3) to produce green chemicals in bioelectrosynthesis (4) or green energy in microbial fuel cells (5)

Altogether, there are already several publications concerning the carbonization of green waste or herbaceous materials. However, publications investigating the carbonized materials as electrode material in electro-biotechnological applications are lacking. The use of electrodes made of different carbonized herbaceous biomass was shown almost exclusively for the application in capacitors (Jain et al., 2020; Kolanowski et al., 2020; Meng et al., 2020; Saning et al., 2019). Deng et al. showed the use of carbonized alfalfa leaves, activated with KOH, as a cathodic catalyst in microbial fuel cells (Deng et al., 2017). The biomass-derived carbon material showed superior current density and long-term stability as well as similar performance characteristics in comparison to a Pt/C cathode catalyst. This exceptional example shows that there is still an enormous research potential. Due to promising properties and significantly lower costs compared to conventional electrode materials, electrodes from green waste might be a profitable alternative for electro-biotechnological applications.

II.8 Conclusions

Our overview shows that many valuable chemicals and materials can be produced from green waste. The direct extraction of lipophilic compounds such as fatty acids, long-chained carbohydrates, and aldehydes from green waste does not seem to be profitable due to the low yields received, while the extraction of proteins as well as lignin, which is released as a by-product during pretreatment, is more promising. As potential transformation products of green waste we have identified levulinic acid, furfural, and HMF from chemical conversion and succinic acid, xylitol, and polyhydroxyalkanoates from fermentative conversion. In particular, the fermentative utilization of green waste shows great potential, whether as a carbon source through sugars received after pretreatment or as a supplement in the form of e. g. press juice. In relation to this, a consolidated bioprocess tailored to green waste is

a promising perspective. Furthermore, there are several publications concerning the carbonization of green waste materials, whereas the application as electrodes is hardly researched. These “green” electrodes could contribute to novel bioelectrochemical processes in a bio-based economy. However, alternative recycling methods of green waste are far from being as extensively researched as other waste streams. Studies on the material utilization of heterogeneous green waste in its entirety are hardly available. Consequently, economic and ecological evaluations of potential alternatives are also lacking. The comparability of methods for green waste processing from pretreatment to conversion is limited, e. g. there is often a lack of quantitative data regarding energy and material consumption. But the profitable realization of the utilization of green wastes will not depend solely on technical approaches. Collecting and logistics must always be considered as well. For green waste to be recycled economically, it must be done as locally as possible. Nevertheless, the reviewed publications show the untapped potential of green waste. Finally, it is likely that only a cascade-like use of the collected green waste will lead to an economic process. In a first step, the green waste would be pressed. The press juice would be subsequently used for fermentative production of high-value substances. In parallel, electrodes could be produced from the remaining solid biomass by carbonization. After use in electro-biotechnological applications, these electrodes could be utilized energetically, e. g. in biogas plants. This cascade-like processing would result in a green waste-biorefinery.

II.9 List of abbreviations

AFEX	Ammonia Fiber Expansion
ARP	Ammonia Recovery Process
CAFI	Biomass Refining Consortium for Applied Fundamentals and Innovation
COSLIF	Cellulose solvent- and organic solvent-based lignocellulose fractionation
CS	Combined severity factor
DES	Deep eutectic solvents
HMF	5-hydroxymethylfurfural
HTC	Hydrothermal carbonization
LTSR	Liquid-to-solid-ratio
MSW	Municipal solid waste
PHA	Polyhydroxyalkanoate
PHB	Polyhydroxybutyrate
SAA	Soaking in aqueous ammonia

II.10 Declarations

Ethics approval and consent to participate: Not applicable.

Consent for publication: Not applicable.

Availability of data and materials: Not applicable.

Competing interests: The authors declare that they have no competing interests.

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Authors' contributions:

All authors contributed to the manuscript conceptualization. MV and AL wrote the manuscript and contributed equally to this review. RL and DH commented on the manuscript and reviewed it. All authors read and approved the final manuscript.

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III. Effects of Pretreatment on the Biocatalysis of Renewable Resources

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III.1 Abstract

Within a biorefinery platform several conversion steps such as pretreatment, saccharification, fermentation and downstream processing are necessary to obtain the final bio-based product(s) from lignocellulosic biomass. The structural composition of the biomass, especially the lignin content, determines the necessary pretreatment steps. To obtain sugar monomers, the hydrolysis of lignocellulosic biomass is an essential step. This work examines the impact of different pretreatments on the sugar release during biocatalysis. Even without prior pretreatment the biocatalysis of low lignin biomass achieves glucose yields of up to 93 %, while the biocatalysis of high lignin biomass requires an upstream hydrothermal procedure to achieve a glucose yield of 74 %.

III.2 Introduction

During the past decades the uncontrolled consumption of fossil-based resources has caused several environmental issues, such as greenhouse gas emissions, climate change and the reduction of natural resources [1]. Further, at present, the energy and organic chemical consumptions are growing incessantly due to the rapid increase of the world's population with improved standards of living [2]. As a consequence, the exploration of new renewable resources to produce energy and chemicals is becoming imperative. In this scenario, lignocellulosic biomasses have been recognized as emerging and sustainable alternative resources because of their relatively low cost, great abundance, and sustainable supply [3]. In addition, an important element in the transition towards a bio-based economy is the biorefinery facility. The International Energy Agency defines biorefining as the conversion of renewable raw materials into a spectrum of marketable products and bioenergy [4]. Among others, lignocellulosic biomasses (LCB) such as municipal green waste and herbaceous materials are particularly attractive as they are one of the major underused waste streams occurring in urban and agricultural areas respectively [5]. In order to use LCB in a biorefinery concept, physical, chemical and catalytic pre-treatment steps followed by (bio)catalytic conversion steps are necessary. The first ones have great influence on the efficiency of the (bio)catalytic conversion steps.

The chemical composition of lignocellulosic biomass determines their potential as biorefinery feedstocks, even if it differs depending on the species, its maturity, and environmental conditions [5, 6]. Lignocellulose is mainly composed of cellulose (30-50 %), hemicellulose (20-35 %) and lignin (15-20 %) [7]. Cellulose is a linear polymer of *D*-glucose linked to each other by β-1,4 glycosidic bonds [8]. Because of its crystalline structure, cellulose is highly resistant to hydrolysis thus impeding efficient conversion of this polymer during biorefining processes [2]. Hemicellulose is an amorphous and branched polymer of five carbon (xylose and arabinose) and six carbon sugars (galactose, glucose and mannose) linked by β-1,4 and β-1,3 glycosidic bonds [2]. Due to their amorphous branched structure and low molecular weight, hemicellulose can be readily hydrolyzed [8]. Lignin is an amorphous heteropolymer network of three-dimensional polymers composed of three different methoxylated

phenylpropane units (coniferyl alcohol, sinapyl alcohol and coumaryl alcohol) that are bonded together by different kinds of linkages [3]. It provides structural support and acts as natural, impermeable barrier to microbial attacks and oxidative stress on plant tissues [3, 8]. As reported by Langsdorf et al., slight differences can be observed with regards to the biomass type [5]. In particular, stems and leaves in municipal waste contain a lower amount of cellulose and hemicellulose and higher concentration of lignin compared to grassy material [5]. In the current study, two different lignocellulosic biomasses were considered for a high-lignin content biomass (municipal green waste) and low-lignin content biomass (perennial ryegrass). The overall structure made up of cellulose, hemicellulose, and lignin exist in complex carbohydrates linkages formed by hydrophobic and covalent interactions between lignin and carbohydrates. All the structure components in biomass cellulose-hemicellulose-lignin are responsible for the recalcitrant character of lignocellulose [7].

To overcome biomass recalcitrance, pretreatment is used for the isolation of cellulosic and hemicellulosic polysaccharides [7]. The main objectives of this process are the breakdown of cell wall structures, the reduction of crystallinity, particle size or degree of polymerization, the increase of porosity and accessibility, as well as the delignification in order to make the cellulose and hemicellulose more accessible to hydrolytic enzymes. Factors such as energy consumption and economics of a pretreatment process are also a key criterion for defining the viability of the process [9]. A generalized classification of pretreatment methods groups them into physical, chemical, biological or combinatorial pretreatment [3, 10].

The production of lignocellulosic value-added products is mainly divided into three steps: pretreatment, hydrolysis (also known as biocatalysis), and fermentation. Enzymatic hydrolysis [11, 12] is one of the most important unit operations, in which polysaccharides are hydrolyzed into monosaccharides that can be further converted to bioproducts. The enzymatic hydrolysis is the result of the synergistic action of multiple enzyme components having different mechanisms of action. The main enzymes employed are cellulases (endoglucanases, exoglucanases and β-glucosidases) and hemicellulases (xylanase, mannanase, arabinose, galactosidase etc.). In the first step of biocatalysis, the binding of the enzymes to the substrate takes place. The bound fraction of endo- and exoglucanases converts cellulose to cellobiose. On the contrary, the unbonded fraction of β-glucosidases converts cellobiose to glucose. The binding step is followed by a hydrolysis reaction which is inhibited by hydrolysis products like cellobiose and glucose [13]. Nevertheless, the biocatalysis process is affected by several substrate related factors, enzymes related factors, presence of inhibitors and feedback inhibition [14]

The aims of this work are to compare different pretreatment methods and conditions on two biomasses with different lignin content and to assess the effects of the procedures on the following

biocatalysis step. For this purpose, a physical pressing step and a chemical organosolv as well as a liquid hot water method was chosen for the pretreatment of municipal green waste and perennial ryegrass. The present work demonstrates that by using different pretreatment procedures on different lignin-content biomass, differences in sugars released can be observed. As expected, the higher sugar amount was obtained by treating the biomasses with more severe processes. Finally, a kinetic study on the low-lignin biomass was performed in order to assess the rate of the polymers degradation over time.

III.3 Materials and methods

III.3.1 Material

Grass cutting from German ryegrass (*Lollium perenne*) was used as representative example for herbaceous lignocellulosic biomass with a low lignin content and was kindly provided by Julius Kühn-Institute, Braunschweig, Germany. A mixture of beech and pine wood chips, originating from local gardens, was used exemplary for recalcitrant lignocellulosic material with a high lignin content.

III.3.2 Mechanical pretreatment

Biomass with a low lignin content was mechanically pretreated using either a tincture press (high-pressure tincture press HP 2 H, Fischer Maschinenfabrik GmbH, Neuss, Germany) or screw press (Angel Juicer 7500, Luba GmbH, Bad Homburg, Germany). The tincture press operates at *p*=440 bar for 20 minutes and the screw press, which is made up of two rotating cylinders, at 82 rpm . The wood chips as an example for biomass with high lignin content were shredded into particles with a size of about 5 mm and further milled to a particle size of about 200 µm. Biomass was dried in a drying cabinet (Memmert GmbH & Co. KG, Schwabach, Germany).

III.3.3 Hydrothermal pretreatment

Biomass was pretreated hydrothermally in a high-pressure reactor BR-500 (Berghof Products + Instruments GmbH, Eningen, Germany). The reaction vessel consisted of Polytetrafluorethylene (PTFE) and had a volume of 0.5 L. The solid:liquor ratio of all experiments was 1:10. Demineralized water was used as solvent for LHW (Liquid-Hot-Water) and 50 % (w/w) ethanol for organosolv pretreatments. The reactor was heated using an electric heating jacket (Berghof Products + Instruments GmbH) with a holding time of 15 minutes at 180 °C. An overpressure of 5 bar N_2 was applied to the reactor vessel and the reaction mixture was stirred at 600 rpm. Solid and liquid fraction of the reaction mixture were separated via centrifugation. The residue was washed three times with demineralized water to remove the solvent and possible by-products of the hydrothermal pretreatment.

III.3.4 Biocatalysis

The pretreated low lignin content biomass was enzymatically hydrolyzed in 0.1 mM sodium-acetate buffer. To prevent microbial contamination, 0.02 % (w/v) sodium-azide was added during hydrolysis

for analytical purposes. If the hydrolysate is used for subsequent fermentations, this needs to be omitted and the substrate autoclaved instead. The reaction was conducted at 50°C and 50 rpm. Two different enzyme mixtures were employed. Ultraflo® Core and Ultraflo® Max (Novozymes A/S, Bagsvaerd, Denmark) were used in a ratio of 50:50 with an enzyme loading of 0.1 $g_{enzyme\ solution}\ g_{biomass}^{-1}$ and a solid content of 50 $g \cdot L^{-1}$. Xylanase 2x and Pectinase L-40 (both ASA Spezialenzyme GmbH, Wolfenbüttel, Germany) were used in a ratio xylanase: pectinase of 60:40. Xylanase 2x contained a mixture of endo-1,4-ß-D-xylanases and endo-1,3-ß-D-xylanase, Pectinase L-40 consisted of polygalacturonase. Enzyme loading was based on the protein concentration in the stock solutions and they were tested using the Bradford (Coomassie Bradford Protein Assay Kit, ThermoFisher Scientific) assay. The protein concentrations were found to be 23.5 mg mL^{-1} for xylanase and 4.2 mg mL^{-1} for pectinase. The enzyme loading was set at 0.16 $g_{enzyme}\ g_{biomass}^{-1}$. with a biomass loading of 10 % (w/w). The hydrolysate samples were taken at 0, 2, 4, 8, 24, 48, 72 and 96 h and were thermally inactivated at 100 °C for 7 minutes, centrifuged and filtered. All the enzymatic hydrolysis were performed at higher enzyme loadings in order to get comparable results between the different biomasses. Thus, the experiments focused on the comparability without considering the optimization process as well as the economic evaluations. The enzymatic hydrolysis was carried out in duplicate and the results have been analyzed as average values and corresponding standard deviation.

III.3.5 Analytical methods and data processing

The carbohydrate composition of the pretreated materials was performed according to the protocol [15]. Sugar monomers were analyzed using a High Performance Liquid Chromatography system (ESA Inc. 542 autosampler (Chelmsford, Massachusetts, USA), Azura pump P 6.1 L (Knauer GmbH, Berlin, Germany)) equipped with a refractive index detector and a BioRad Aminex HPX- 87H column (300 x 7.8 mm) (Hercules, California, USA). In the chromatographic analysis, the samples were diluted with deionized water, filtered, and thus 20 µL of each sample was injected into the chromatograph under the conditions of a column temperature (80 °C), and 2.5 mM H_2SO_4 as mobile phase at a flow rate of 0.6 mL min^{-1}. The yield of the enzymatic hydrolysis was calculated as the amount of sugar released over the total carbohydrate content in the biomass. The percentage was calculated as follow:

$$yield\ [\%]\ = \frac{C_S - C_{S0}}{\alpha_s C_{i0} M_{P0}} * 100 \qquad (1)$$

Where C_s is the concentration [g L^{-1}] of solubilized sugar in the supernatant, C_{S0} is the initial sugar concentration [g L^{-1}], αs is the molecular weight of glucose to glucan monomer (αs= 1.11), C_{i0} is the initial concentration [g L^{-1}] of insoluble solids, and M_{P0} is the initial mass fraction of the polymer in the insoluble solids.

III.4 Results and discussion

Pretreatment of lignocellulosic material is necessary to obtain carbohydrates which can be applied for further uses such as carbon source for fermentations. Due to its varying composition, different strategies are necessary for different biomass types. As lignin plays an important role in the digestibility of lignocellulosic biomass, the lignin content can serve as a decision criterion to select an appropriate pretreatment method.

III.4.1 Pretreatment of biomass with low lignin content (grass biomass)

Herbaceous material is one example for a biomass with low lignin content. Therefore, the lignocellulosic structure is less recalcitrant. Due to a higher moisture content, the biomass can be pressed, resulting in a liquid and a solid fraction [16]. The pressing process can be carried out in decentralized facilities (e.g. on a farm) to obtain a solid fraction with reduced water content, resulting in a better shelf life and lower transport costs due to the lower weight. However, the press juice must be used immediately, e.g. as an additive for biogas fermentation. The solid press cake must be further pre-treated, for example in a central biorefinery.

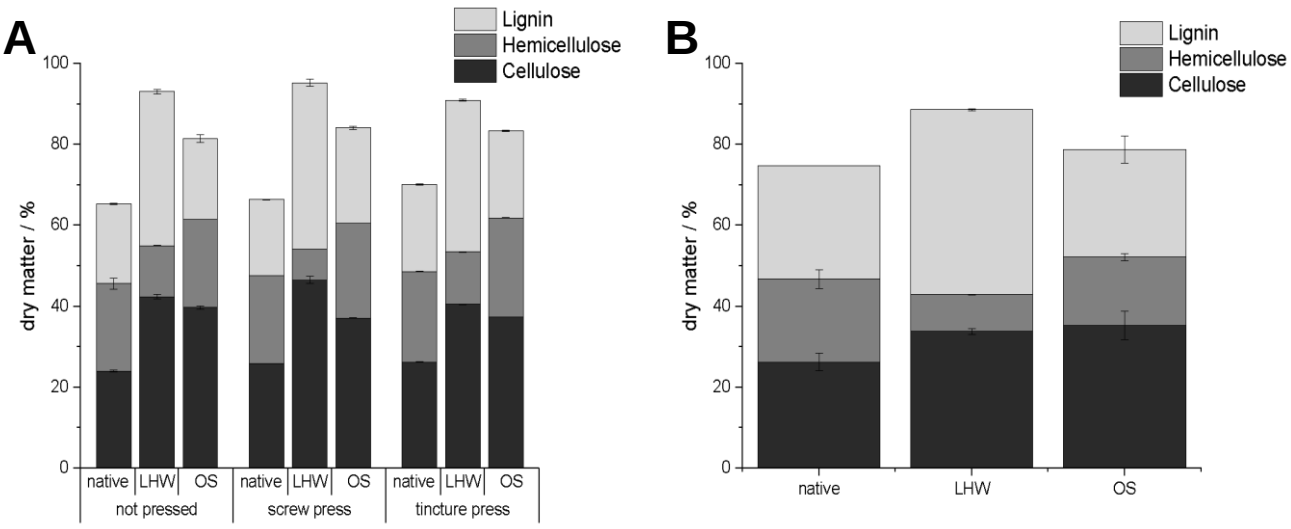

Figure 1: Influence of different pretreatment procedures on the composition of the dried biomass of grass cutting (A) and mixed wood chips (B). Native: no further pretreatment

Grass cutting was pressed using either a screw press or a tincture press. This was followed by a hydrothermal pretreatment as described in chapter 2.3 and [17] using water (LHW) or 50 % (w/w) ethanol (OS) as solvent. As can be seen in figure 1, the influence of a pressing step on the composition of the residue can be neglected. Neither for the native grass, which was not further pretreated after pressing, nor for the hydrothermally processed grass there is a significant difference in the composition of the dried biomass between substrates which were pressed by screw or tincture press or not pressed at all. Compared to the native composition, the hydrothermal procedure increases the share of cellulose in the residue. This effect is stronger for LHW pretreated grass, with an increase of up to 70 %,

compared to an increase of up to 50 % for the organosolv pretreatment. At the same time, the LHW procedure reduces the share of hemicellulose in average by 50 %, while it does not change significantly during organosolv pretreatment. The percentage increase of lignin during the LHW pretreatment procedure can be explained by the removal of other soluble compounds during the pretreatment, leading to lignin to account for a bigger share. Dimos et al. report a 58 % increase of the lignin share during hydrothermal pretreatment of cotton stalks, and a 74 % decrease during an organosolv process using formic acid [18]. This increase of lignin does not occur during organosolv pretreatment with ethanol due to the increased solubility of lignin in this solvent compared to the solubility in water [19].

III.4.2 Pretreatment of biomass with high lignin content (woody biomass)

Biomass with a high lignin content such as wood chips usually is more solid and also has a lower moisture content than grass cuttings. Consequently, a pressing step is either mechanically not possible (e.g. for the screw press) or does not yield a notable amount of press juice and was thus neglected in this work. However, the surface area of the material was increased by crushing and grinding to achieve a better yield in the processes used.

The effects of applying a hydrothermal procedure on biomass with a low lignin content could be confirmed for woody biomass with a lignin content of 30 %. In accordance with the findings for grass cutting, the hydrothermal pretreatment of wood chips increased the cellulose content in the residue by up to 35 %. In contrast to the previous shown results, there is no significant difference in the increase of cellulose content between LHW and organosolv pretreated biomass. The hemicellulose content decreases by 55 % and 18 % during the LHW and organosolv pretreatment, respectively. Kabir et al. report a hemicellulose decrease of 28.5 % during an organosolv process of forest residues [20]. The percentage increase of lignin previously described can also be observed, with an increase of 63 % for LHW pretreated wood chips. In contrast, during the organosolv procedure, the lignin content decreases by 5 %. Again, this can be explained by the solubility of lignin in ethanol [19].

III.4.3 Biocatalysis of biomasses with different lignin contents

Grass cutting was digested enzymatically in a native condition as well as after pressing with either a screw or a tincture press. All biomass samples were saccharified both right after cutting or pressing, respectively, and after drying to constant weight. Similar to commonly used enzyme preparations like Cellic CTec the enzyme mixture of Ultraflo® Max and Ultraflo® Core consists of ß-glucanases, xylanases and cellulases and is therefore promising for the degradation of lignocellulosic biomass. Weiermueller achieved a glucose yield of 87 % from grass cuttings with these enzymes [21].

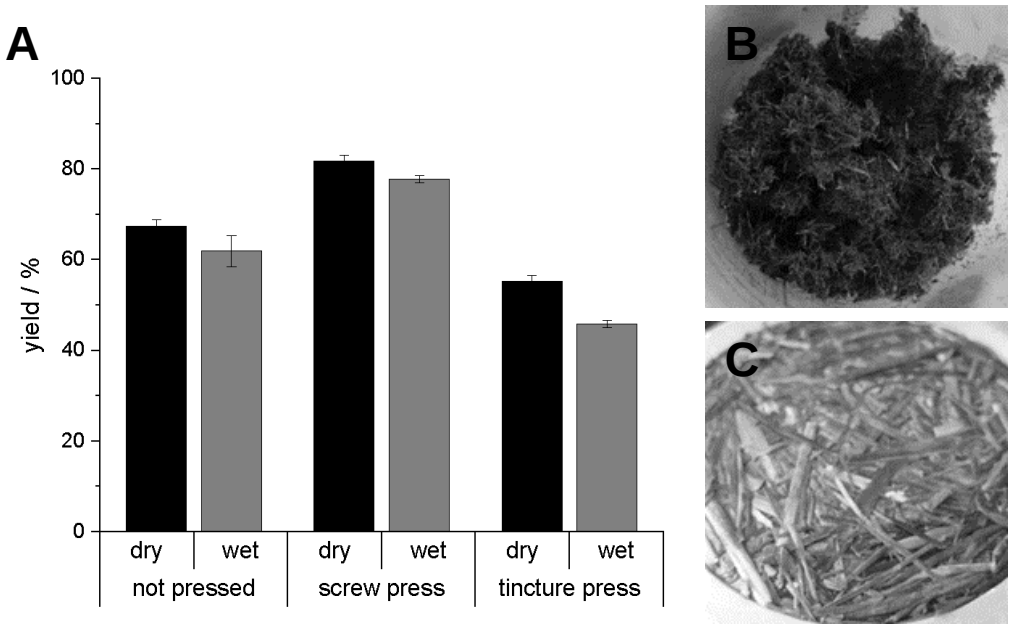

Figure 2: Conversion of cellulose during the biocatalysis step of untreated and pressed grass cuttings in wet or dry conditions (A). Press cake after pressing by screw press (B) or tincture press (C). Enzymatic hydrolysis was carried out with Ultraflo® Max (50%) and Ultraflo® Core (50%).

As can be seen in figure 2, slightly higher conversion rates were achieved when the biomass was dried to constant weight at 50°C prior to the enzymatic hydrolysis. The best result was obtained from screw pressed grass with a conversion rate of 82 % of the total amount of glucose based on the NREL-protocol, followed by native grass with 67 %. Biocatalysis of grass pretreated by tincture press yielded 55 % cellulose conversion. This can be explained by the influence of the pressing mechanism. The tincture press applies pressure of up to 440 bar, resulting in a strong compression of the biomass. Thus, the produced press cake is very dense and compact as can be seen in figure 2C. In the screw press however, the material is crushed between two rotating cylinders. The result is a very fluffy grass cut (figure 2A). The screw press thereby changes the integrity of the grass cuttings. Konan et al. also discuss the possibility of extrusion, breaking up the lignocellulose structure by the shearing forces of rotating screws, as a potential pretreatment procedure for lignocellulosic biomass [22]. The fibers are shredded and partially broken up. This results in a significantly enlarged surface. The pretreatment step aims to disrupt the cell wall as well as to reduce the particle size in order to make the cellulose and hemicellulose available to hydrolytic enzymes. According to this, the press cake obtained from the screw press is more accessible to the enzymes than the compact press cake from the tincture press.

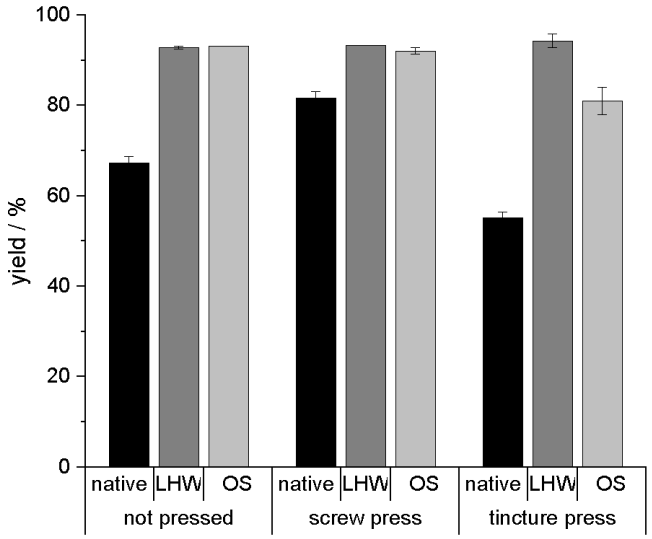

Figure 3: Conversion rate of cellulose after the biocatalysis step of differently pressed and hydrothermally pretreated grass cutting. Native: no further pretreatment. Enzymatic hydrolysis was carried out with Ultraflo® Max (50%) and Ultraflo® Core (50%).

Figure 3 shows the influence of different pretreatment methods on the cellulose conversion during the biocatalysis process. A hydrothermal process step increases the cellulose conversion rate both for pressed and not pressed biomass. Regardless of the prior pretreatment method, the conversion rate of the pretreated biomass is on average 93 % with no significant difference between different pressing or hydrothermal procedures. Only the enzymatic hydrolysis of organosolv pretreated grass, which was pressed via tincture press, yields a lower amount of 81 %. The hydrothermal procedure increases the glucose yield for grass which was not pressed by 38 %, while the yield for screw pressed grass only increased by 13 % as the conversion rate for this pressing method was already at 81 % without an additional hydrothermal step. The hydrothermal process is a time- and energy-consuming procedure. Thus, the small increase of the glucose yield does not justify the expenditure. A pressing step with a screw press, which mechanically breaks up the structure of the biomass, is a sufficient pretreatment method for herbaceous biomass with a low lignin content of up to 20 %. A further hydrothermal procedure is economically not worthwhile.

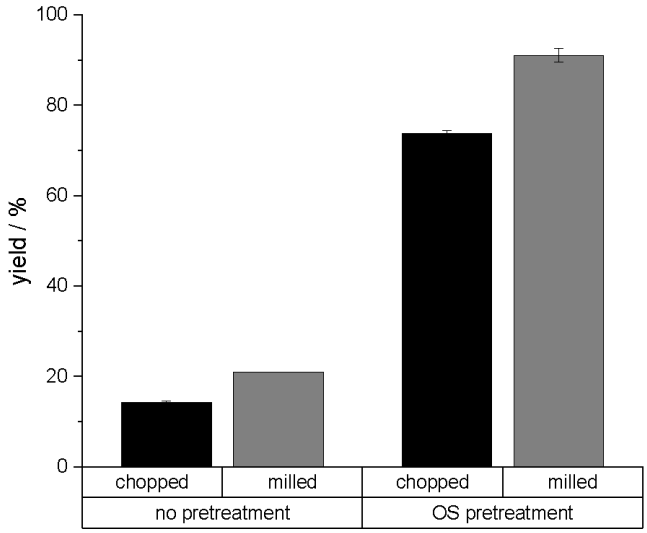

Figure 4: Conversion rate of cellulose after the biocatalysis step of differently pretreated wood chips. Enzymatic hydrolysis was carried out with Xylanase 2x (60 %) and Pectinase L-40 (40 %).

For more recalcitrant biomass with a higher lignin content of around 30 % however, the situation is different. Kirui et al. observed about twice as much lignin-cellulose interactions in woody biomass than in grasses. This leads to the mechanical strength and also the reduced accessibility for enzymes of biomass with a higher lignin content [23]. The cellulose conversion rate of chopped wood chips is only 14 %. This can be increased by 5 % if the biomass is milled prior to the enzymatic hydrolysis. The smaller particle size facilitates the access of saccharolytic enzymes to cellulose. Csiszar et al. examined the hydrolysis of linen and cotton powders by a cellulase after different ultrasound pretreatments. The authors demonstrated a preferential hydrolysis of smaller particles by cellulases as well as a higher saccharification rate of substrates with a smaller particle size [24]. Fernandes et al. found an increase in glucose yield during enzymatic hydrolysis of sugarcane bagasse of 76 % with particles smaller than 60 mesh compared to native particle size [25]. However, the achieved glucose yield of milled biomass particles is still unsatisfactory. An organosolv pretreatment of wood chips increases the glucose yield by 414 %, reaching a cellulose conversion rate of 74 %. This can be further increased to a final conversion rate of 91 % by milling the organosolv pretreated residue prior to the enzymatic hydrolysis to improve the cellulose accessibility (figure 4). An overview of different sugar yields obtained from various lignocellulosic feedstocks using different pretreatment processes and biocatalysis steps is given in table 1.

Table 1: Comparison of sugar yields obtained from different lignocellulosic feedstocks after pretreatment and biocatalysis using varying enzymes

Substrate	Pretreatment	Biocatalysis	Yield	Reference
Eucalyptus wood chips	LHW and extrusion	CelliCTec2 solid loading: 10 % (w/v) enzyme loading: 20 mg $g_{cellulose}^{-1}$	79.6 %	[26]
Sugarcane bagasse	LHW	Cellulase, Xylanase solid loading: 5% (w/v) enzyme loading: 15 FPU $g_{substrate}^{-1}$	55.6 %	[27]
German ryegrass	LHW	Ultraflo Max, Ultraflo Core 1:1 solid loading: 50 $g \cdot L^{-1}$ enzyme loading: 0.1 g $g_{dry\ matter}^{-1}$	87 % glucose	[21]
Sorghum sweet biomass	Organosolv	CelliCTec2 solid loading: 27.7 mg g_{solid}^{-1} enzyme loading: 10 FPU $g_{dry\ matter}^{-1}$	42.1 % total sugars	[28]
Poplar sawdust	Organosolv	CelliCTec2 solid loading: 2 % (w/v) enzyme loading: 20 FPU $g_{cellulose}^{-1}$	78 % total sugars	[29]
Perennial ryegrass	LHW Organosolv	Ultraflo Max, Ultraflo Core 1:1 solid loading: 50 $g \cdot L^{-1}$ enzyme loading: 0.1 g $g_{dry\ matter}^{-1}$	93 % glucose	this work
Mixed beech and pine wood chips	Organosolv	Xylanase 2x, Pectinase L-40 3:2 solid loading: 10 % (w/w) enzyme loading: 0.16 g $g_{dry\ matter}^{-1}$	74 % glucose	this work
Spruce and birch chips	Organosolv	CelliCTec2 solid loading: 5 % (w/v)	69.1 %	[30]

		enzyme loading: 10 mg g_{solids}^{-1}		
Energy crop	Organosolv	Cellulase solid loading: 5 % (w/v) enzyme loading: 20 FPU $g_{dry\ maatter}^{-1}$	87.5 %	[31]

III.4.4 Kinetic of the biocatalysis of low-lignin content biomass

The enzymatic hydrolysis of low-lignin content biomass was conducted in order to study the sugar release over time. Figure 5 reports the yield of glucose and xylose released as a function of the time. Sugars were released fast at the beginning of the hydrolysis and progressively reached a steady-state. In particular, the glucose released changed little after 24 h, suggesting that the hydrolysis of glucan was almost completed. At the same time, the amount of xylose was still increasing thus indicating that the xylanase needs more time to completely hydrolyze the xylan. The results are shown in figure 5.

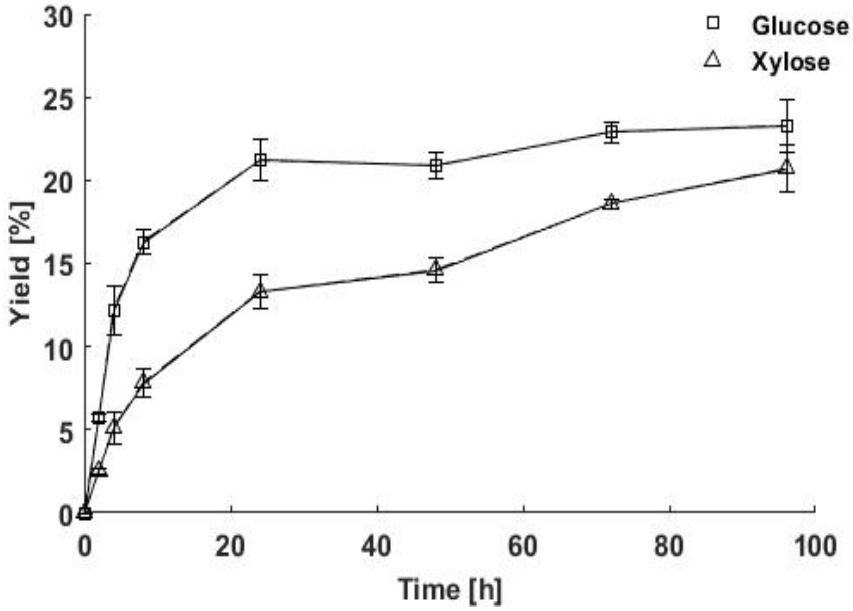

Figure 5: Glucose and xylose yield from mechanical pre-treated low-lignin content biomass over time during biocatalysis. Enzymatic hydrolysis was carried out with Xylanases (60 %) and Pectinases (40 %). All experiments were performed in duplicates.

The overall results were expected as the raw material was pretreated using a weak procedure, thus not completely disrupting the recalcitrant structure of lignocellulosic biomass and reducing the efficiency of the hydrolysis process. As mentioned above, the main goals of the pretreatment are the breakdown of the cell wall and the increase of accessibility for the enzymes. Harsh process methods with chemicals (acids, bases, organic solvents) are extensively used in industrial production and ensure higher sugar release during the enzymatic hydrolysis step. However, the use of strong conditions

caused the formation of undesirable products which inhibit the fermentation process. For all these reasons, the physical and chemical pretreatment procedures are usually combined because no pretreatment technique alone can ensure the goals mentioned above [7].

Another explanation for the present result could be the high biomass loading. As reported by Niglio et al., the increase of biomass loading causes high viscosity that makes it more difficult for the solution to access and wet the biomass. Moreover, enzyme inactivation and product inhibition could hinder the enzymatic hydrolysis process [32].

In the usual single-substrate-enzyme-catalyzed reactions (Eq. 2), the relationship between the initial reaction rate and the substrate concentration assumes the form of a saturation curve (figure 5) [33]. A mathematical model to describe the kinetic is the Michaelis-Menten equation (Eq. 3):

$$E + S \leftrightarrow ES \rightarrow E + P \qquad (2)$$

$$v = \frac{V_{max}\,[S]}{K_M + [S]} \qquad (3)$$

Where *v* is the product formation rate, V_{max} is the maximum velocity achieved at the maximum substrate concentrations, and K_M is the substrate concentration at which the velocity is 50 % of the V_{max}.

The initial rate of reaction is calculated by correlating the substrate concentration or product concentration change and the reaction period insofar as their time course is estimated as a linear relationship [33]. In this work, as showed in figure 6, the linear range was found within the first 8 h. To experimentally define the kinetic parameters V_{max} and K_M, the Michaelis-Menten equation is modified using the integration method. The integration method was applied by assuming that the reverse reaction is negligible and that the product does not affect the reaction rate [33]. According to this, the it is possible to integrate the Michaelis-Menten equation (Eq.2):

$$\int_{S_0}^{S_t} \frac{[S]+K_M}{[S]} = -\int_0^t V_{Max}\,dt \qquad (4)$$

$$\frac{1}{t}\,ln\,ln\,\frac{S_0}{S_t} = -\frac{1}{K_M}\frac{(S_0 - S_t)}{t} + \frac{V_{Max}}{K_M} \qquad (5)$$

Equation 4 can be recognized as an equation of a line in which the data are plotted as S_0-St/t *versus* (1/t)ln(S_0/S_t).

Since the calculation is based on the decreasing substrate concentration over time, the substrate concentration was calculated as:

$$S_{(t)} = Total\ carbohydrate\ content\ according\ to\ NREL - c(t)_{glu} - c(t)_{xyl} \qquad (6)$$

The resulting curve (figure 6) is a line with a negative slope. From equation 3, V_{max} and K_M result to be 0.6 mmol min^{-1} and 93.4 mmol L^{-1}, respectively. The spectrum of experimentally determined K_M values of enzymes varies depending on the experimental setup and the substrate. Typical K_M values of cellulases range from 0.45 to 6.7 mmol L^{-1} for the model substrate carboxymethyl cellulose [34, 35]. For xylanases, K_M values between 0,03 mmol L^{-1} for beechwood xylan [36] and 7,7 mmol L^{-1} for birchwood xylan [36, 37] are reported. Polygalactoronases display a slightly lower range of K_M values from 0,02 mmol L^{-1} to 0,6 mmol L^{-1} [38, 39]. The K_M value of the enzyme mixture of Xylanase 2x and Pectinase L-40 used in the present experiments is in the same order of magnitude as the reported literature data for these kinds of enzymes. The slightly higher K_M value, signifying a lower affinity between enzymes and substrate, can be explained by the different substrates used for the experiments.

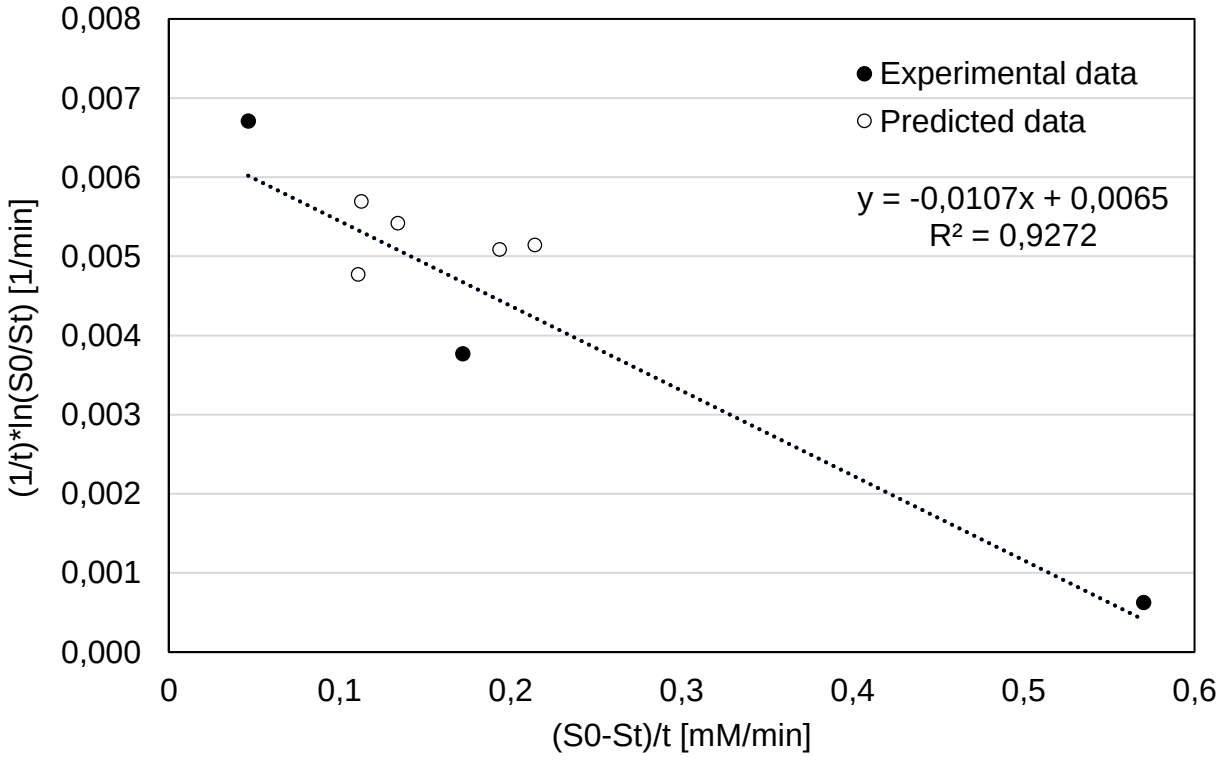

Fig. 6: Integrated Michaelis-Menten method to determine the kinetic parameters V_{max} and $K_{M.}$. Full circles: experimental data; empty circles: predicted data.

III.5 Conclusion

Lignocellulosic biomass is a potential carbon source for various purposes. However, a pretreatment procedure is indispensable prior to its use. Complex carbohydrates are broken down into fermentable monosaccharides by saccharolytic enzymes. Depending on the lignin content of the biomass, an additional hydrothermal process is necessary to break up the recalcitrant structures. Cellulose from grass biomass can be converted with an efficiency of 93 % without prior hydrothermal pretreatment. In contrast to this, the cellulose conversion rate of woodier biomass such as wood chips without a hydrothermal processing is 14 %, whereas an organosolv procedure increases the cellulose conversion rate to 74 %. The overall results demonstrated that the saccharification step depends on the pretreatment process used, as it aims to disrupt the biomass structure in order to make the biomass more accessible for the hydrolytic enzymes. To make a biorefinery process more efficient, lignocellulosic biomass should be separated according to its lignin content. In this way, the energy consuming hydrothermal pretreatment is only applied to biomass which requires this procedure for an effective saccharification. Finally, kinetic studies were carried out on the grass material over time. With this regard, the time-course of the enzymatic hydrolysis revealed that after 24 h the hydrolysis reached the steady-state. In order to study the kinetic parameters, the Michaelis-Menten equation was integrated by considering the time window where product concentration and the reaction time had a linear relationship. The parameters of V_{max} and K_M were estimated to be 0.6 mmol min^{-1} and 93.4 mmol L^{-1} respectively, which are in line with the data reported in literature.

III.6 Acknowledgement

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III.7 Symbols used

p [bar] Pressure

rpm [min^{-1}] Revolution per minute

C_S *[g L^{-1}]* Concentration of solubilized sugar in the supernatant

C_{S0} *[g L^{-1}]* Initial sugar concentration

αs [-] Molecular weight of glucose to glucan monomer

C_{i0} *[g L^{-1}]* Initial sugar concentration of insoluble solids

M_{P0} *[-]* Initial mass fraction of the polymer in the insoluble solids

K_M *[mmol L^{-1}]* Michaelis-Menten constant

V_{max} *[mmol min^{-1}]* maximum velocity achieved at the maximum substrate concentrations

v [mmol min^{-1}] Product formation rate

III.8 Abbreviations

LCB Lignocellulosic biomass

PTFE Polytetrafluorethylene

HPLC High-Performance Liquid Chromatography

LHW Liquid-Hot-Water

OS Organosolv

FPU Filter Paper Unit

NREL National Renewable Energy Laboratory

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IV. New Insights into the Influence of the Pre-culture on the Solvent Production of *C. acetobutylicum*

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Keywords: Butanol, ABE, *C. acetobutylicum*, Acid crash, Metabolic shift, Pre-culture

IV.1 Abstract

Clostridia are known for their solvent production, especially the production of butanol. Concerning the projected depletion of fossil fuels, this is of great interest. The cultivation of clostridia is known to be challenging, and it is difficult to achieve reproducible results and robust processes. However, existing publications usually concentrate on the cultivation conditions of the main culture. In this paper, the influence of cryo-conservation and pre-culture on growth and solvent production in the resulting main cultivation are examined. A protocol was developed that leads to reproducible cultivations of *C. acetobutylicum*. Detailed investigation of the cell conservation in cryo-cultures ensured reliable cell growth in the pre-culture. Moreover, a reason for the acid crash in the main culture was found, based on the cultivation conditions of the pre-culture. The critical parameter to avoid the acid crash and accomplish the shift to the solventogenesis of clostridia is the metabolic phase in which the cells of the pre-culture were at the time of inoculation of the main culture; this depends on the cultivation time of the pre-culture. Using cells from the exponential growth phase to inoculate the main culture leads to an acid crash. To achieve the solventogenic phase with butanol production, the inoculum should consist of older cells which are in the stationary growth phase. Considering these parameters, which affect the entire cultivation process, reproducible results and reliable solvent production are ensured.

IV.2 Key points

- Both cryo- and pre-culture strongly impact the cultivation of *C. acetobutylicum*
- Cultivation conditions of the pre-culture are a reason for the acid crash
- Inoculum from cells in stationary growth phase ensures shift to solventogenesis

IV.3 Introduction

Clostridial fermentation was an extensively used method in the 20th century to produce acetone, ethanol, and butanol (ABE), but it was successively replaced by a growing petrochemical industry (Jones, 2001). However, the projected depletion of fossil resources currently leads to rising oil prices. This results in a newly increasing interest in biological production processes, if possible, based on renewable resources. Butanol, the main product of most solventogenic clostridial fermentations, is an important platform chemical. It is used as a solvent in lacquer or for the formation of more valuable butyl-esters (Mascal, 2012). Moreover, the component butanol is viewed as a promising alternative to ethanol as a biofuel because it has very similar physical properties as gasoline and offers a high heating value and energy content (Ibrahim et al., 2017). Therefore, the application of butanol as fuel or fuel additive is feasible in unmodified engines. Also, the ABE mixture was investigated concerning its combustion properties and has the potential for application as a biofuel (Lee et al., 2016).

Saccharolytic clostridia are capable of using a wide variety of carbon sources as substrate, such as cellulose, hemicellulose, and starch after hydrolysis into monomers. This is of special interest as they

can use various lignocellulosic biomasses as feedstock (Riaz et al., 2022). Therefore, solvent production with clostridia does not cause the typical food or feed dilemma of first-generation biofuels that use agricultural products.

The most commonly used organism for ABE fermentation is *Clostridium acetobutylicum*. It is a strictly anaerobic, gram-positive bacterium that can be found ubiquitously, but mostly in sediments. As all solventogenic clostridia, *C. acetobutylicum* displays a two-phased metabolism (Monot et al., 1982) and produces the solvents acetone, butanol, and ethanol typically in the molar ratio of 3:6:1 (Jones and Woods, 1986). At the first stage of the metabolism, organic acids, mainly butyric acid, are produced. After this so-called acidogenesis, the secreted acids are partly reassimilated and, together with saccharides, used for the formation of solvents in solventogenesis. The particular reason for the onset of the solventogenic phase is not fully resolved yet, but several parameters are connected to the phase shift. These include the pH, the acid concentration, the glucose concentration, and the concentration of nutrients such as nitrogen and phosphate (Kumar et al., 2013). If the cultivation does not enter the solventogenic phase, the produced acids accumulate and lead to a so-called acid crash. This phenomenon is characterised by a drop in the pH and metabolic stagnancy (Maddox et al., 2000). This process is not fully understood yet and is one reason why the cultivation of clostridia is often challenging.

The course of a cultivation depends on various factors such as the available nutrients as well as process parameters like temperature, pH, and agitation. The pre-culture generally also has crucial effects on the growth and productivity of the subsequent main culture and the robustness of the entire process (Becker et al., 2023). Shida et al. examined the influence of the size and growth phase of *Achromobacter delmarvae* and *Escherichia coli* inoculums on the lag phase of the respective main cultures and reported a shorter lag phase for bacteria from the exponential phase (Shida et al., 1977). The adaptation of *E. coli* to a new culture medium during the pre-culture was analysed by Kim et al. (Kim et al., 2021). They showed an increased growth rate after sequential transfers to the new medium and proved the presence of genetically adapted cells in the pre-culture. Sandoval et al. investigated the influence of the metabolic phase of the inoculum for *Clostridium beijerinckii* and reported higher growth rates when cells from the exponential growth phase were used as inoculum (Sandoval-Espinola et al., 2015). Moreover, Gutierrez et al. found a connection between the cell motility of the inoculum and the solvent production of *C. acetobutylicum* and suggested using cells with high motility as inoculum (Gutierrez and Maddox, 1986).

Despite the crucial effects of the pre-culture on the main culture, its impact is not much investigated yet. This especially applies to the impact of the pre-culture on the product formation in the main culture. Therefore, in this work, the pre-culture has been studied concerning the inoculum, the optimal

pH conditions, and the fermentation duration. For all parameters concerning the pre-culture the impact on the main culture was studied. As a result, connections were found between the metabolic phase of the pre-culture at the time of inoculation and the occurrence of the acid crash in the subsequent main culture. By avoiding the acid crash and enabling the shift to the solventogenesis, solvent production in the main culture can be ensured. In this work, a cultivation process of *C. acetobutylicum* is presented. The method ensures reliable cell growth and an efficient and robust butanol production.

IV.4 Materials and methods

IV.4.1 Microorganism

Clostridium acetobutylicum DSM 792 was purchased from DSMZ (Deutsche Stammsammlung für Mikroorganismen und Zellkulturen, German Collection of Microorganisms and Cell Cultures GmbH, Braunschweig, Germany). Cells of *C. acetobutylicum* were cryo-conserved in a 40 % glycerol solution at -80 °C under anerobic conditions.

IV.4.2 Cultivation conditions

C. acetobutylicum was cultivated at 37 °C and 50 rpm in serum flasks with a fermentation volume of 150 mL. Anaerobic conditions were ensured by sparging the medium for 40 minutes with nitrogen. Cryo-cultures consisted of 4 mL cultivation broth mixed with 4 mL of 80 % glycerol as a cryo-protective agent. Pre-cultures were inoculated with 1 vol.% of cryo-culture and cultivated in a modified PY+X medium derived from the PY+X medium of DSMZ. Modified PY+X medium contained per liter: 5 g tryptone, 10 g yeast extract, 5 g peptone from meat, 5 g glucose, and 40 mL salt solution. Salt solution consisted of 0.25 g $CaCl_2$, 0.5 g $MgSO_4x7H_2O$, 1 g KH_2PO_4, 1 g K_2HPO_4, 10 g $NaHCO_3$, and 2 g NaCl per liter. Main cultures were inoculated with 10 vol.% of pre-culture and cultivated in MP2opt medium from Engel et al. MP2opt medium contained per liter: 2.2 g ammonium acetate, 0.5 g KH_2PO_4, 0.5 g K_2HPO_4, 0.2 g $MgSO_4x7H_2O$, 0.015 g $Fe(II)SO_4x7H_20$, 0.01 g $MnSO_4x7H_2O$, 1 mL of vitamin solution and 45 g glucose (Engel et al., 2018). Vitamin solution was prepared with 2 mg p-aminobenzoic acid, 2 mg thiamine-HCl, and 0.01 mg D-biotin per liter. The pH of the MP2opt medium was adjusted to 6.8 before inoculation. 1 mL samples were taken periodically to measure the optical density (OD), pH, and substrate and product concentration. After determining the first two parameters, the sample was centrifuged (centrifuge 5418, rotor FA-45-18-11, Eppendorf SE, Hamburg, Germany) for 2 min at 16873 g and the supernatant sterile filtered using polyamid filters with a pore size of 0.20 µm (Chromafil Xtra PA20/13, Macherey Nagel, Düren, Germany). Samples were stored at -20 °C until further analysis.

IV.4.3 Analytical methods

The substrate and product concentration in the supernatant of cultivation samples was analysed using an HPLC system consisting of a Merck Hitachi L-6200 pump (Merck KGaA, Darmstadt, Germany), a

Midas cool autosampler (Spark Holland B.V. Emmen, The Netherlands), a Jetstream II plus column thermostat (Duratec, Hockenheim, Germany), an Aminex HPX-87H-column (300 mm x 7.8 mm, Bio-Rad Laboratories GmbH, Feldkirchen, Germany), and a RI detector (PN3140, Postnova, Landsberg am Lech, Germany). The column temperature was 80 °C and the mobile phase consisted of 2.5 mM H_2SO_4 at a flow rate of 0.6 mL·min^{-1}. Measured values of the glucose concentration were normed to the weighed in concentration. Cell density was measured by the OD_{600} with the photometer Lambda Bio+ (Perkin Elmer, Rodgau, Germany). The pH was measured with the pH-meter 221 from Hanna Instruments (Vöhringen, Germany).

IV.5 Results

To enable a reliable cultivation of *C. acetobutylicum* with a high butanol production, several factors were examined. First, the influence of the cryo-culture was analysed to establish an optimised procedure. This then served as the basis for analysing the influence of the initial pH and the metabolic phase of the pre-culture on the main culture.

IV.5.1 Optimal preparation of cryo-cultures

Culture degeneration is a known problem of clostridial fermentations, where the strain loses its ability to produce solvents. This is usually the result of subculturing, repeated batch or continuous fermentation of clostridia, that lead to a genetic change (Maddox et al., 2000). A suitable method for cell preservation is therefore of great importance. A common procedure to conserve cell cultures is the preparation of cryo-cultures, where cells are mixed with glycerol and frozen at -20 to -80 °C (Julca et al., 2012). Usually, conserving actively growing cells from the exponential growth phase results in fast-growing pre-cultures (Shida et al., 1977). To examine the optimal time for cell extraction, four different points in cultivation time were examined concerning the solvent production in the subsequent cultivation of the conserved cells. Fig. 1 shows the cultivation of *C. acetobutylicum* in the complex medium PY+X (A) with an initial glucose concentration of 5 g·L^{-1}, which is recommended by DSMZ for the cultivation of *C. acetobutylicum*, and in the defined medium MP2opt (B) with initially 45 g·L^{-1} of glucose, which was optimised for the cultivation of this strain (Engel et al., 2018). Cells were extracted after 25, 30, 48, and 100 h of cultivation in PY+X medium and after 9.5, 26, 51, and 116 h of cultivation in MP2opt medium and used for cryo-conservation. The slight variations in the times of sampling result from the different behaviour of the cells in the different media. This is explained in more detail below. The numbered dashed lines in Fig. 1 indicate the extraction of cells. The hitherto irregular growth behaviour of clostridia prevents the use of cultivation times for the differentiation of the growth phases. Also, the utilisation of the OD is not optimal to detect the shift to the stationary growth phase as cells tend to agglomerate in the synthetic medium, which hampers the OD measurement (Engel et al., 2018). However, the different phases of the clostridial metabolism can be distinguished based on glucose consumption and product formation, which can be monitored based

on pH changes (Fig. 1). The exponential growth phase corresponds to the rapid uptake of glucose and the production of acids, which leads to a drop in the pH. In the stationary phase, glucose consumption is limited. In cultivations in which the metabolic shift to solventogenesis takes place, the conversion of butyric acid to ABE products also occurs mainly during the stationary phase. The uptake of acids and their transformation into solvents causes an increase of the pH. The cells were extracted at the end of the exponential growth phase ①, at the shift from exponential to stationary growth phase ②, and at the early ③ and late ④ stationary phase when grown in a complex medium containing 5 $g \cdot L^{-1}$ glucose (Fig. 1, a), and at the start of cell growth ⑤, at the shift of exponential to stationary phase ⑥, and at early ⑦ and late ⑧ stationary phase when grown in defined medium containing 45 $g \cdot L^{-1}$ glucose (Fig 1, b). Due to the different media compositions, the time to reach the mentioned metabolic phases differs in the two cultivations, which is why the cells from the MP2opt medium were extracted earlier than the ones grown in PY+X medium.

The maximum OD measured in PY+X medium was 3.85. With higher glucose concentration in the MP2opt medium, a maximum OD of 4.1 was measured. Due to the higher amount of available carbohydrates, the cultivation in defined MP2opt medium also results in higher butanol and total solvent concentrations compared to complex PY+X medium. Al-Shorgani et al. established a direct correlation between glucose and ABE concentration up to an initial glucose concentration of 50 $g \cdot L^{-1}$ (Al-Shorgani et al., 2016). The extracted cells from these cultivations were firstly cryo-conserved for at least 24 h and subsequently used for the inoculation of a pre-culture, with which a cultivation in MP2opt medium was inoculated. This medium is chosen due to its higher glucose content than the PY+X medium. Also, it is an already established medium for main cultivations. Cultivation conditions were the same for all cryo-conservated cells. A pre-culture was inoculated with 1 vol.% of cryo-culture and incubated for 48 h. With these pre-cultures, a main culture was inoculated with 10 vol.%.

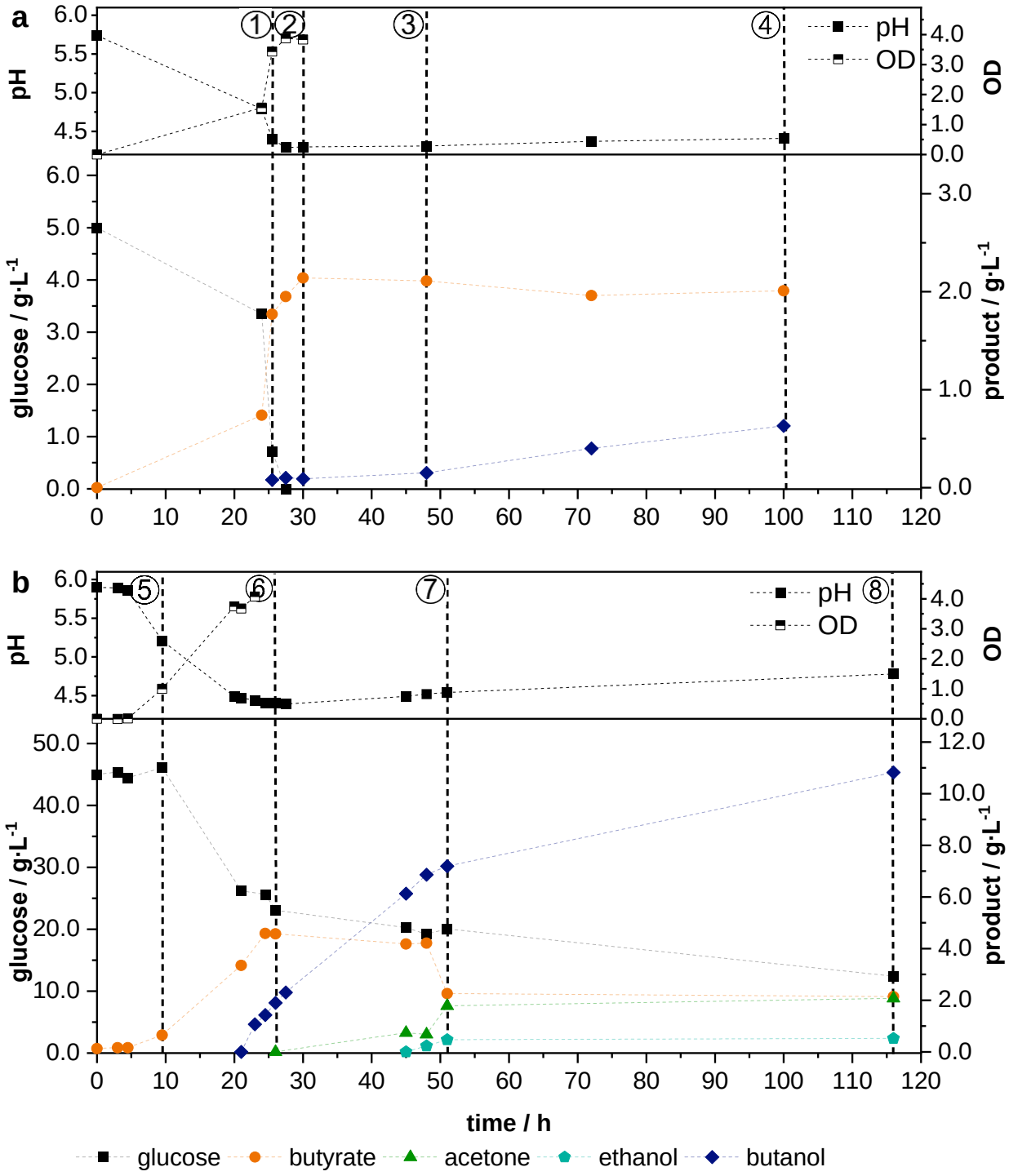

Fig. 1: Cultivation of *C. acetobutylicum* for the production of cryo-cultures. Dashed lines indicate the time of cell harvest for cryo-conservation, the numbers correspond to the entries in Table 1; a: Cultivation in PY+X medium, 37°C, 50 rpm, V=100 mL, inoculum size: 1 vol.%; b: Cultivation in MP2opt medium, 37°C, 50 rpm, V=150 mL, inoculum size: 10 vol.%; standardized glucose concentration, n = 1.

Table 1 shows the maximum butanol and total ABE concentration obtained from the subsequent main cultivation in MP2opt medium depending on the time of extraction of the conserved cells. Initial ODs of the main cultures were in a range from 0.32±0.01 which excludes the influence of different cell densities on productivity.

Table 1: Effect of cultivation medium and metabolic phase of cells used for cryo-conservation on solvent production in the subsequent fermentation of *C. acetobutylicum* cultivated in Mp2opt medium. The course of the fermentation of the cells used for cryo-conservation as well as the sampling time is indicated in Fig. 1, cultivation of pre-culture in PY+X medium: 37°C, 50 rpm, V=100 mL, inoculum size: 1 vol.%, initial pH= 5.5, fermentation time= 48 h, cultivation of main culture in MP2opt medium: 37°C, 50 rpm, V=150 mL, inoculum size: 10 vol.%, n=2.

Cultivation medium of cells for cryo-conservation	Metabolic phase at time of harvest	Max. butanol in resulting cultivation / $g \cdot L^{-1}$	Max. ABE in resulting cultivation / $g \cdot L^{-1}$
PY+X medium	① late exponential phase	8.15±0.55	11.42±0.60
	② shift to stationary phase	6.53±2.73	9.22±2.92
	③ early stationary phase	9.18±0.38	13.16±0.39
	④ late stationary phase	9.45±0.27	13.6±0.28
MP2opt medium	⑤ start of cell growth	no cell growth	no cell growth
	⑥ shift to stationary phase	10.31±0.37	14.65±0.41
	⑦ early stationary phase	9.84±0.39	14.11±0.51
	⑧ late stationary phase	11.22±0.30	16.08±0.46

The longer the cells were cultivated before cryo-preservation, the higher the butanol and solvent concentration of the subsequent cultivations. Working with cryo-conservated cells from PY+X medium, cultivations based on cells from the late exponential phase resulted in a maximal butanol concentration of 8.15 $g \cdot L^{-1}$ and a maximum total solvent concentration of 11.42 $g \cdot L^{-1}$ in the main culture. These concentrations increased by 16 % and 19 % to 9.45 and 13.6 $g \cdot L^{-1}$, respectively, when the cryo-conserved cells originated from the late stationary phase. Cells conserved from MP2opt medium display the same tendency. Here, the butanol and total solvent concentrations increased by 9 % and 10 %, respectively, and reached 11.22 and 16.08 $g \cdot L^{-1}$in the subsequent main culture.

IV.5.2 Influence of the initial pH of the pre-culture

Based on the preparation of cryo-cultures, which was described previously, the growth of these cells in pre-culture must be ensured. To examine the optimal pH to grow cryo-conservated cells of *C. acetobutylicum* in the pre-culture, different initial pH values were adjusted. Fig. 2 shows the resulting course of pH and OD in pre-cultures.

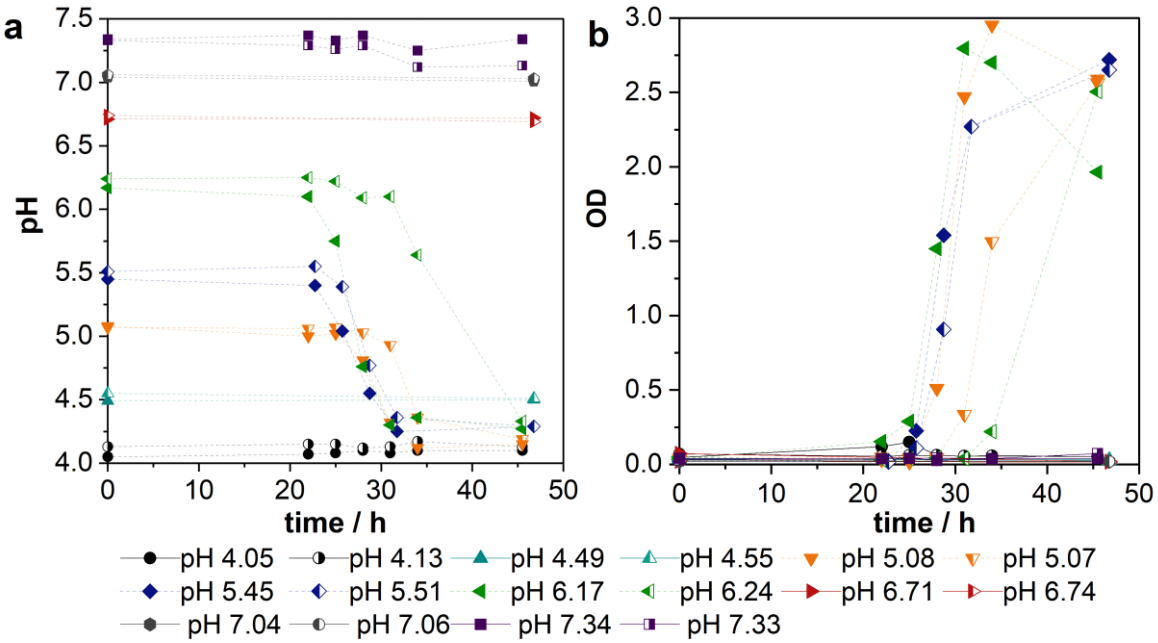

Fig. 2: Course of a: pH and b: OD in pre-cultures of *C. acetobutylicum* cultivated with different initial pH; PY+X medium, 50 rpm, V=100 mL, inoculum size: 1 vol.%, n = 1.

For pH values of 6.8 and higher as well as 4.5 and lower, no change in the pH could be observed during a cultivation time of 45 h. This indicates that no acids were produced and therefore no cell growth took place in these cultivations. This is confirmed by the course of the OD (Fig. 2, b), where no change can be detected over 48 h. However, for experiments with an initial pH value between 5.0 and 6.2, the pH drops after around 25 h, which indicates the production of acids. In all the growing pre-cultures butyrate was produced in a concentration of 1.91±0.10 $g \cdot L^{-1}$. Acetate and lactate were produced in lower concentrations of 1.09±0.05 and 0.98±0.18 $g \cdot L^{-1}$, respectively. The acid production is linked to the exponential growth phase of clostridia, which can also be seen by the increasing OD. The drop of the OD after the exponential growth phase, which can be seen for the pre-cultures with initial pH of 5.08 and 6.17, is a result of the agglomeration of cells, which was observed visually by inspecting the serum flasks.

Above all, the solvent production in the resulting main cultures is of interest. For this reason, all growing pre-cultures were used to inoculate a main culture after a cultivation time of 48 h. Regarding all main cultivations, a good solvent production could be observed: on average a final butanol concentration of 11.46±1.01 $g \cdot L^{-1}$ was measured. Moreover, a concentration of combined ABE products of 16.27±1.56 $g \cdot L^{-1}$ was detected.

IV.5.3 Influence of the metabolic phase of the inoculum on product formation

The growth phase of the pre-culture can influence growth and productivity in the resulting main cultivation. This correlation was investigated based on cultivations that were inoculated with pre-cultures of different cultivation times. The same pre-culture (Fig. 3, a, pre-culture A) was used after

different cultivation times of 26, 28, and 32 h to inoculate a main culture. As the volume of the pre-culture was limited, an additional pre-culture (Fig. 3, a, pre-culture B) with comparable growth behaviour was used as inoculum after a cultivation time of 48 h. A cultivation time of pre-culture of 26 h and 28 h represents cells that were in the exponential growth phase, which was attended by acid production and can be detected by the pH drop. ODs of the pre-culture at the times of 26 h and 28 h were 1.46 and 2.16. At a cultivation time of 32 h, the shift to the stationary growth phase occured, which was marked by the decline of acid production and the flattening of the pH decrease until it became constant. An OD of 3.18 was reached after a cultivation time of 32 h. At a cultivation time of 48 h acid production and cell growth fully stopped and an OD of 3.08 was measured, which indicated the stationary growth phase. By this approach, cells from the exponential growth phase, the shift to stationary growth phase, and the stationary growth phase were used as inoculum for a main culture. To evaluate the productivity of the resulting main cultivations, the solvent concentration was determined (Fig. 3, b). In some cultivations, final solvent concentrations of up to 17.78 $g \cdot L^{-1}$ were measured, but only after a cultivation time of almost 10 days. This results from a commonly known phenomenon of clostridial fermentations, the so-called “recommencement”, where the acid crash occurs but the acids are degraded slowly and the shift to solventogenesis is possible after an exceptionally long fermentation time (Li et al., 2020; Maddox et al., 2000). This is why the time frame of 48 h was selected here for comparison, to focus on cultivations with high productivity.

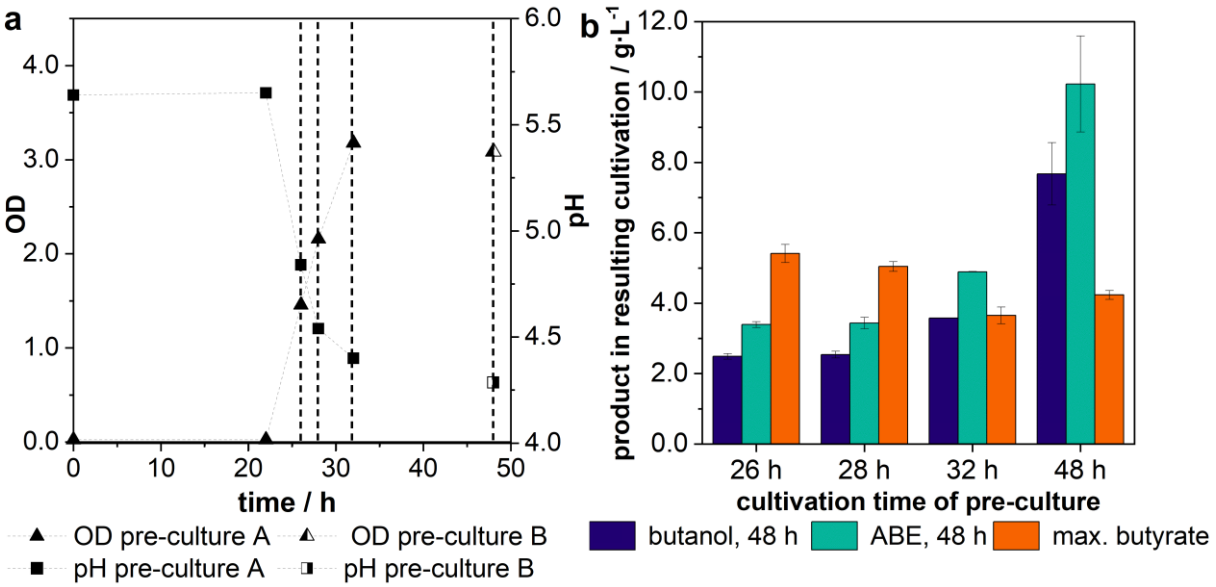

Fig. 3: Effect of the cultivation time of the pre-culture on the resulting production in the main culture of *C. acetobutylicum* after 48 h of cultivation; a: OD and pH of pre-cultures used for inoculation, dashed lines indicate extraction time of inoculum, b: butanol, total ABE and max. butyrate concentration in main cultures after 48 h inoculated with pre-cultures after different cultivation times; pre-culture: PY + X medium, 50 rpm, V=100 mL, inoculum size: 1 vol.%, pH_0=5.5; main culture: MP2opt medium, 50 rpm, V=150 mL, inoculum size: 10 vol.%, n=2.

A trend to higher solvent production with higher cultivation time of pre-culture is visible. With a cultivation time of the pre-culture of 48 h, the maximal ABE concentration of 10.22±1.36 $g \cdot L^{-1}$ and a butanol concentration of 7.67±0.88 $g \cdot L^{-1}$ after 48 h was reached in the main culture. In contrast, in a main culture that was inoculated with a pre-culture of a short cultivation time of 26 h, only 2.48±0.08 $g \cdot L^{-1}$ of butanol and a total ABE concentration of 3.39±0.08 $g \cdot L^{-1}$ could be measured. Also, with a cultivation time of 48 h in the pre-culture, a maximal butyrate concentration of 4.23±0.12 $g \cdot L^{-1}$ was measured in the main culture, which is more than 20% less butyrate compared to a main culture that was inoculated with a pre-culture in the exponential growth phase. A Tukey-Test was carried out and showed that the resulting butanol concentrations measured from pre-culture cells cultivated for 26, 28, and 32 h significantly differ ($\alpha<0,05$) to the concentrations measured for pre-culture cells cultivated for 48 h.

For better comparability, the course of the resulting main cultivation from pre-cultures with short and long cultivation times of 26 and 48 h are exemplarily represented in Fig. 4.

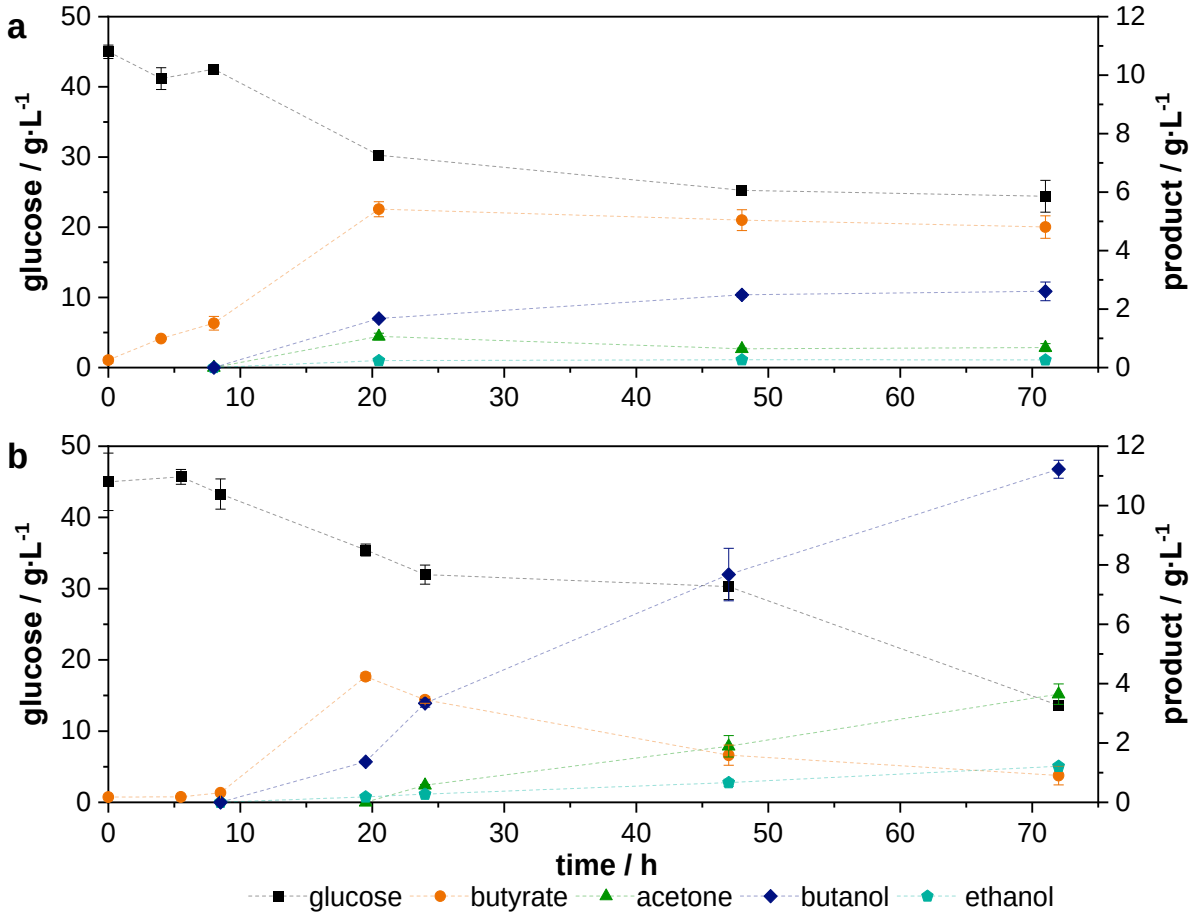

Fig. 4: Resulting cultivation of *C. acetobutylicum* using pre-cultures with different cultivation times, a: Cultivation time of pre-culture: 26 h; b: Cultivation time of pre-culture: 48 h; pre-culture: PY+X medium, 50 rpm, V=100 mL, inoculum size: 1 vol.%, pH_0=5.5; main culture: MP2opt medium, 50 rpm, V=150 mL, inoculum size: 10 vol.%; standardized glucose concentration, n=2.

In the resulting cultivation from the pre-culture with a cultivation time of 26 h (Fig. 4, a) an “acid crash” was observed, which means that butyrate is produced in high concentrations but no shift to solventogenesis is visible (Maddox et al., 2000). Moreover, the production of butyrate can already be observed after a cultivation time of 4 h, which indicates a very short lag phase. The fast butyrate production leads to a high butyrate concentration of 5.41±0.26 $g \cdot L^{-1}$ and a low pH of 4.38±0.01. The "acid crash" was also observed in the main culture of the pre-culture with a cultivation time of 28 h. With a longer cultivation time of the pre-culture, the shift to solventogenesis in the main culture occurs (Fig. 4, b). In these cultivations, the production of acids starts later at a cultivation time of 9.5 h. Butyrate is produced in lower concentrations and within the solventogenesis, acid is degraded and the solvent products are built.

IV.6 Discussion

With this work, a specific cultivation method for *C. acetobutylicum* is examined, which ensures reliable and robust cell growth and the shift to solvent production. Investigations were made regarding the whole cultivation process, including cryo-conservation and pre-cultivation, and the effects of different cultivation conditions on the subsequent main cultivation. The results will be discussed in the following.

The time of cell extraction for cryo-conservation can have an effect on cell growth in the subsequent cultivation (Shida et al., 1977). Besides the growth in pre-cultures, the effect on the solvent production in the main culture is of particular interest, which is why cells of different growth stages were examined concerning the solvent production of resulting main cultures. Here, the cells conserved from a late stationary phase display higher productivity in comparison to cells from earlier cultivation stages. This trend applies for both media tested in this work. The cultivation of cryo-conserved cells from the late stationary phase which were originally grown in MP2opt medium results in a 15% increase in total ABE concentration compared to conserved cells in the same metabolic phase originating from PY+X medium. The statistical significance between the two media was confirmed via a Tukey test ($p<0.06$). Péter et al. observed higher survival rates of cells from various microorganisms harvested in the stationary phase for cryo-conservation compared to cells from the late exponential phase (Péter and Reichart, 2001). For the cultivation of clostridia, there is the additional effect of sporulation, that is initiated by solventogenesis (Ravagnani et al., 2000). The formation of spores due to the solvent production in the defined medium could therefore result in higher stability against the conservation process (Diallo et al., 2021). These findings underline the influence of the metabolic phase in which the cells are conserved for subsequent cultivations, and lead to the recommendation to use cells from a late stationary phase grown in MP2opt medium for cryo-preservation.

The pH is a commonly known factor that can influence the growth of bacteria (Stolp et al., 1981). The pH suggested by DSMZ for the cultivation of *C. acetobutylicum* in PY+X medium is 6.8-7 (DSMZ). Another commonly used medium for pre-cultures of *C. acetobutylicum* is the Reinforced Clostridial Medium, where a pH of 6.6 to 7 is applied (Merck KGaA). Other authors refer to using lower pH for pre-cultivation of different strains of *C. acetobutylicum* like 5.8 or 6 (Hartmanis et al., 1986; Li et al., 2011). Based on our investigation, growing pre-cultures could only be observed in a pH range between 5.0 and 6.2. In these cultivations, the OD increased parallel to the decrease of the pH. With a pH of 6.8, which is suggested for clostridia cultivation in PY+X Medium by DSMZ, there could not be achieved any cell growth based on cryo-preserved cells in this work. Also important is the effect on solvent production. For all growing pre-cultures, butanol production in a range from 11.46±1.01 $g \cdot L^{-1}$ was observed, which is near the maximal possible butanol concentration of 14 $g \cdot L^{-1}$ (Cho et al., 2011). Hence, this work could narrow the initial pH recommended for clostridia down to a range between 5 and 6.2 to achieve reliable growth in the pre-culture. For a pH of 5.5, the fermentation course of independent cultivations was more consistent than for fermentations with a pH closer to the threshold where growth is still possible. This is why a pH of 5.5 is suggested for further cultivations.

The great influence of the metabolic phase of the pre-culture on the solvent production in the main culture was also shown in this work. The butanol and ABE concentration in the main culture could be increased by nearly 70 % by using a pre-culture of the stationary growth phase instead of a pre-culture of the exponential growth phase. Regarding the increase in cell number with the cultivation time of the pre-culture, it might be reasonably expected that the higher solvent production could be related to a higher cell number. But the fact that there was no increase in cell number after 32 h contradicts this assumption. As the main culture is inoculated using 10 vol.% of pre-culture, not only cells, but also metabolites are transferred to the main culture. However, the maximum total acid concentration in the pre-culture is below 5 $g \cdot L^{-1}$, which leads to a negligible acid concentration in the main culture after the dilution through the inoculation. Also, there is a natural correlation between the composition of the inoculum and the metabolic phase of the pre-culture, which emphasizes the influence of the latter. Furthermore, with an increasing cultivation time of pre-culture, a decrease in butyrate production in the resulting main culture goes along. Hence, the metabolic phase the cells are in during inoculation has a great effect on acid and solvent production in the main culture. This influence is clearly shown in Fig. 4. Cultures inoculated with cells from exponential growth phase result in a short lag phase and rapid acid production, which leads to a low pH. The resulting high concentration of undissociated acids has an inhibitory effect on the metabolic activity (Cho et al., 2011; Gottwald and Gottschalk, 1985; Maddox et al., 2000). Sandoval-Espinola et al. investigated the impact of the harvest time of cells of *Clostridium beijerinckii* on the growth of the cells in the resulting culture (Sandoval-Espinola et al., 2015). Harvested cells from the exponential and late exponential growth phases resulted in high

maximum specific growth rates (Sandoval-Espinola et al., 2015). This also correlates with the results in this work, as high growth rates are related to high acid production. The rapid acid production then leads to the “acid crash”. Sandoval-Espinola et al. also found that the harvest of cells in the stationary growth phase results in lower specific maximum growth rates in the following culture (Sandoval-Espinola et al., 2015). With this investigation, a longer lag phase and a lower acid production for cultures that were inoculated with cells from stationary growth phase was observed. The lower acid production prevents the occurrence of an acid crash and therefore enables the shift to the solventogenesis. For these cultures a good solvent production was observed. This enables a reliable solvent production with clostridia even without pH regulation, which is the common method to avoid acid crashes (Capilla et al., 2021).

Based on these findings, the recommended cultivation method for *C. acetobutylicum* is described in the following passage: Cryo-cultures should be prepared from cells that were cultivated in MP2opt medium to reach a late stationary phase. The shift to solventogenesis should be ensured because cells from the stationary growth phase display an increased resistance when cryo-conservated, which could be caused by the sporulation. Cryo-cultures were stored in a 40 % glycerol solution at -80 °C. Pre-cultures were then inoculated with 1 vol.% of said cryo-culture. The initial pH range of the pre-culture could be narrowed down to a value between 5.0 and 6.2 and the culture was incubated for approximately 48 h to make sure the stationary growth phase was achieved. This can be detected by following the pH. Main cultures were then inoculated with 10 vol.% of pre-culture. Following this protocol ensured not only a stable growth of the clostridia culture, but also reliable solvent production even without external pH regulation. This underlines the importance of the pre-culture for cultivation processes.

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IV.8 Declarations

IV.8.1 Data availability

All data supporting the findings of this study are available in the article, supporting information, or upon request from the corresponding author.

IV.8.2 Ethics approval

Not applicable.

IV.8.3 Conflict of interest

The authors declare no competing interests.

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V. Municipal Green Waste as Carbon Source for the Fermentative Production of Platform Chemicals

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J. Bohlender	Investigation, formal analysis, visualization
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Keywords: Circular bioeconomy, Biomass valorization, Lignocellulose, Biobutanol, Green waste

V.1 Abstract

In Germany alone, more than $5 \cdot 10^6$ tons of municipal green waste is produced each year. So far, this material is not used in an economically worthwhile way. In this work, grass clippings and tree pruning as examples of municipal green waste were utilized as feedstock for the microbial production of platform chemicals. A pretreatment procedure depending on the moisture and lignin content of the biomass was developed. The suitability of grass press juice and enzymatic hydrolysate of lignocellulosic biomass pretreated with an organosolv process as fermentation medium or medium supplement for the cultivation of *Saccharomyces cerevisiae*, *Lactobacillus delbrueckii* subsp. *lactis*, *Ustilago maydis*, and *Clostridium acetobutylicum* was demonstrated. Product concentrations of 9.4 $g_{ethanol} \cdot L^{-1}$, 16.9 $g_{lactic\ acid} \cdot L^{-1}$, 20.0 $g_{itaconic\ acid} \cdot L^{-1}$, and 15.5 $g_{solvents} \cdot L^{-1}$ were achieved in the different processes. Yields were in the same range as or higher than those of reference processes grown in established standard media. By reducing the waste arising in cities and using municipal green waste as feedstock to produce platform chemicals, this work contributes to the UN sustainability goals and supports the transition towards a circular bioeconomy.

V.2 Introduction

Although frequently used in the literature, there is no standardised definition of municipal green waste. In general, green waste includes all compostable organic fractions of generated waste, including bio-waste from food processing. In this study, the term municipal green waste includes all lignocellulosic biomass generated in urban municipal areas, while excluding food waste. Examples of this municipal green waste are grass clippings, hedge pruning, and tree cuttings including bark as well as leaves (Borisova et al. 2022). This material occurs in municipal areas for example in public gardens and parks, cemeteries, and as roadside greenery (Eades et al. 2020). In the European waste catalog, it is summarized under the waste code 200201 as “biodegradable garden and park wastes, including cemetery waste” (Commission decision 2014). To prevent negative effects on the global environment, the EU council directive obliges all member states to reduce the amount of biodegradable waste in landfills (Official Journal of the European Communities 1999; Borisova et al. 2022). According to the classification, $5.7 \cdot 10^6$ tons of municipal green waste were collected in Germany in 2020 (German Federal Statistical Office Destatis 2020). The depletion of fossil fuels combined with the increasing demand for energy and food emphasizes the need for alternative feedstocks. Following the ideal of a circular economy, this inefficiently used waste stream of municipal green waste constitutes a promising resource with so far untapped potential. According to Medick et al. (Medick et al. 2017), this resource could be much more productive than the current waste management report indicates. They determined the technical biomass potential of the city of Berlin, Germany, to be more than 120,000 tons of herbaceous fresh matter per year. This includes leaves and grass cuttings which could technically be harvested. So far, the collected biomass is mainly composted or used for the production

of biogas. However, this application has not yet been economically viable (Inghels et al. 2016, Pick et al. 2012). In a previous review, alternative valorization methods for municipal green waste were already discussed (Langsdorf et al. 2021).

On the molecular level, the main component of green waste is lignocellulose, a complex structure of cellulose, hemicellulose, and lignin. Cellulose consists of glucose, while hemicellulose is a heteropolymer of hexoses and pentoses. Lignin comprises different phenolic compounds. Additionally, green waste contains proteins, lipids, and minerals (Mohapatra et al. 2017). The exact composition varies greatly. One important factor is the type of biomass. Woodier biomass has a higher lignin content, while herbaceous biomass usually has a lignin content below 25% (Langsdorf et al. 2021). Besides the species, the season as well as the condition of the soil on which it is grown also influence the composition of the biomass. With up to 60%, carbohydrates are the dominating fraction of lignocellulosic biomass (Álvarez et al. 2016). Therefore, green waste is a promising growth substrate for microorganisms.

Every valorization of municipal green waste requires a pretreatment of the raw material. Especially the recalcitrance of lignin is often challenging. Established procedures employ physical, chemical, or biological methods as well as combinations. Recently a lot of publications extensively reviewed different pretreatments of biomass with their advantages and disadvantages (Broda et al. 2022; Banu J et al. 2021; Martín et al. 2022; Gallego-García et al. 2023; Sołowski et al. 2020; Duque et al. 2021).

There is a long tradition of using lignocellulosic biomass as feedstock. To bypass the food or feed dilemma, most processes use agricultural waste streams such as straw. As described above, municipal green waste is not only lignocellulosic biomass and arises in large amounts, but has no other application so far besides the unprofitable biogas production and is therefore in no competition with other usages.

In literature, there are various reports of using lignocellulosic biomass as feedstock for fermentations. Most of these relate to agricultural waste streams, but there are also publications dealing with material that can be compared to municipal green waste. For example, Schoeters et al. used press juice from a mixture of *Festulolium* and perennial ryegrass for the co-cultivation of microalgae to produce high-protein biomass (Schoeters et al. 2023). Langsdorf et al. prepared growth media from grass clippings either by using a juice extractor or by blending the feedstock and produced α-humulene with *Cupriavidus necator* without any further additives (Langsdorf et al. 2022). The press juices of *Miscanthus x giganteus* (MxG), *Lolium perenne*, and grass clover mixtures were also already used for cultivcations (Boakye-Boaten et al. 2016; Hull et al. 2014; Santamaria-Fernandez et al. 2019).

The aim of this study is to examine the potential of municipal green waste as a feedstock for microbial production of different platform chemicals. Therefore, suitable methods for the pretreatment of lignocellulosic biomass were selected and a process scheme for the usage of municipal green waste was developed. The resulting components were analyzed and used as solely cultivation media or as media supplements. To cover various fermentation requirements, the cultivation of *Saccharomyces cerevisiae*, *Lactobacillus delbrueckii* subsp. *lactis*, *Ustilago maydis*, and *Clostridium acetobutylicum* was tested for the production of platform chemicals such as the solvents acetone, butanol, and ethanol as well as lactic acid and itaconic acid. Thus, this work contributes to the UN sustainability development goals (SDG), especially to provide affordable and clean energy (SDG 7) as well as to develop sustainable cities and communities (SDG 11).

V.3 Results and Discussion

V.3.1 From parks to biorefineries

In municipal areas, green waste arises mainly in public gardens and parks as well as through roadside greenery. Collected by the city authorities, it can be directly used for a biorefinery application without further pre-processing. However, it might be necessary to remove urban contaminations (e. g. plastics, printed paper) or stones which occur in municipal parks.

V.3.1.1 Pretreatment of municipal green waste for the use in biorefineries

A suitable pretreatment of the biomass is necessary to exploit the carbohydrates and nutrients comprised in municipal green waste for further use in a biorefinery. A process schematic for the pretreatment of municipal green waste according to the different properties of the lignocellulosic material for the use in microbial cultivations was established. The different pathways are summarized in Figure 1. The most important factor for the pretreatment is the moisture content of the raw material. Feedstock with a dry matter content of less than 40% can be pressed. In this way, the biomass is separated into two different fractions, namely a solid press cake, and a liquid press juice. The latter can directly be used as a medium or medium supplement for microbial cultivations. Due to its low moisture content, the press cake is easier to store and transport than the initial biomass. This simplifies the logistics behind a biorefinery concept that uses municipal green waste and makes its implementation more realistic. A dry matter share of more than 40% is usually accompanied by an elevated lignin content. For example, wood cuttings of the hardwood beech contain 26% lignin (Smit et al. 2022), and the softwood pine 37% lignin (Kundu et al. 2021), while the lignin content of herbaceous biomass with a lower dry matter share is typically below 25% (Langsdorf et al. 2021).

If the pressing step of biomass with a high moisture content includes a harsh mechanical pretreatment, as is the case with for example a screw press system, an additional hydrothermal process step is not necessary (Varriale et al. 2022). The solid residue of the pressed biomass, the so-called press cake, can

be saccharified enzymatically, without further processing, and together with the residue of the low-moisture pathway. Biomass with a high lignin content needs to undergo hydrothermal pretreatment. Hereby, the recalcitrant structure of lignocellulose is broken up through the application of elevated temperature and pressure. As lignin is more soluble in organic solvents (Jääskeläinen et al. 2017), these are used during hydrothermal pretreatments to effectively remove lignin from the remaining biomass in so-called OrganoSolv-processes (Paulsen Thoresen et al. 2023; Parot et al. 2022). Varriale et al showed a 41.1% increase in carbohydrate yield from enzymatic hydrolysis of mixed wood chips after an organosolv treatment compared to untreated material (Varriale et al. 2022). The solid residue of hydrothermal procedures is washed to remove by-products such as degraded carbohydrates.

To obtain sugar monomers, the residue is saccharified enzymatically. In this enzymatic hydrolysis, saccharolytic enzymes such as cellulases and hemicellulases are employed, to break down complex carbohydrates. Østby et al. also describe deacetylases, esterases, and lytic polysaccharide monooxygenases for the saccharification of lignocellulosic biomass (Østby et al. 2020). As it is shown in this paper, the resulting hydrolysate can be used as a medium or medium supplement for microbial cultivations without further processing steps.

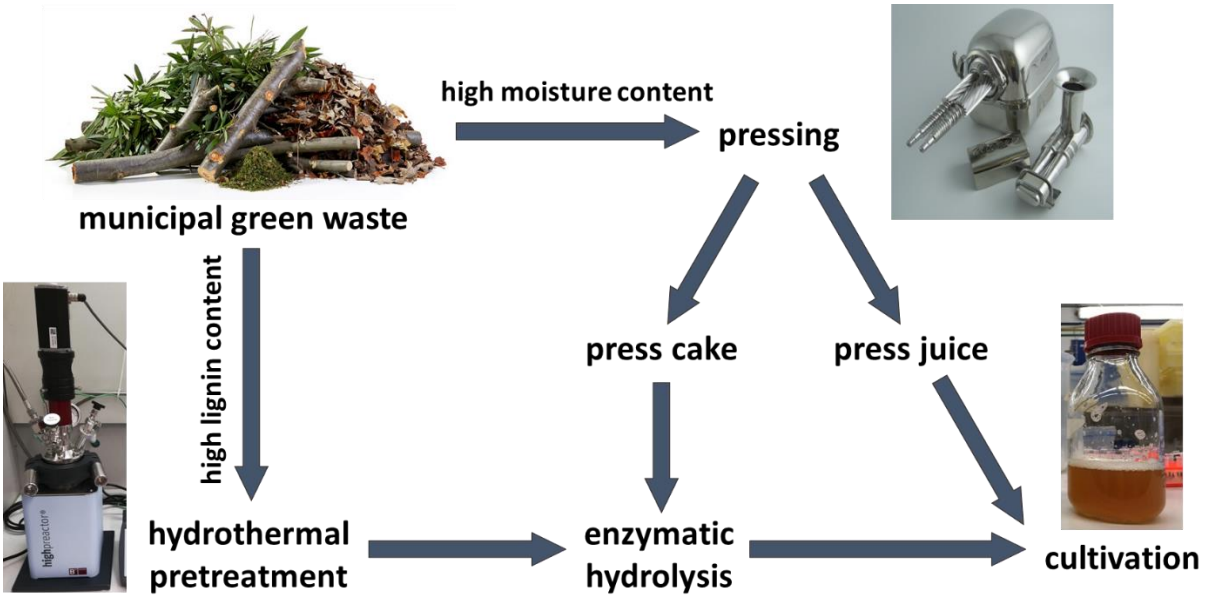

Figure 1: Process schematic of different pretreatment methods of municipal green waste. Biomass with a high lignin content is pretreated using an organosolv process to break up the recalcitrant lignocellulose structure. The residue is saccharified enzymatically before the use in cultivations. Biomass with a high moisture content is separated into a liquid press juice and a solid press cake. The press juice can directly be used for cultivations, the press cake undergoes enzymatic saccharification together with the residue of the high lignin biomass pathway after organosolv pretreatment.

V.3.1.2 Analysis of renewable media supplements

One challenge of working with municipal green waste is the seasonality of the feedstock. At a macroscopic level, the seasonal change is obvious, with a higher proportion of hedge trimmings in spring and a significant amount of leaves in autumn. According to Boldrin and Christensen, the garden waste in Aarhus, Denmark, consisted of more than 90% of material such as grass and flowers in September, while in winter up to 45% of the garden waste consisted of woody material (Boldrin and Christensen 2010). But the composition of municipal green waste also changes on the molecular level. Over the course of the seasons, the distribution of carbohydrates, lipids, and proteins in plants varies greatly. The cell walls of young grasses make up 35% of the plant, while the cell content accounts for 65%. This ratio is inverted to 60% cell walls and 40% cell content in mature plants. As the cell walls comprise mostly lignocellulose, the overall carbohydrate content increases with maturity, while the share of proteins and lipids decreases (Sharma et al. 2011). This development also affects the composition of the media supplements derived from the biomass. The press juice obtained from mixed grass cuttings changes greatly depending on the season (see Table 1). The carbohydrate content of press juice from grass cut in spring was 53 $g \cdot L^{-1}$, while the press juice from fall grass contained 30.5 $g \cdot L^{-1}$ of carbohydrates. The grass stemmed from private gardens in Lohnsfeld, Germany. Although the carbohydrate content of mature plants is higher than that of young grass, the saccharides are mostly incorporated in the rigid structure of the cell walls, resulting in a lower sugar yield in the press juice from herbaceous biomass harvested in fall. The amount of press juice extracted from grass cuttings also changes depending on said factors. On average, 53% of the fresh matter can be obtained as grass press juice.

There are already publications on the use of grass press juice as feedstock for fermentations. Schoeters et al. report 6.7 $g \cdot L^{-1}$ of total carbon and 0.5 $g \cdot L^{-1}$ of total nitrogen in press juice from a mixture of *Festulolium* and perennial ryegrass (Schoeters et al. 2023), while Langsdorf et al. measured 20.0 $g \cdot L^{-1}$ total organic carbon and 1.2 $g \cdot L^{-1}$ total nitrogen in press juice from grass clippings (Langsdorf et al. 2022).

Santamaria-Fernandez et al. reported glucose and fructose concentrations of 9-19 $g \cdot L^{-1}$ and 7-17 $g \cdot L^{-1}$, respectively, in mixed grass-clover press juice (Santamaria-Fernandez et al. 2019). Hull et al. found 61.1 $g \cdot L^{-1}$ of combined glucose, fructose, sucrose, and fructan in press juice from *Lolium perenne* (Hull et al. 2014).

Additionally to carbohydrates and proteins, grass press juice also contains various minerals and ions. The press juice of Italian ryegrass contained, among others, 2.8 $g \cdot L^{-1}$ chloride, 6.6 $g \cdot L^{-1}$ potassium, 997.7 $mg \cdot L^{-1}$ sulfate, 677.6 $mg \cdot L^{-1}$ phosphate, and 155.2 $mg \cdot L^{-1}$ magnesium. Both Boakye-Boaten et al.

and Xiu et al. give an example of the rich content of different organic acids and metabolites in *Miscanthus* press juice (Boakye-Boaten et al. 2016; Xiu et al. 2017).

For the use in cultivations, all components need to be sterilized. This also applies to media components or media supplements. However, press juice is a complex mixture containing both carbohydrates and proteins. When autoclaving amino groups together with reducing sugars, maillard reactions are to be expected (Cardoso et al. 2023). This was shown exemplarily for one for one type of grass press juice. As can be seen in Table 1, the press juice of Italian ryegrass loses around 25% of carbohydrates and around 70% of proteins during an autoclaving process. Therefore, either the loss of nutrients has to be compensated for the use as a cultivation medium or another means of sterilization should be chosen, for example, sterile filtration. The latter has the benefit of conserving the full carbohydrate content of the media supplement. However, the procedure is time consuming and rather expensive, which hampers the transfer to an economical industrial process.

Table 1: Carbohydrate and protein content of press juices from different grass feedstocks

	Glucose / $g \cdot L^{-1}$	Fructose / $g \cdot L^{-1}$	Cellobiose/ Saccharose / $g \cdot L^{-1}$	Protein (Bradford) / $g \cdot L^{-1}$
Mixed grass cutting (spring)	19.9	29.9.	3.2	0.9
Mixed grass cutting (fall)	8.5	15.0	7.0	1.0
Italian ryegrass (native)	9.0	31.1	not quantified	1.7
Italian ryegrass (autoclaved)	7.7	22.8	not quantified	0.5

Both the solid residue after a pressing step and material too dry for pressing can be used as feedstock as well. Material with a high lignin content was pretreated with an organosolv procedure before enzymatic saccharification, and the press cake of herbaceous biomass was directly hydrolyzed enzymatically (see Figure 1). Hereby, we achieved a hydrolysate containing carbohydrate concentrations of around 33 $g \cdot L^{-1}$ from mixed wood chips as shown in table 2. This feedstock has a cellulose content of 26%, which corresponds to a cellulose yield of around 74% after enzymatic hydrolysis (see also (Varriale et al. 2022). Around 21% of the applied bio dry mass can be obtained as sugars in the hydrolysate. In the literature, the composition of the resulting hydrolysate used for cultivations is rarely given; mostly cellulose conversion rates are indicated. Bay et al. obtained glucose yields between 44.6 and 49.3% from poplar and pine wood as well as rice straw after phosphoric acid treatment and enzymatic hydrolysis (Bay et al. 2022). Cui et al. report glucose yields of up to 46.3% from paper mulberry (*Broussonetia papyrifera*) after pretreatment with deep eutectic solvents at elevated temperatures followed by saccharification (Cui et al. 2022). The hydrolysis yield of beech chips after microwave-assisted pretreatment with 60% ethanol and 1% sulfuric acid and enzymatic hydrolysis performed by Mikulski et al. is 56.8% (Mikulski and Kłosowski 2023). During hydrothermal

pretreatments, sugar degradation products such as 5-(hydroxymethyl)furfural (5-HMF) and furfural as well as other phenolic compounds and acids are formed. Mikulski et al. report 5-HMF and furfural concentrations of 51.5 and 6289.7 $mg \cdot L^{-1}$, respectively, for wheat straw, which was pretreated microwave-assisted with 60% ethanol and 1% sulfuric acid, and 433.4 and 5945.9 $mg \cdot L^{-1}$ for equally processed beech chips (Mikulski and Kłosowski 2023). In this study, the hydrolysate of mixed wood chips contained 4.1 $mg \cdot L^{-1}$ HMF and 4.2 $mg \cdot L^{-1}$ furfural, while the hydrolysate of a mixture of wood chips and grass only comprises 2.3 and 3.8 $mg \cdot L^{-1}$, respectively. These degradation products inhibit fermentation processes by intercalating into biological membranes (Palmqvist and Hahn-Hägerdal 2000; Kordala et al. 2023). Different detoxification strategies such as the use of laccase, chemical and physical methods, or the *in situ* removal by a fermentative organism are discussed in the literature (Fillat et al. 2017; Jönsson et al. 2013; Ujor and Okonkwo 2022). In this work, a washing step between the organosolv process and the enzymatic saccharification was employed, which explains the low concentration of less than 9 $mg \cdot L^{-1}$ of sugar degradation products left in the final hydrolysate. As the grass press cake was not pretreated hydrothermally, no sugar degradation products should be expected in the hydrolysate.

The pretreatment method described above, the enzymatic hydrolysis of biomass pretreated with an organosolv procedure, not only results in a hydrolysate with a 60% higher glucose yield compared to the literature, but additionally does not require an expensive detoxification step to remove sugar degradation products. Therefore, it is a suitable procedure to obtain cultivation media from municipal green waste.

Table 2: Composition of enzymatic hydrolysate of different lignocellulosic feedstocks. Mixed wood chips and the wood-grass mixture were pretreated using an organoyolv procedure before enzymatic hydrolysis. Organosolv pretreatment with 50% (w/w) EtOH and 10% solid loading for 15 min at 180°C, enzymatic hydrolysis with 0.16 $g_{enzyme}/g_{biomass}$ and 5% solid loading for 72 h at 50°C. Mean deviation from n=2 if indicated.

Component	**Mixed wood chips (organosolv pretreatment)**	**3:1 grass and wood chips (organosolv pretreatment)**	**Grass press cake**
Glucose / $g \cdot L^{-1}$	21.3	17.6	10.8 ± 0.1
Xylose/galactose/mannose / $g \cdot L^{-1}$	12.3	9.2	6.7 ± 0.1
5-HMF / $mg \cdot L^{-1}$	4.1	2.3	n.d.
Furfural / $mg \cdot L^{-1}$	4.2	3.8	n.d.

In the following chapters, the general suitability of media supplements derived from municipal green waste for the use in microbial cultivations is demonstrated. For industrial processes however, the carbohydrate concentration of the resulting media is too low to obtain satisfying product yields. One

possibility to further increase the glucose concentration of the hydrolysate is the the optimization of the pretreatment processes. For example, the enzyme cocktail can be modified and augmented with cellulases. Another possibility is to increase the solid loading during the enzymatic hydrolysis, which can increase the carbohydrate yield due to the additional polysaccharides present. However, a higher solid loading also increases the liquefaction time and requires more energy and possibly other equipment. Amândino et al. determined a solid loading of 11% (w/v) for the enzymatic hydrolysis of *Eucalyptus globulus* bark as an optimal trade-off between a higher sugar concentration and the hydrolysis conversion efficiency (Amândio et al. 2023). An optimization of the hydrolysis procedure is necessary to render the process more cost-effective.

V.3.2 Cultivation of four different microorganisms with media supplements based on municipal green waste

The suitability of media supplements derived from municipal green waste was tested using several microorganisms. The organisms were chosen diversely to cover a broad range of cultivation requirements. Both bacteria and fungi were tested, as well as aerobic and anaerobic organisms. Table 3 summarizes various publications examining the cultivation of the organisms employed here using media based on different lignocellulosic feedstocks as a comparison.

Table 3: Overview of publications using different lignocellulosic waste fractions as feedstock for the microbial production of various products

Substrate	Product	Organism	Reference
press juice from a mixture of *Festulolium* and perennial ryegrass	1.01 $g_{algal\ biomass} \cdot L^{-1}$ with 41% protein content	co-cultivation of microalgae *Chlorella sorokiniana* and *Acutodesmus obliquus*	(Schoeters et al. 2023)
extracted juice from mixed grass clippings	2 $mg_{\alpha\text{-humulene}} \cdot L^{-1}$	*Cupriavidus necator*	(Langsdorf et al. 2022)
press juice of *Lolium perenne*	22.5 $g_{ethanol} \cdot L^{-1}$, 7.89 $mg_{squalene} \cdot g_{dry\ mass}^{-1}$	*S. cerevisiae* YUG37-ERG1	(Hull et al. 2014)
cashew apple juice	38.6 $g_{ethanol} \cdot L^{-1}$ 0.47 $g_{ethanol} \cdot g_{sugar}^{-1}$	*S. cerevisiae*	(Pacheco et al. 2010)
simulated corn stover hydrolysate	53.4 $g_{ethanol} \cdot L^{-1}$ 0.498 $g_{ethanol} \cdot g_{total\ sugars}^{-1}$	engineered *S. cerevisiae* strain	(Wang et al. 2022)
100% press juice from mixed grass cuttings without	9.4 $g_{ethanol} \cdot L^{-1}$ 0.61 ± 0.03 $g_{ethanol} \cdot g_{sugar}^{-1}$	*S. cerevisiae*	This work

additional components			
unsterile press juice of *Miscanthus x giganteus* (MxG) with the addition of saccharolytic enzymes	11.91 $g_{lactic\ acid} \cdot L^{-1}$, 0.29 $g_{ethanol} \cdot L^{-1}$ ethanol 0.63 to 1.38 $g_{acetuc\ acid} \cdot L^{-1}$	heterolactic co-culture of *Lactobacillus plantarum* and *Lactobacillus brevis*	(Boakye-Boaten et al. 2016)
press juice of grass clover mixtures	22 $g_{lactic\ acid} \cdot L^{-1}$	*L. salivarius* BC 1001	(Santamaria-Fernandez et al. 2019)
hydrothermally pretreated sugarcane bagasse	52.4 $g_{lactic\ acid} \cdot L^{-1}$ 0.66 $g_{lactic\ acid} \cdot g_{sugar}^{-1}$	*B. coagulans* under non-sterile conditions	(Cox et al. 2023)
chemically and enzymatically pretreated *P.australis* reed stems	28.3 $g_{lactic\ acid} \cdot L^{-1}$	*B. coagulans*	(Schroedter et al. 2020)
100% press juice from mixed grass cuttings without supplements	16.93 $g_{lactic\ acid} \cdot L^{-1}$ 1.36 ± 0.04 $g_{lactic\ acid} \cdot g_{sugar}^{-1}$	*Lactobacillus delbrueckii* subsp. *lactis*	This work
beech wood xylan	0.08 $g_{itaconate} \cdot L^{-1}$	*U. maydis* MB215P_{oma}xyn11A	(Geiser et al. 2016)
Cellobiose	5.2 $g_{itaconate} \cdot L^{-1}$	*U. maydis* MB215$_{oma}$bgl1	(Geiser et al. 2016)
hydrolyzed hemicellulose fraction from beech wood	0.36 $g_{itaconic\ acid} \cdot L^{-1}$	*U. maydis* MB215	(Klement et al. 2012)
hydrolyzed hemicellulose fraction from beech wood with added glucose	8.5 $g_{itaconic\ acid} \cdot L^{-1}$	*U. maydis* MB215	(Klement et al. 2012)

brewer's spent grain, hydrothermally pretreated and enzymatically hydrolyzed	6 $g_{itaconic\ acid}·L^{-1}$ 0.38 $g_{itaconic\ acid}·g_{sugar}^{-1}$	*U. maydis* MB215 ΔCyp3 P_{etef}Ria1	(Weiermüller et al. 2021)
Mineral salt medium + 70% (v/v) press juice of mixed grass cuttings	19.18 $g_{itaconic\ acid}·L^{-1}$ 0.51 $g_{itaconic\ acid}·g_{glucose}^{-1}$	*U. maydis* MB215 ΔCyp3 P_{etef}Ria1	This work
Mineral salt medium with 40% (v/v) enzymatic hydrolysate of mixed wood chips after organosolv pretreatment	17.2 $g_{itaconic\ acid}·L^{-1}$ 0.40$g_{itaconic\ acid}·g_{glucose}^{-1}$	*U. maydis* MB215 ΔCyp3 P_{etef}Ria1	This work
enzymatic hydrolysate of alkaline pretreated coffee silverskin	3.2 $g_{ABE}·L^{-1}$ 0.1 $g_{ABE}·g_{sugar}^{-1}$	*C. acetobutylicum*	(Niglio et al. 2019)
detoxified wood hydrolysate and alfalfa juice with buffering agents, vitamins and minerals	0.17 $g_{solvents}·g_{total\ sugars}^{-1}$	*C. acetobutylicum*	(Mechmech et al. 2016)
detoxified pine wood hydrolysates	5.7 $g_{butanol}·L^{-1}$	*C. acetobutylicum*	(Maddox and Murray 1983)
mineral salt medium with 30% (v/v) enzymatic hydrolysate of mixed wood chips after organosolv pretreatment	15.5 $g_{ABE}·L^{-1}$ 0.31 ± 0.01 $g_{ABE}·g_{glucose}^{-1}$	*C. acetobutylicum*	This work

V.3.2.1 Ethanol production of Saccharomyces cerevisiae in grass press juice

In the literature are reports of various media used for the cultivation of *S. cerevisiae*. Most of the media derived from waste material are based on enzymatic hydrolysates from pretreated lignocellulosic biomass, but there are also few publications using material without prior enzymatic hydrolysis. For example, Boakye-Boaten et al. showed the suitability of *Miscanthus* grass press juice for the cultivation of *S. cerevisiae* and achieved a 75% increase in cell dry weight under anaerobic conditions compared to the growth in standard yeast medium broth. However, they did not determine the formation of ethanol using press juice (Boakye-Boaten et al. 2016). Pacheco et al. cultivated *S. cerevisiae* in cashew apple juice supplemented with media components and achieved an ethanol concentration of 38.6 $g \cdot L^{-1}$, but obtained a yield of 0.47 $g_{ethanol}/g_{sugar}$ (Pacheco et al. 2010). Further optimization can be achieved by genetic modifications of the organism. Wang et al. engineered a *S. cerevisiae* strain to also metabolize xylose and yielded 0.498 $g_{ethanol}/g_{total\ sugars}$ with an ethanol concentration of 53.4 $g \cdot L^{-1}$ in a simulated corn stover hydrolysate (Wang et al. 2022).

In this work, autoclaved grass press juice was used as medium for the anaerobic cultivation of *Saccharomyces cerevisiae* for the fermentative production of ethanol. Due to the turbidity of the autoclaved press juice, reliable measurement of the optical density (OD_{600}) was not always possible. Cell growth could be observed by determining the bio-dry mass, and the metabolism could be analyzed by the substrate and product concentrations. Before a scaleup, different cultivation setups with and without additives were tested in 100 mL Schott flasks (see Table 4).

S. cerevisiae can both grow and produce ethanol in pure grass juice. According to the course of the ethanol and sugar concentrations (see Figure 2B), the metabolism of the organisms is slightly slower in grass press juice, but there is no prolonged lag phase.

The press juice used for the cultivations of *S. cerevisiae* contained 5.7 $g \cdot L^{-1}$ glucose, 13.4 $g \cdot L^{-1}$ fructose, and 4.8 $g \cdot L^{-1}$ saccharose. During a cultivation in pure grass press juice without any additives, both glucose and fructose were consumed almost completely (see Figure 2B).

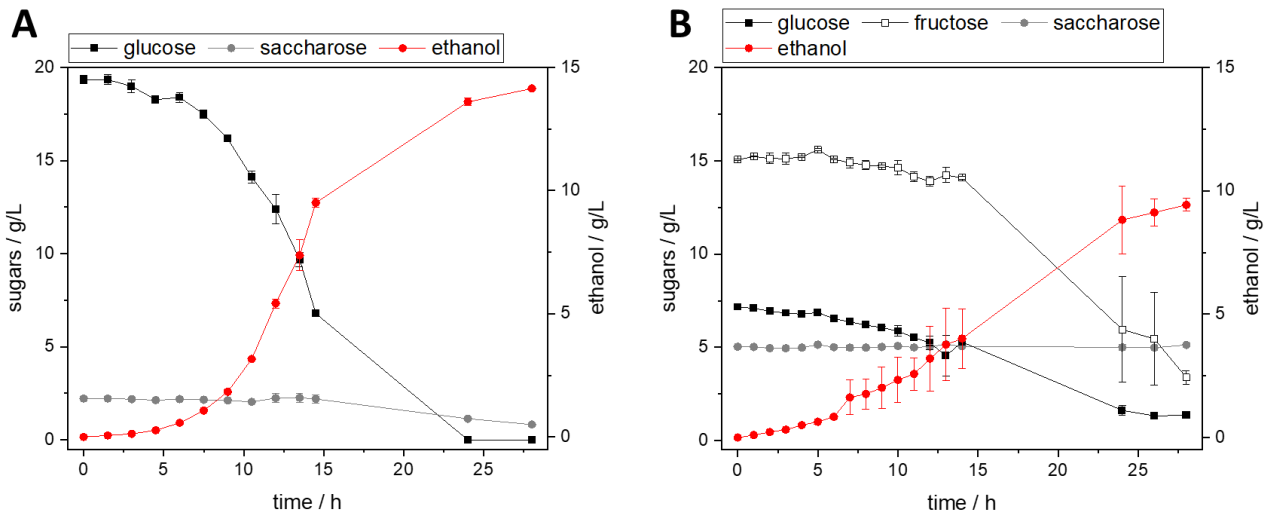

Figure 2: Cultivation of *S. cerevisiae* in (A) standard complex medium in Schott flasks and (B) 100% autoclaved grass press juice without any additives in a bioreactor system. n=2, lines serve as a guide to the eye.

In contrast, saccharose was not metabolized. The addition of invertase into cultivation broths is the established basis of simultaneous saccharification and cultivation processes. Molina et al. for example report an increase in mannitol yield of 70% produced by *Apilactobacillus kunkeei* NFICC 2128 cultivated in a mixture of apple pulp and sugar beet molasse by adding invertase to the fermentation broth (Molina et al. 2023). Therefore, the addition of invertase and media components to the grass press juice to improve the productivity and yield of the fermentation was examined in a preliminary test. While the addition of the enzyme led to the degradation of saccharose, the overall yield of the fermentation increased by only 4% (see Table 4), although components of the reference medium were supplemented as well. The marginal increase in ethanol yield does not justify the use of expensive enzymes or the addition of media components. Therefore, the scale-up in a bioreactor with a working volume of 0.4 L was performed in pure grass press juice without any additional components, which is shown in Figure 2B. With 9.4 $g \cdot L^{-1}$ the final ethanol concentration in grass press juice is lower than in a reference cultivation in standard medium with 14.1 $g \cdot L^{-1}$, but with 0.61 and 0.68 $g \cdot g_{sugar}^{-1}$ the product yields of both cultivations are in the same range. The maximum theoretical yield of ethanol produced from glucose by *S. cerevisiae* is 0.51 $g_{ethanol}/g_{glucose}$ due to the production of 2 mol CO_2. The higher yield obtained in this work traces back to the fact that the fermentation broth contained not only glucose but also fructose and other complex components which were metabolized by the microorganism. The scaleup from 50 mL in Schott flasks (see Table 4) to 0.4 L in a bioreactor system with pH control and otherwise unchanged parameters resulted in the yield increasing by 3%. This shows that pure grass press juice is a suitable medium for the cultivation of *S. cerevisiae* and can be used for the production of ethanol. For an industrial application however, the ethanol concentration of the cultivation broth needs to be increased to render the process economically feasible.

Table 4: Yield of ethanol for cultivations of *S. cerevisiae* in reference media and grass press juice with and without additional supplements. Mean deviation from n=2 if indicated, experiments conducted in shake flasks if not stated otherwise.

Cultivation conditions	$g_{ethanol}/g_{sugar}$
General Yeast Medium	0.68 ± 0.01
100% grass press juice without additional components	0.59
100% grass press juice with all complex media components + invertase	0.71
100% grass press juice without additional components, 0.4 L stirred tank bioreactor	0.61 ± 0.03

V.3.2.2 Lactic acid production of Lactobacillus delbrueckii subsp. lactis in grass press juice

After the successful production of ethanol using *S. cerevisiae*, the cultivation of the homofermentative *Lactobacillus delbrueckii* subsp. *lactis* using grass press juice as cultivation medium was tested for the production of lactic acid. The fermentation of grass and other lignocellulosic biomass using lactobacilli has long been known for the production of silage. Here, the acidification of foliage by an unspecific anaerobic fermentation leads to the preservation of fodder. More recent literature also shows the use of herbaceous biomass as feedstock for the cultivation of specific lactobacilli in order to produce lactic acid. Using a heterolactic co-culture of *Lactobacillus plantarum* and *Lactobacillus brevis* in unsterile *Miscanthus* press juice with the addition of saccharolytic enzymes, Boakye-Boaten et al. achieved a lactic acid concentration of 11.91 $g \cdot L^{-1}$, together with 0.29 $g \cdot L^{-1}$ ethanol and 0.63 to 1.38 $g \cdot L^{-1}$ acetic acid (Boakye-Boaten et al. 2016). Santamaria-Fernandez et al. obtained lactic acid concentrations of up to 22 $g \cdot L^{-1}$ with the organism *L. salivarius* BC 1001 in press juice of a grass-clover mixture (Santamaria-Fernandez et al. 2019).

In this work, different parameters were examined in Schott flasks before a scaleup in a bioreactor (see Table 5). Calcium carbonate was added to both the standard complex medium and the grass press juice as a buffering agent to prevent pH shifts due to the production of lactic acid.

Table 5: Yield of lactic acid for cultivations of *L. delbrueckii* subsp. *lactis* in reference media and grass press juice with and without additional supplements. Experiments were conducted in Schott flasks if not stated otherwise. Mean deviation from n=3 if indicated.

Cultivation conditions	$g_{lactic\ acid}/g_{sugar}$
Complex medium + $CaCO_3$	1.35
100% grass press juice + $CaCO_3$	1.27
100% grass press juice with media supplements + invertase	1.85
100% grass press juice without supplements, 0.4 L stirred tank bioreactor	1.36 ± 0.04

It is possible to cultivate *L. delbrueckii* in pure grass press juice and obtain lactic acid as a product. In pure press juice, the onset of the production of lactic acid is after about five hours, whereas in the standard medium, the production starts after two hours (see Figure 3). The addition of media components and invertase to the press juice results in the onset of lactic acid production in a comparable amount of time to in standard medium.

The yields of all experiments were above the maximum theoretical yield of 1 $g_{lactic\ acid}/g_{glucose}$. This is due to complex media components in the reference medium and the grass press juice which are consumed by the organism without being considered for the calculation of the yield. The addition of reference media components and invertase to convert saccharose into monosaccharides resulted in a 46% increase in yield. But as almost no degradation of saccharose could be observed, the effect is mainly attributed to the supplementation of nutrients in the form of media components. Therefore, the scaleup was performed without invertase. Moreover, due to the automatic pH regulation, calcium carbonate as a buffer could be omitted in the bioreactor cultivation. The scaleup increased the yield by 7% compared to the cultivation in Schott flasks and also resulted in a 40% increased lactic acid concentration of 16.9 $g \cdot L^{-1}$ (see Figure 3B). The product concentration in pure grass press juice is still lower than when cultivated in the standard medium with a final lactic acid production of 25 $g \cdot L^{-1}$ and needs to be increased for economical industrial processes. However, this shows that grass press juice is a suitable medium for the cultivation of *L. delbrueckii* even without any supplements. The addition of media components is a possibility to increase the lactic acid yield.

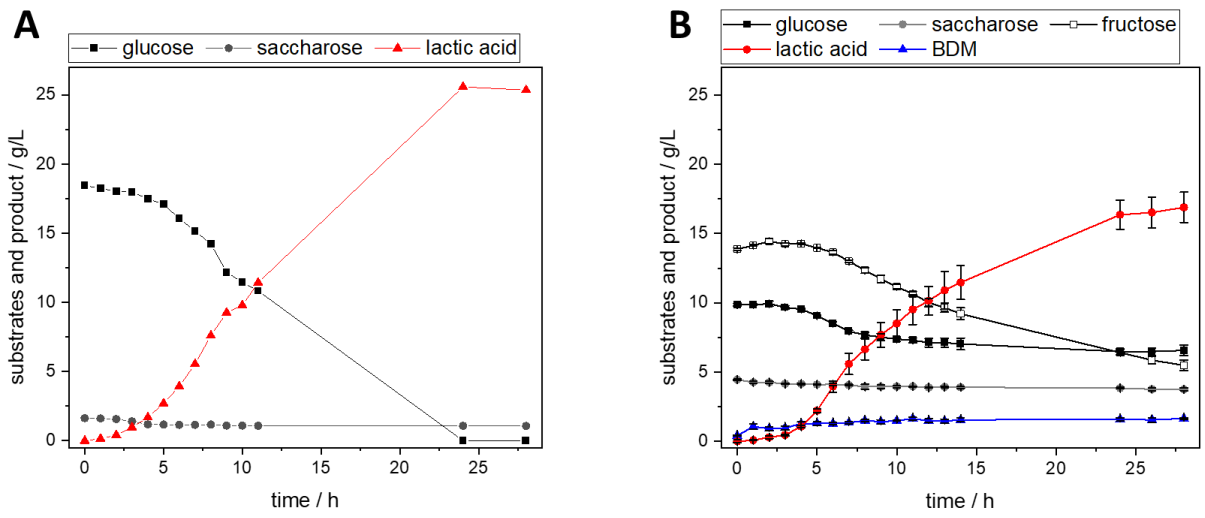

Figure 3: Cultivation of *L. delbrueckii* subsp. *lactis* in (A) standard complex medium with $CaCO_3$ in Schott flasks and (B) 100% autoclaved grass press juice without any additives in a bioreactor system. Mean deviation from n=3 if indicated, lines serve as a guide to the eye. BDM, bio dry mass.

V.3.2.3 Itaconic acid production of Ustilago maydis with supplements of grass press juice and enzymatic hydrolysate

Ustilago maydis is a parasitic fungus that causes the so-called corn smut. Recently, the organism received more attention as it can produce itaconic acid (Geiser et al. 2014; Wierckx et al. 2020). Compared to *Aspergillus terreus*, the common producer of itaconic acid, *U. maydis* has the advantage of unicellular growth and high resistance to impurities. Therefore, *U. maydis* can be regarded as a highly robust production strain (Becker et al. 2023).

In contrast to the other organisms tested in this work, there are not many publications about the cultivation of *U. maydis* based on non-glucose carbon sources or even lignocellulosic biomass. However, as a plant pathogen, *U. maydis* is expected to be able to grow on media based on lignocellulosic biomass. Geiser et al. obtained an itaconate concentration of 0.08 $g \cdot L^{-1}$ with an endoxylanase-producing strain MB215P_{oma}xyn11A from beech wood xylan. With the strain MB215$_{oma}$bgl1, which overexpresses a β-glucosidase, they achieved 5.2 $g \cdot L^{-1}$ on cellobiose (Geiser et al. 2016). Klement et al. cultivated *U. maydis* MB215 on a hydrolyzed hemicellulose fraction from beech wood. With this hydrolysate being the only carbon source, the final itaconic acid concentration was 0.36 $g \cdot L^{-1}$. The addition of glucose increased the concentration to up to 8.5 $g \cdot L^{-1}$ (Klement et al. 2012). Using a medium based on hydrothermally pretreated and enzymatically hydrolyzed brewer's spent grain, Weiermüller et al. achieved a yield of 0.38 g/g_{sugar}, equaling an itaconic acid concentration of 6 $g \cdot L^{-1}$ (Weiermüller et al. 2021).

Here, the *U. maydis* standard medium was supplemented with grass press juice as well as enzymatic hydrolysate of wood chips pretreated with an organosolv process in different concentrations. Due to the solubility of the buffer used for the standard medium, the amount of the supplement was limited to 70% (v/v). The glucose concentration of all experiments with media supplements was adapted to be in the range of the standard medium. *U. maydis* was successfully grown in 70% (v/v) grass press juice (see Figure 4B).

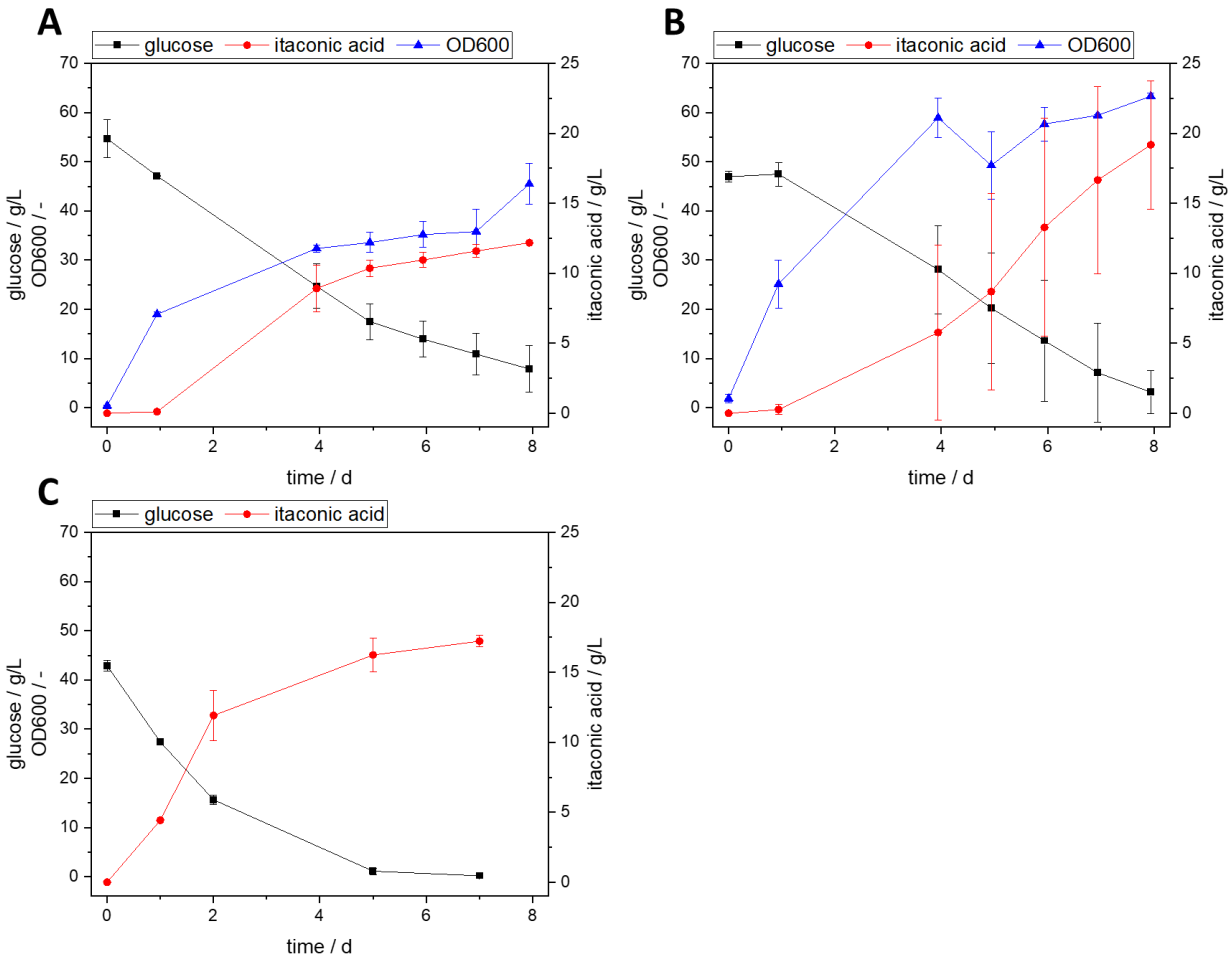

Figure 4: Cultivation of *U. maydis* in (A) modified Tabuchi standard medium, (B) modified Tabuchi medium supplemented with 70% (v/v) autoclaved grass press juice, and (C) modified Tabuchi medium supplemented with 40% (v/v) sterile filtered enzymatic hydrolysate of mixed wood chips pretreated with an organosolv process; n=2, lines serve as a guide to the eye

The supplement did not affect the length of the lag phase, but the final OD reached after 8 days increased by 28%. Compared to the standard medium, the yield of itaconic acid increased by 82% to 0.51 $g_{itaconic\ acid} \cdot g_{glucose}^{-1}$. This considerable increase can be explained by the surplus of nutrients provided by the press juice supplementation. The press juice contains not only glucose, but also fructose and complex components, which can be metabolized by *U. maydis*, but were neither considered for adjusting the sugar concentration nor for the calculation of the yield. Klement et al. demonstrated that *U. maydis* can metabolize xylose (Klement et al. 2012), and preliminary experiments indicated that *U. maydis* is able to use fructose (data not shown). So even though it is not possible to determine the precise influence on the yield, it is evident that grass press juice is a suitable medium for the cultivation of *U. maydis* and the production of itaconic acid.

Subsequently, the standard medium was supplemented with different concentrations of up to 40% of enzymatic hydrolysate of lignocellulosic biomass pretreated with an organosolv process (see Figure

4C). Again, growth was possible and the supplementary nutrients were beneficial for the production of itaconic acid. The determination of the OD was not possible due to the formation of agglomerates when grown in the hydrolysate. However, in contrast to the cultivation in standard medium, itaconic acid is already produced within the first 24 h. Therefore, the lag phase is expected to be shorter. The yield was in the range of 0.35 to 0.42 $g_{itaconic\ acid} \cdot g_{glucose}^{-1}$, on average 35% higher than in the reference cultivation. Contrary to the cultivations of the previously mentioned organisms, the final product concentration of itaconic acid in cultivations with supplements of renewable media components is also higher than the concentration obtained in standard media. While the reference cultivation resulted in 12 $g \cdot L^{-1}$, the concentration of itaconic acid in supplemented cultivations reached up to 20 $g \cdot L^{-1}$. There is no clear trend in the itaconic acid concentration or the product yield for the experiments with 20, 30, and 40% hydrolysate supplementation. Preliminary tests hint that the growth of *U. maydis* in pure grass press juice as well as enzymatic hydrolysate without any additional media components is also possible (data not shown).

Table 6: Yield of itaconic acid for cultivations of *U. maydis* in media with different supplements derived from municipal green waste. Mean deviation from n=2.

Cultivation medium	$g_{itaconic\ acid}/g_{glucose}$
Mineral salt medium	0.28 ± 0.02
Mineral salt medium + 70% (v/v) grass press juice	0.51 ± 0.00
Mineral salt medium + 20% (v/v) hydrolysate	0.35 ± 0.00
Mineral salt medium + 30% (v/v) hydrolysate	0.42 ± 0.06
Mineral salt medium + 40% (v/v) hydrolysate	0.40 ± 0.04

V.3.2.4 Solvent production of Clostridium acetobutylicum with supplements of grass press juice and enzymatic hydrolysate

The organism *Clostridium acetobutylicum* is known for its so-called ABE fermentation, the fermentative production of the solvents acetone, butanol, and ethanol, typically in the ratio of 3:6:1 (Jones and Woods 1986). Especially in light of the rising demand for fuels, butanol gains in importance as a so-called biofuel. There is a long tradition of using media derived from lignocellulosic biomass for the cultivation of Clostridia (Jones and Woods 1986). Riaz et al. extensively review the use of different agro-waste feedstocks for the production of butanol using different Clostridia strains (Riaz et al. 2022). Niglio et al. focused on the use of coffee silverskin. By fermenting the enzymatic hydrolysate of this alkaline pretreated by-product from the coffee industry, they achieved an ABE concentration of 3.2 $g \cdot L^{-1}$, corresponding to 0.1 g_{ABE}/g_{sugar} (Niglio et al. 2019).

Media supplements derived from municipal green waste were successfully used to cultivate *C. acetobutylicum*. Both the addition of grass press juice and sterile filtered enzymatic hydrolysate of mixed wood chips pretreated with an organosolv process enabled the growth of the organism. For all

experiments with media supplements, the final glucose concentration was adjusted to be in the same range as the reference media. Taking about 15 h, the lag phase of cultivations with green waste media supplements was prolongated by 50% compared to the standard medium. The growth phase itself lasted for 25 h, 40% longer than in the reference cultivation. The addition of media supplements also led to higher OD_{600} values of 7 and 5.5 for press juice and hydrolysate, respectively, compared to a maximum of 4.5 in the reference medium (OD_{600} data not shown). However, only marginal amounts of solvents were produced when press juice was added (see Table 7). This is likely due to a phenomenon called acid crash. Clostridia undergo a two-phased metabolism with an acid-producing acidogenesis and a solventogenesis, where the produced acids are re-assimilated and used for the formation of solvents. The acid crash describes the situation in which the metabolic switch to the second phase does not occur or the solventogenesis is terminated rapidly (Maddox et al. 2000; Kumar et al. 2013). The pH decreasing to 4.5, but especially the high concentrations of butyric acid of up to 7.7 $g \cdot L^{-1}$ in experiments with grass press juice supports the thesis of an insufficient metabolic switch. Two different grass press media were prepared. The standard medium components were either dissolved in 30% (v/v) press juice, hence adding additional nutrients to the standard medium recipe, or the press juice was added to readily prepared standard medium, hence diluting the standard medium components. Both the addition as well as the dilution lead to comparable results with low solvent and high acid yields (see Table 7). Therefore, the acid crash is not caused by a lack of nutrients from the media, but by the press juice itself. The triggers for the metabolic switch to solventogenesis as well as the reasons for the acid crash phenomenon are not finally understood yet. Although a low pH value is necessary for the onset of solventogenesis (Hüsemann and Papoutsakis 1988), an internal pH value in the cells below 5.5 hampers the enzyme activity necessary for the switch to solvent production (Gottwald and Gottschalk 1985). One possibility for the acid crash in press juice could be a reduced buffering capacity of the medium in which the pH drop cannot be compensated by the organisms through the uptake of the produced acids. As growth itself was not hampered by the press juice and butyric acid concentrations of up to 7.7 $g \cdot L^{-1}$ were obtained, high solvent yields should be achieved by controlling the pH value during the cultivation to prevent an acid crash.

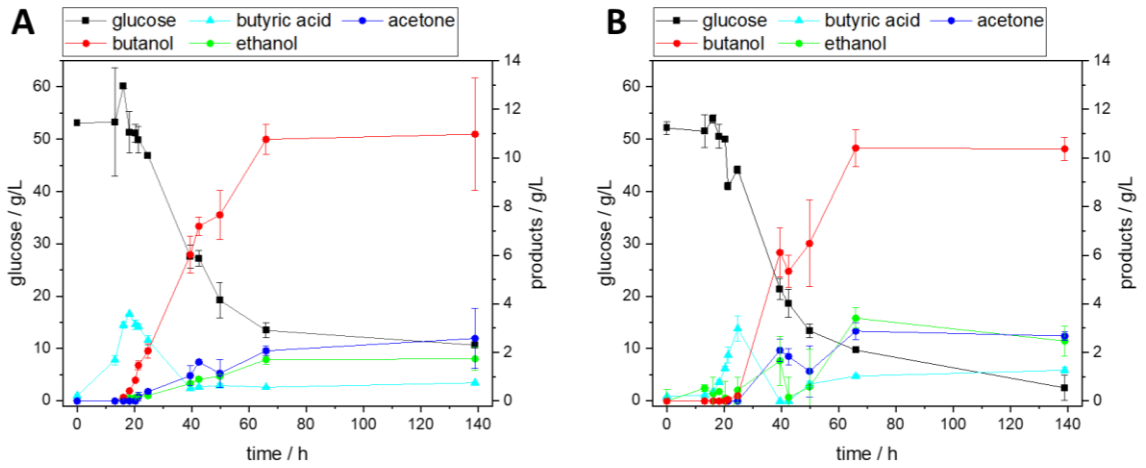

Figure 5: Cultivation of *C. acetobutylicum* in (A) Mp2opt standard medium, and (B) standard medium supplemented with 30% (v/v) sterile filtered enzymatic hydrolysate of mixed wood chips pretreated with an organosolv process; n=2, lines serve as a guide to the eye

The addition of 30% (v/v) enzymatic hydrolysate of mixed wood chips pretreated with an organosolv process had no negative influence on the growth or productivity of the organism. Apart from a slightly extended lag phase, the cultivation is nearly identical to the cultivation in the standard medium (see Figure 5), achieving similar solvent concentrations and solvent yields (see Table 7a). As the yield only refers to the consumed glucose and the hydrolysate contains also other sugars and complex components, the yield is probably slightly overestimated. Still, the enzymatic hydrolysate is a promising media supplement. While in this case only glucose was partly replaced by the supplement, the rest of the standard media components could likely be reduced or even replaced completely by the hydrolysate as well, reducing the costs for the cultivation medium.

Mechmech et al. cultivated *C. acetobutylicum* with wood hydrolysate as carbon source and alfalfa juice as nitrogen source together with buffering agents as well as vitamins and minerals and obtained a solvent yield of 0.17 $g/g_{total\ sugars}$. However, the wood hydrolysate was detoxified by flocculation before its use in fermentations (Mechmech et al. 2016). Maddox et al. also report the necessity to detoxify pine wood hydrolysates. Without this pretreatment, no cultivation of *C. acetobutylicum* was possible, whereas with a combined anion and cation exchange pretreatment they achieved a butanol concentration of 5.7 $g \cdot L^{-1}$ (Maddox and Murray 1983). In this work, however, the only detoxification procedure was a simple washing step of the hydrothermally pretreated material before enzymatic saccharification, simplifying the procedure and making it more economically feasible. Furthermore, with a butanol concentration of 10.3 $g \cdot L^{-1}$ and an overall ABE concentration of 15.5 $g \cdot L^{-1}$, the process presented here is also competitive.

Table 7: Yield of ABE products for cultivations of *C. acetobutylicum* in media with different supplements derived from municipal green waste. Mean deviation from n=2.

Cultivation medium	**Yield**
Standard medium (see Figure 5A)	0.34 ± 0.11 $g_{ABE}/g_{glucose}$
Reduced standard medium + 5% (v/v) grass press juice	0.18 ± 0.03 $g_{ABE}/g_{glucose}$
	0.09 ± 0.02 $g_{butyric\ acid}/g_{Glu}$
Standard medium + 30% (v/v) grass press juice	0.02 ± 0.01 $g_{ABE}/g_{glucose}$
	0.19 ± 0.07 $g_{butyric\ acid}/g_{glucose}$
Reduced standard medium + 30% (v/v) grass press juice	0.04 ± 0.01 $g_{ABE}/g_{glucose}$
	0.30 + 0.01$g_{butyric\ acid}/g_{glucose}$
Standard medium + 30% (v/v) hydrolysate (see Figure 5B)	0.31 ± 0.01 $g_{ABE}/g_{glucose}$

V.4 Conclusions

In this work, the applicability of municipal green waste as feedstock for microbial cultivations was examined. Due to the recalcitrant lignocellulose structure, pretreatment of the biomass is necessary. It should be adapted according to moisture and lignin content. It could be shown that both press juice from herbaceous biomass and enzymatic hydrolysate of high-lignin biomass pretreated with an organosolv process are suitable as a medium or medium supplement for various organisms for the production of a broad product spectrum. *S. cerevisiae* and *L. delbrueckii* were able to grow in pure grass press juice without any additives and produced 9 $g \cdot L^{-1}$ ethanol with a yield of 0.61 $g_{ethanol}/g_{sugar}$ and 16 $g \cdot L^{-1}$ lactic acid with a yield of 1.36 $g_{lactic\ acid}/g_{sugar}$, respectively. The higher than theoretically possible yield is due to additional carbohydrates in the media supplements. Thus, the yields are comparable to cultivations in standard media, although the product concentrations are lower. As the addition of further media components did not improve the cultivation process significantly, grass press juice contains enough nutrients for the growth of both organisms and the production of ethanol and lactic acid. *U. maydis* can use supplements of both grass press juice and enzymatic hydrolysate of biomass pretreated with an organosolv process as a source of nutrients for the production of itaconic acid, and the supplementation increased both product concentration and product yield from 12.0 $g \cdot L^{-1}$ and 0.28 $g_{itaconic\ acid}/g_{glucose}$ to up to 20 $g \cdot L^{-1}$ and 0.51 $g_{itaconic\ acid}/g_{glucose}$. The cultivation of *C. acetobutylicum* is also possible when replacing up to 30% of the standard media components with grass press juice or enzymatic hydrolysate. With the latter, product concentration and yield are similar to reference cultivations with 10.4 $g \cdot L^{-1}$ butanol and 0.31 $g_{ABE}/g_{glucose}$. The use of grass press juice yields high butyric acid concentrations of up to 7.7 $g \cdot L^{-1}$ and requires pH regulation for the production of butanol. The present study shows the applicability of different municipal waste fractions as a feedstock for a broad range of microorganisms. Depending on the microorganism, little to no further nutrients need to be added to the media derived from municipal green waste. This demonstrates the potential of municipal green waste as a promising feedstock for microbial fermentations.

V.5 Materials and methods

V.5.1 Raw material

Grass cuttings as well as wood chips were obtained from private gardens in Lohnsfeld and Kaiserslautern, Germany (49°33’3’’ N, 7°51’12’’ E and 49°26’5’’°N, 7°43’18°E) as a mixture of lignocellulosic biomass representative for municipal green waste. If not stated otherwise, the grass stems from meadows with a blend of grass species characteristic of typical garden and park lawns. The wood chips mainly stem from beech and pine trees.

V.5.2 Production of media supplements from municipal green waste

V.5.2.1 Mechanical and physicochemical pretreatment

The grass was pressed using a screw press Angel Juicer 7500 (Luba GmbH, Bad Homburg, Germany), operating at 82 rpm. Biomass with a low moisture content was pretreated with an organosolv process in a high-pressure reactor BR-500 (Berghof Products + Instruments GmbH, Eningen, Germany) with a volume of 0.5 L. To prevent deformation of the reaction vessel of polytetrafluorethylene (PTFE) an overpressure of 5 bar N_2 was applied. The reactor was heated to 180°C for a holding time of 15 min using an electric heating jacket (Berghof Products + Instruments GmbH). 50% (w/w) ethanol was used as solvent with a solid:liquid ratio of 1:10. The stirring speed was 600 rpm. After cooling down, the residue was washed three times with demineralized water to remove solvent and hydrothermal degradation products.

V.5.2.2 Enzymatic pretreatment

Biomass pretreated with an organosolv process was enzymatically hydrolyzed using a mixture of different enzymes as described in (Varriale et al. 2022). Xylanase 2x and Pectinase L-40 (both ASA Spezialenzyme GmbH, Wolfenbüttel, Germany), containing different xylanases and polygalacturonase, were used in a ratio of 60:40. Enzyme loading based on the protein content of the enzyme solutions was set at 0.16 g_{enyzme} $g_{biomass}^{-1}$ with a biomass loading of 5%. The biomass was autoclaved in the reaction vessel before the addition of the enzymes to prevent microbial contamination and the hydrolysis was conducted at 50°C and 50 rpm for 72 h.

V.5.3 Analysis

The carbohydrate and lignin content of the biomass was determined according to the protocol of the National Renewable Energy Laboratory (Sluiter et al. 2012). Quantitative analysis of sugar monomers and fermentation products was conducted using an HPLC system (ESA Inc. 542 autosampler (Chelmsford, Massachusetts, USA), Azura pump P 6.1 L (Knauer GmbH, Berlin, Germany)) with a refractive index detector. The Bio-Rad Aminex HPX-87H column (300x7.8 mm, Hercules, California, USA) had a temperature of 80°C. 2.5 mM H_2SO_4 with a flow rate of 0.6 mL min^{-1} was used as the mobile phase. The separation of xylose, galactose and mannose was not possible with this system. As it is the

most common sugar of the three, the respective peaks were identified as xylose, but presumably it is a mixture of the pentoses.

V.5.4 Organisms

Saccharomyces cerevisiae (DSM 3799), *Lactobacillus delbrueckii* subsp. *lactis* (DSM 20072), and *Clostridium acetobutylicum* (DSM 792) were obtained from DSMZ (Deutsche Sammlung von Mikroorganismen und Zellkulturen, Braunschweig, Germany). *Ustilago maydis* MB215 ΔCyp3 P_{etef}Ria1 was kindly provided by Prof. Lars Blank from RWTH Aachen. All organisms were stored as cryo cultures at -80°C in the respective culture medium with 40% glycerol.

S. cerevisiae was cultivated anaerobically in 100 mL Schott flasks with butyl septa at 32°C and 120 rpm in 50 mL General Yeast Medium containing 5 $g \cdot L^{-1}$ casein peptone, 3 $g \cdot L^{-1}$ yeast extract, 3 $g \cdot L^{-1}$ malt extract, and 20 $g \cdot L^{-1}$ glucose at pH 6.5.

L. delbrueckii subsp. *lactis* was cultivated anaerobically in 100 mL Schott flasks with butyl septa at 45°C and 120 rpm in 50 mL MRS medium as recommended by the DSMZ containing 10 $g \cdot L^{-1}$ tryptically digested casein peptone, 10 $g \cdot L^{-1}$ meat extract, 5 $g \cdot L^{-1}$ yeast extract, 2 $g \cdot L^{-1}$ potassium phosphate, 5 $g \cdot L^{-1}$ sodium acetate, 2 $g \cdot L^{-1}$ tri-ammonium citrate, 0.2 $g \cdot L^{-1}$ magnesium sulfate heptahydrate, 0.05 $g \cdot L^{-1}$ manganese sulfate monohydrate, 1 $g \cdot L^{-1}$ polysorbate 80, and 20 $g \cdot L^{-1}$ glucose at pH 7.0.

U. maydis was cultivated aerobically in 500 mL shake flasks with baffles at 30°C and 120 rpm. Pre-cultures were grown in 150 mL YEPG medium containing 10 $g \cdot L^{-1}$ yeast extract, 20 $g \cdot L^{-1}$ casein peptone, and 20 $g \cdot L^{-1}$ glucose. They were used to inoculate 200 mL of modified Tabuchi medium to OD_{600} 0.3. The medium contains 19.5 $g \cdot L^{-1}$ MES buffer, 50 $g \cdot L^{-1}$ glucose, 0.8 $g \cdot L^{-1}$ ammonium chloride, 0.5 $g \cdot L^{-1}$ monopotassium phosphate, 0.2 $g \cdot L^{-1}$ magnesium sulfate heptahydrate, 0.1 $g \cdot L^{-1}$ iron sulfate heptahydrate, vitamins and trace elements at pH 6.5 (Geiser et al. 2014). When supplementing the medium with grass press juice or enzymatic hydrolysate, the final glucose concentration of the medium was adjusted to the standard concentration of 50 $g \cdot L^{-1}$.

C. acetobutylicum was cultivated anaerobically in 250 mL serum flasks with butyl septa at 37°C and 50 rpm. Pre-cultures were grown in 100 mL modified PY+X medium containing 5 $g \cdot L^{-1}$ tryptone, 5 $g \cdot L^{-1}$ peptone, 10 $g \cdot L^{-1}$ yeast extract, 5 $g \cdot L^{-1}$ glucose, and 40 $mL \cdot L^{-1}$ salt solution, consisting of 0.25 $g \cdot L^{-1}$ calcium chloride dihydrate, 0.5 $g \cdot L^{-1}$ magnesium sulfate heptahydrate, 1 $g \cdot L^{-1}$ potassium dihydrogen phosphate, 1 $g \cdot L^{-1}$ dipotassium phosphate, 10 $g \cdot L^{-1}$ sodium bicarbonate, 2 $g \cdot L^{-1}$ sodium chloride. Experimental cultivations were inoculated with 10% (v/v) and grown in 150 mL MP2opt medium according to (Engel 2019) containing 2.2 $g \cdot L^{-1}$ ammonium acetate, 0.5 $g \cdot L^{-1}$ potassium dihydrogen phosphate, 0.5 $g \cdot L^{-1}$ dipotassium phosphate, 0.2 $g \cdot L^{-1}$ magnesium sulfate heptahydrate, 0.015 $g \cdot L^{-1}$ iron sulfate heptahydrate, 0.01 $g \cdot L^{-1}$ manganese sulfate monohydrate and 1 $mL \cdot L^{-1}$ vitamin solution,

consisting of 2 mg·L^{-1} 4-aminobenzoic acid, 2 mg·L^{-1} thiamine HCl, 0.01 mg·L^{-1} D-biotin. The final glucose concentration of the medium was adjusted to 45 g·L^{-1} for all experiments when grass press juice or enzymatic hydrolysate was added.

The scaleup of selected cultivations was conducted in a Biostat Q plus reactor system (Sartorius AG, Göttingen, Germany) with a total volume of 0.4 L with integrated temperature probe and pH probe (Easy Ferm Bio HB K8 120, Hamilton, Bonaduz, Switzerland).

V.6 List of abbreviations

5-HMF 5-(hydroxymethyl)furfural

BDM	bio dry mass
DM	dry matter
MxG	*Miscanthus x giganteus*
OD_{600}	optical density at 600 nm
SDG	sustainability development goals

V.7 Declarations

V.7.1 Ethics approval and consent to participate

Not applicable.

V.7.2 Consent for publication

Not applicable.

V.7.3 Availability of data and materials

All data supporting the findings of this study are available in the article, supporting information, or upon request from the corresponding author.

V.7.4 Competing interests

The authors declare that they have no competing interests.

V.7.5 Funding

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V.7.6 Authors’ contributions

MV – conceptualization, data curation, formal analysis, investigation, visualization, writing – original draft preparation, writing – review & editing; ALM, MW, JB – investigation, data curation; AL – conceptualization, writing – review & editing; DH – conceptualization, funding acquisition, project

administration, supervision, writing – review & editing; RU – conceptualization, funding acquisition, project administration, resources, supervision, writing – original draft preparation, writing – review & editing. All authors read and approved the final manuscript.

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VI. Low Oxygen Availability Increases Itaconate Production by *Ustilago maydis*

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VI.1 Abstract

Itaconic acid is a monomer for high performance polymers. While produced in industry with the filamentous fungi *Aspergillus terreus*, the production with the smut fungus *Ustilago maydis* was proposed recently. The strict aerobic process suffers from high power input via gassing and stirring. Here, we investigated in detail possible scenarios for the reduction of energy use during cultivation. In contrast to fermentations with other organic acids, in which even small oxygen concentrations in the medium hinders production, low oxygen availability correlated with increased itaconic acid titer and yield. This is somewhat surprising, as not only the sensitivity of itaconic acid producing *U. maydis* to oxygen deprivation was previously reported, but also the lower degree of reduction compared to glucose is not directly arguing for improved yield. Scale ups from 0.4 L to 30 L using different criteria confirmed the positive impact of low availability of oxygen on itaconic acid production, suggesting a shift in metabolic pathways under restricted oxygen conditions. Oxygen limitation, encountered more likely in industrial fermenters, can be effectively used as a process control strategy to enhance itaconic acid production in *U. maydis*, offering a new approach for improving the efficiency of industrial-scale biotechnological production.

VI.2 Introduction

Itaconic acid is an unsaturated dicarboxylic acid. It is primarily used as a co-monomer for acrylic polymers and rubbers (Teleky and Vodnar 2021), but also for the production of binders, thickeners, resins and coatings (Willke and Vorlop 2001). Due to its structural similarity to acrylic acid and methacrylic acid, it is suitable to replace these petrochemical-based components (Kuenz and Krull 2018). This makes it a promising platform chemical for a sustainable industry. So far, compared to the global demand of $6.2 \cdot 10^6$ t for acrylic acid, the market volume of itaconic acid is rather small with 41,400 t per year (Kuenz and Krull 2018). However, the current market value of 117.8 mio US$ (2024) is projected to grow by 7.8% until 2029 (Market Data Forecast 2023). The commercial production of itaconic acid happens exclusively via biotechnological routes (Willke and Vorlop 2001; Kuenz and Krull 2018). The established organism for this purpose is the filamentous fungus *Aspergillus terreus*, enabling itaconic acid titers of up to 160 $g \cdot L^{-1}$, production rates of 1.53 $g \cdot L^{-1} \cdot h^{-1}$, and yields of 0.63 $g_{ITA} \cdot g_{GLU}^{-1}$, which is close to the maximum theoretical yield of 0.72 $g_{ITA} \cdot g_{GLU}^{-1}$ (Becker et al. 2020a; Krull et al. 2017). However, due to its filamentous growth and the resulting shear sensitivity (Tehrani et al. 2019; Klement et al. 2012), the cultivation of *A. terreus* is challenging. In the course of the development towards a sustainable industry, the inclusion of residual flows such as agricultural residues is of particular interest. The sensibility of *A. terreus* towards impurities in the medium prevents the use of agricultural residues as renewable feedstock (Bafana and Pandey 2018). This is

why the smut fungus *Ustilago maydis* is currently considered to be an alternative itaconic acid producer (Becker et al. 2020a). Compared to *A. terreus*, *U. maydis* has some advantages as a production organism. Firstly, it is able to grow in single cells instead of filamentously. Furthermore, it has also been shown that it can grow on sustainable substrates (Weiermüller et al. 2021; Volkmar et al. 2023). As a model organism for plant pathogenesis (Kämper et al. 2006), it displays a dimorphic life cycle (Bölker 2001). The haploid spores are non-pathogenic and grow yeast-like, making it possible to cultivate them in media (Börner 2009). The conjugation of haploid spores leads to the infectious, filamentous dicaryot (Kahmann and Kämper 2004). Besides itaconic acid, it also produces 2-hydroxyparaconic acid, glycolipids, iron-chelating siderophores, indol pigments, and other organic acids such as ustilagic or malic acid (Becker et al. 2020a; Becker et al. 2020b; Bölker 2001; Bölker et al. 2008; Zambanini et al. 2017; Budde and Leong 1989; Zuther et al. 2008). The organism *U. maydis* MB215 ΔCyp3 P_{etef}Ria1 used in this work was genetically modified to suppress side product formation. The deletion of the cyp3 gene prevents the degradation of itaconic acid to 2-hydroxyparaconic acid, while the promotor exchange leads to an overexpression of the gene cluster for itaconic acid production (Tehrani et al. 2019). Figure 1 shows a simplified metabolism of *U. maydis* for the production of itaconic acid. Cis-aconitate from the tricarboxylic acid (TCA) cycle is exported from the mitochondrion via the mtt1 transporter, converted into itaconic acid in the cytoplasm and exported to the extracellular space via the Itp1 transporter (Zambanini et al. 2017; Geiser et al. 2016b). In contrast to *A. terreus*, where itaconic acid is produced directly from cis-aconitate, itaconic acid production in *U. maydis* takes place in two steps via the intermediate trans-aconitate (Geiser et al. 2016b).

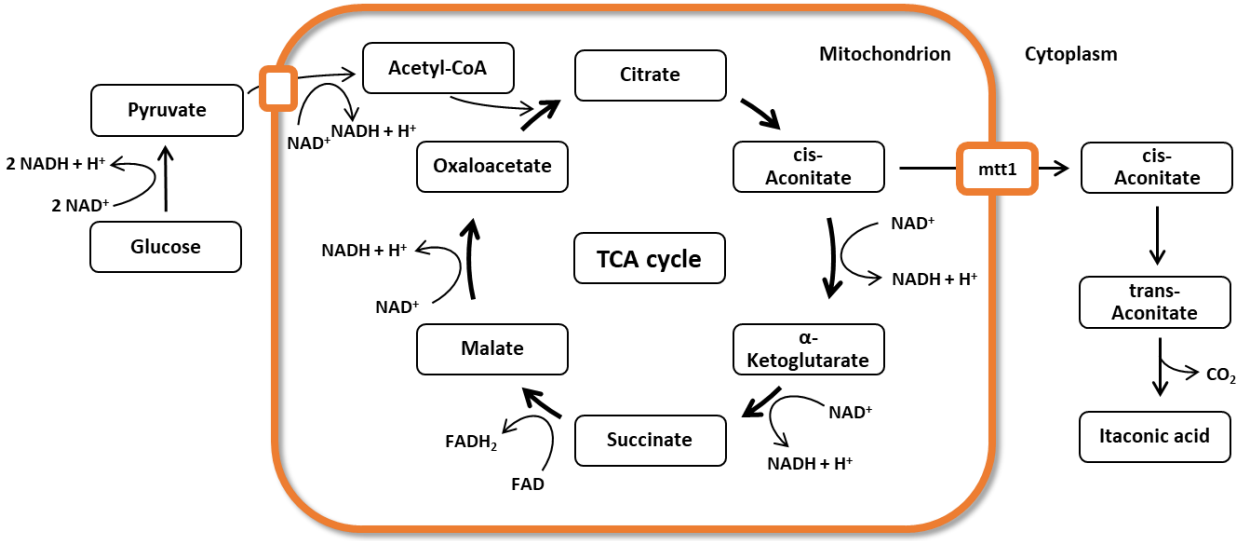

Figure 1: Simplified scheme of itaconic acid production in *U. maydis*, based on (Geiser et al. 2016b).

To consider itaconic acid production via *U. maydis* as economically worthwhile process, a scale up of the fermentation is essential to increase the production volume. However, the scale impacts biological

factors such as the number of cell divisions during inoculum preparation and the sensitivity to contaminations. Chemical and physical factors are affected by the scale as well, for example the means of pH control, the redox potential, temperature control, aeration, and agitation (Junker 2004). Common scale up approaches include constancy of volumetric mass transfer coefficient (k_La), power input per volume of medium, mixing time, and impeller tip speed (Mello et al. 2024; Junker 2004). One scale up with *U. maydis* has been performed based on constant dissolved oxygen tension (DO) (Helm et al. 2024). However, applied DO levels for fermentations with *U. maydis* fluctuate widely in the literature between 30 to 80% (Helm et al. 2024; Becker et al. 2020b; Geiser et al. 2016a; Ullmann et al. 2021), indicating a lack of clarity of the exact influence of DO. Therefore, this paper aims to investigate scale up criteria for the production of itaconic acid using *U. maydis* and explores the influence of oxygen availability during fermentation.

VI.3 Materials and Methods

VI.3.1 Cultivation

The genetically modified strain *Ustilago maydis* MB215 ΔCyp3 P_{etef}Ria1 was designed in the working group Blank (RWTH Aachen, Germany) (Tehrani et al. 2019). It was stored as cryo culture at -80 °C with 40% glycerol. Pre-cultures were grown in shake flasks with baffles at 30 °C and 120 rpm for 48 h in YEPG medium containing 10 $g \cdot L^{-1}$ yeast extract, 20 $g \cdot L^{-1}$ casein peptone, 20 $g \cdot L^{-1}$ glucose, and 167 $mg \cdot L^{-1}$ streptomycin sulfate. For screening experiments, pre-cultures were grown in 500 mL shake flasks while the pre-cultures for scale up experiments were cultivated in 1 L shake flasks. In both approaches, the flasks were filled to 30% with YEPG medium. Main cultures were inoculated to OD_{600} 0.5 and grown in modified Tabuchi medium. It contains 19.5 $g \cdot L^{-1}$ MES buffer, 50 $g \cdot L^{-1}$ glucose, 0.8 $g \cdot L^{-1}$ ammonium chloride, 0.5 $g \cdot L^{-1}$ monopotassium phosphate, 0.2 $g \cdot L^{-1}$ magnesium sulfate heptahydrate, 0.1 $g \cdot L^{-1}$ iron sulfate heptahydrate, vitamins, and trace elements at pH 6.5 (Geiser et al. 2014). Screening experiments were conducted in the 0.5 L reactor system Biostat Q+ from Sartorius (Göttingen, Germany) with a working volume of 0.4 L. The scale up was carried out in the 42 L reactor system Biostat C+ (Sartorius, Germany) with a working volume of 30 L. A dimensioned sketch of the reactors including the reactor internals is depicted in figure S1. Both reactors have six-bladed Rushton impellers and a ring sparger. The Biostat C+ reactor is equipped with a VISIFERM DO 120 probe (Hamilton, Bonaduz, Switzerland) and a BioPAT Xgas sensor (Sartorius, Germany), measuring CO_2 via infrared and O_2 via lambda probe in the off-gas. Antifoam was added manually (Biostat Q+) or automatically (Biostat C+) as needed.

VI.3.2 Analytical methods

The volumetric oxygen transfer coefficient k_La was determined via degassing method in distilled water and modified Tabuchi medium at 30 °C. Oxygen concentration was determined using an OxyFerm FDA

120 probe (Hamilton, Bonaduz, Switzerland) in both reactor systems. k_La was calculated by integrating the general oxygen equation of an aerobic fermentation as shown in equation (1).

$$\ln\left(\frac{{c_0}^*}{{c_0}^* - c_0}\right) = k_L a \cdot t \quad (1)$$

With ${c_0}^*$ = saturation concentration of the liquid phase and $c_0 = O_2$ concentration in the liquid phase.

The k_La corresponds to the slope of the graphical representation of equation (1) over time.

Biomass growth was monitored by off-line measuring the optical density at 600 nm (OD_{600}) using a CO8000 Cell Density Meter (Biochrom Ltd, Cambridge, England; 1 OD_{600} = 0.204 $g_{CDW} \cdot L^{-1}$) or a LAMBDA Bio + spectrophotometer (Perkin Elmer Corporation, Waltham, USA; 1 OD_{600} = 0.139 $g_{CDW} \cdot L^{-1}$), with OD_{600} values converted to cell dry weight (CDW) based on the respective device-specific correlation). Substrate and product concentration were analyzed via a HPLC system (ESA Inc. 542 autosampler (Chelmsford, Massachusetts, USA), Azura pump P 6.1 L (Knauer GmbH, Berlin, Germany)) with a refractive index detector (RI 101 Shodex, Showa Denke KK, Tokyo, Japan). The Bio-Rad Aminex HPX-87H column (300 × 7.8 mm, Hercules, California, USA) had a temperature of 80 °C. 2.5 mM H_2SO_4 with a flow rate of 0.6 $mL \cdot min^{-1}$ was used as the mobile phase. As the buffer substance of the medium MES is chemically stable and is not metabolized during the cultivation, its concentration was used as internal standard to account for any changes in concentration due to evaporation or dilution by correctives. The correction factor was used for the concentration of glucose and itaconic acid as well as for the CDW.

VI.3.3 Calculation

The amount of carbon bound in the biomass and the volume flow and amount of CO_2 were determined to calculate the carbon balance. To calculate the former, the established relation between optical density and cell dry weight was calculated according to equation (2)

$$CDW = OD_{600} \cdot c_{CDW} \quad (2)$$

with an obtained proportionality factor c_{CDW} of 0.20395±0.01717 $g \cdot L^{-1}$. The amount of carbon bound in the biomass was adopted from Klement et al., reporting a share of 0.579 $g \cdot g^{-1}$ (Klement et al. 2012), which leads to equation (3)

$$m_{C,CDW}(t_E) = \left(OD_{600}(t_E) - OD_{600}(t_0)\right) \cdot c_{CDW} \cdot V_R \cdot 0.579\ g \cdot g^{-1} \quad (3)$$

t_E = time of measurement, t_0 = start time, V_R = reaction volume

The volume flow $\dot{V}_{CO_2}(t)$ was calculated according to equation (4), considering an exhaust gas flow of $5 \cdot 10^{-4}$ m³·s^{-1}.

$$\dot{V}_{CO_2}(t) = 5 \cdot 10^{-4} \mathrm{m^3 \cdot s^{-1}} \cdot X^{(v)}_{CO_2}(t) \quad (4)$$

$X^{(v)}_{CO_2}(t)$ = measured volume fraction of CO_2 in the exhaust gas

To calculate the flow of CO_2 in the exhaust gas, equation (5) was applied, assuming an exhaust gas temperature of 20°C due to the exhaust gas cooler.

$$\dot{n}_{CO_2}(t) = \frac{p \cdot \dot{V}_{CO_2}}{R \cdot T} = \frac{10^5 \mathrm{Pa} \cdot \dot{V}_{CO_2}(t)}{R \cdot (20 + 273.15) \mathrm{K}} \quad (5)$$

$\dot{n}_{CO_2}(t)$ = flow of amount of substance, p = pressure in the head space of the reactor, R = universal gas constant

Integration of equation (5) lead to equation (6) yielding the amount of CO_2.

$$n_{CO_2}(t_E) = \sum_{t_i = t_0}^{t_E} \dot{n}_{CO_2}(t_i) \cdot \Delta t \quad (6)$$

VI.4 Results and Discussion

VI.4.1 Investigating aeration of *U. maydis* for high itaconic acid production

Screening experiments were conducted in 0.4 L bioreactors to find suitable process parameters for the cultivation of *Ustilago maydis*. The aim of the screening was to increase the concentration and yield of itaconic acid. The varied parameters were gassing rate and impeller speed. The impeller speed was varied in five steps from 300 to 1200 rpm with a flow of pressured air of1 and 2.5 vvm. The tested range was analyzed according to Doran concerning impeller flooding and complete gas dispersion. The points of impeller flooding are 140 rpm for 1 vvm and 190 rpm for 2.5 vvm. Complete gas dispersion occurs from 330 rpm and 200 rpm, respectively. (Doran 2013). Therefore, no impeller flooding took place in the observed range. Both impeller speed and gassing rate influence the oxygen availability in the reactor, which is likely to impact the itaconic acid production due to the aerobic metabolism of *U. maydis* (Kuenz and Krull 2018). Table 1 summarizes amount of biomass, yield of itaconic acid per consumed glucose, space time yield after 40 hours (time point chosen to make sure that there is no substrate depletion, which started for some experiments after 48 h), final concentration of itaconic acid after 108 h, and k_La.

The course of the cultivation at 1 vvm and 300 rpm, which resulted in the highest final itaconic acid concentration, is shown as an example in Figure 3A. Cell growth started for all experiments within the first twelve hours. For the experiment depicted in Figure 3A, biomass increased until around 99 h,

while for most other parameter combinations from Table 1 the maximum CDW was reached at around 24 h. The substrate concentration decreased linearly until 114 h. For the experiment with 2.5 vvm and 300 rpm, glucose was depleted after 72 h, while for the rest of the tested combinations substrate depletion occurred after 48 h. *U. maydis* is a potential candidate to produce itaconic acid using media based on renewable resources, which often do not exceed carbon concentrations of 50 $g \cdot L^{-1}$. This is why the focus in this work was on lower initial glucose concentrations. Itaconic acid production started after around 12 h. For most experiments, the itaconic acid concentration further increases after the depletion of glucose, which is accompanied with a decrease in CDW. This leads to the assumption that the production of itaconic acid is based on the degradation of the biomass. The literature reports the ability of *U. maydis* to metabolize nutrients that are released from dead biomass (Petkovic et al. 2021). Another explanation could be the digestion of storage lipids (Aguilar et al. 2017). After a linear decrease in the cultivation with 300 rpm and 1 vvm, oxygen availability was low (pO_2 < 5%) after 12 h. For the cultivations with 1 vvm, no oxygen limitation was detected above 600 rpm. Interestingly, in the test series with 2.5 vvm, the same was observed only for the experiments with impeller speeds above 1000 rpm despite the higher gas flow. There was no foam formation observed which could affect mass transfer.

Table 1: Summary of the screening experiments in 0.4 L scale. Experiments are grouped by gassing rate, with separate sections for constant impeller speed and for constant DO. Shown are biomass growth (CDW), yield, space time yield (40 h), and concentration of itaconic acid produced. n=1. n.d.: not determined

gas flow	**impeller speed** / rpm	**max CDW** / $g_{CDW} \cdot L^{-1}$	**product yield** / $g_{ITA} \cdot g_{GLU}^{-1}$	**STY (40 h)** / $g \cdot L^{-1} \cdot h^{-1}$	**final ITA conc.** / $g \cdot L^{-1}$	**k_La** / h^{-1}
1 vvm	300	5.75	0.48	0.17	26.3	40.4
	600	9.40	0.35	0.24	17.4	77.0
	800	9.14.	0.32	0.23	16.5	96.6
	1000	9.61	0.32	0.22	16.4	123.3
	1200	9.22	0.31	0.21	16.2	142.7
2.5 vvm	300	6.55	0.47	0.21	22.9	58.2
	600	9.34	0.35	0.19	16.9	98.2
	900	9.16	0.35	0.27	19.6	162.5
	1000	9.10	0.34	0.21	17.1	183.3
	1200	8.06	0.33	0.21	19	236.6
1 vvm	53-929 (30% DO)	8.62	0.32	0.21	16.9	n.d.

Regardless of the gas flow, both the highest itaconic acid concentration of 26.3 $g \cdot L^{-1}$ (1 vvm) and 22.9 $g \cdot L^{-1}$ (2 vvm) and the highest itaconic acid yield of 0.48 $g \cdot g^{-1}$ (1 vvm) and 0.47 $g \cdot g^{-1}$ (2 vvm) was achieved at an impeller speed of 300 rpm for both test series. At the same time, the lowest amount of biomass was produced under these conditions, with an CDW of 5.05 and 6.55 $g \cdot L^{-1}$, respectively. Generally, a higher impeller speed led to a higher biomass concentration, reaching an CDW of up to 9.61 $g \cdot L^{-1}$ (1 vvm, 1000 rpm). However, both final product concentration and itaconic acid yield decreased at higher impeller speeds. At the same time, a higher gas flow does not necessarily cause a higher itaconic acid yield or concentration, the comparison of the two test series does not demonstrate a clear correlation between the product formation and the gas flow. The same applies for the biomass production. In contrast to the final concentration, the maximum space time yield after 40 h of 0.24 $g \cdot L^{-1} \cdot h^{-1}$ (1 vvm) and 0.27 $g \cdot L^{-1} \cdot h^{-1}$ (2.5 vvm) was not achieved at 300 rpm, but at higher impeller speeds for both gassing rates. Jost et al. observed a similar correlation between oxygen availability and the production of biomass and succinic acid using the obligatory aerobic yeast *Yarrowia lipolytica*. When providing a constant gas flow of 0.4 $L \cdot min^{-1}$ during the fermentation, DO fell to 0% after 24 h. This led to a 528% increase in product formation and a reduction of 70% of the biomass concentration compared to a fermentation with a constant DO of 50% (Jost et al. 2015). Krull et al. also reported an increased itaconic acid production together with a reduced biomass concentration with lower aeration rates using *Ustilago rabenhorstiana*. Increasing the gas flow from 0.1 to 1.0 vvm increased the biomass concentration by 36%, while reducing the product concentration by 21% (Krull et al. 2020). In this work, increasing the gas flow from 1 vvm to 2.5 vvm at 300 rpm lead to a biomass increase of 14%, and a reduction in product concentration by 13%.

To benchmark the strategy of constant impeller speed and allow for comparison with the common literature approach, a cultivation controlled at a constant DO level of 30% was also performed. The DO setpoint was reached after 13 h, triggering an increase in impeller speed from 300 rpm to a maximum of 929 rpm after 13 h, followed by a gradual decrease to 53 rpm by 140 h. Although oxygen availability was successfully maintained, the resulting itaconic acid concentration and yield were similar to results achieved at higher impeller speeds, but lower than those at a constant impeller speed of 300 rpm. This outcome further supports the conclusion that lower oxygen availability favors itaconic acid production while limiting biomass formation. Given that similar productivities can be achieved at constant impeller speed and under DO control with varying speeds, a strategy employing a constant impeller speed is recommended due to its operational simplicity.

The results from lab scale fermentations are highly promising for scale-up, as high aeration rates and impeller speeds are either costly or just impossible to reach. Additionally, employing a constant impeller speed can simplify process control and reduce operational complexity. A scale up to a 30 L

scale using the k_La value as scale up criterion was chosen based on the demonstrated influence of oxygen availability on *U. maydis*. Therefore, the screening conditions were characterized with regard to the k_La. Interestingly, a correlation can be drawn between the k_La value and both the biomass and the product formation. This is shown in Figure 2, where the results (CDW, final concentration, and STY after 40 h) of the screening experiments were plotted against the respective k_La value of the experimental conditions.

A clear trend towards a higher biomass concentration with higher k_La can be observed until a k_La of 77 h^{-1}. Despite the aerobic metabolism, the highest itaconic acid concentration was achieved at the relatively low k_La value of 40 h^{-1}. This hints to a shift in metabolic flux, which is discussed in the last chapter. An increase of the k_La, caused by either higher gas flow or higher impeller speed, does not increase the final itaconic acid concentration. However, the space time yield after 40 h does increase further with a higher k_La, with an outlying maximum STY (40 h) at 163 h^{-1}. But similar to the behavior of the biomass concentration, no clear trend for an increasing STY (40 h) for k_La values above 77 h^{-1} could be identified. The most prominent finding when correlating the screening results with the respective k_La values is the high itaconic acid concentration at a relatively low k_La value.

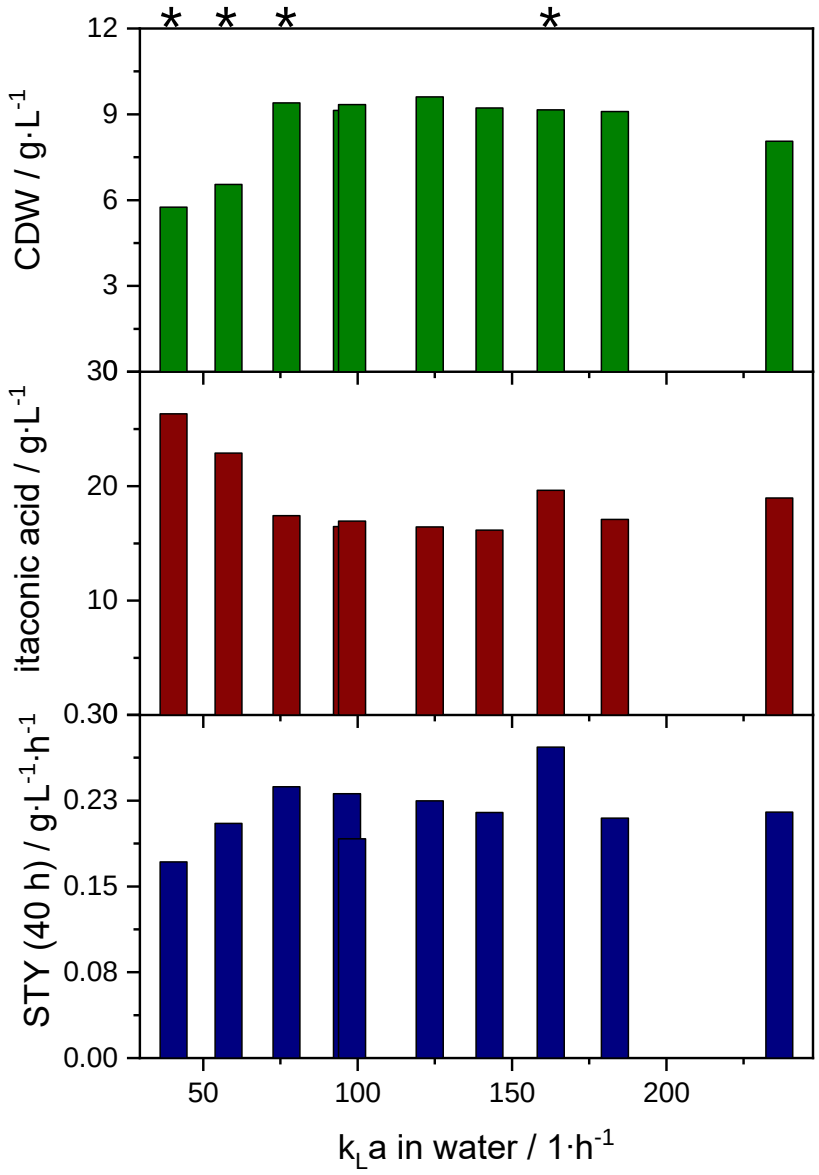

Figure 2: Results of the screening experiments from Table 1 in correlation to the k_La value. For every screening experiment, max. CDW, final itaconic acid concentration and STY (40 h) are plotted against the respective k_La value of the experiment. Fermentation conditions of screening experiments used for scale up are marked with *. n=1

VI.4.2 Scale up via different criteria

The results from the screening experiments lead to a conflict of objectives with regard to the scale up, as the highest itaconic acid yield and the highest STY (40 h) were obtained under different conditions. This is why different operating points were used for the scale up (labeled in Figure 2 with *). Both screening conditions leading to the highest product yield and the highest STY were used, as well as a third point which forms a compromise between the two criteria. As mentioned above, the k_La value was selected as scale up criterion. Additionally, the impeller tip speed (ITS) was used as scale up

criterion for comparison. Both k_La and ITS are commonly used criteria for the scale up of aerobic fermentation processes because they directly influence oxygen transfer efficiency and mixing intensity, which is critical for microbial growth and productivity. Maintaining a similar k_La and impeller tip speed helps to ensure consistent oxygen and shear conditions (Bernemann et al. 2024; Margaritis and Zajic 1978). The results of the performed scale up cultivations, together with the screening conditions on which the scale up was based on, are shown in Table 2.

The screening experiment with the highest product yield (1 vvm, 300 rpm) is scaled up via both impeller tip speed and k_La (114 rpm and 175 rpm in 30 L scale). The screening conditions of 1 vvm and 600 rpm resulted in the second highest STY with a moderately high product yield at the same time, which might constitute a promising compromise between the two objectives. A scale up of this operating point via ITS results in an impeller speed of 229 rpm at the 30 L scale. The screening experiment with 2.5 vvm and 300 rpm resulted in the second highest product yield of all tested conditions. Scaling up this operating point via k_La results in the same impeller speed of 229 rpm at the 30 L scale. An impeller speed of 469 rpm at the 30 L scale is the scale up of the screening conditions with the highest STY via k_La. While a high productivity shortens fermentation time and in consequence leads to lower operational costs, a high final product concentration reduces downstream processing. Therefore, a techno-economic analysis needs to be performed prior to industrially relevant fermentations to maximize overall process efficiency.

The deviation in the maximum CDW, the itaconic acid yield and STY of the scale up from the underlying screening experiment lies between 3.0 and 12.1% for ITS as scale up criterion, and between 3.2 and 18.7% for the final itaconic acid concentration. Although some parameters show a lower deviation when applying k_La as criterion, the range of deviations here goes up to 52.8%. This shows that the ITS is the preferred scale up method which leads to reproducible results of the screening experiments. Reasons for the low validity of the scale up via k_La could be an imprecise correlation of the two reactor systems. Sparger, impeller and oxygen probe are not positioned at exactly the same place. It is assumed that the reactor is not perfectly mixed as this is usually only the case in mathematical models. Therefore, the positioning of the elements is likely to have an influence on the k_La value. As the k_La is affected by every aspect of the reactor geometry while the ITS is a fixed value without dependencies on the surroundings, the positioning of the reactor internals has no effect on the ITS, but a strong effect on the k_La. Additionally, the scale up was based on k_La values determined in water. In a previous experiment in the 0.4 L scale it was shown that the k_La in water corresponds well with the k_La in standard medium (data not shown).

Table 2: Summary of the scale up experiments at a gas flow of 1 vvm and impeller speeds from 114 to 469 rpm in 30 L scale and comparison with the respective screening experiment on which the scale up was based. The scale up experiments at 114 rpm and 175 rpm were based on the same screening experiment (1 vvm, 300 rpm) but with different scale up criteria. The scale up experiment at 229 rpm was the result of the scale up of 1 vvm and 600 rpm via ITS and of 2.5 vvm and 300 rpm at the same time, For every scale up experiment, the deviation from the underlying screening experiment is given. n=1. ITS: impeller tip speed; STY: space time yield; ITA: itaconic acid; GLU: glucose; CDW: cell dry weight

Impeller speed of scale up experiment	**114 rpm**	**175 rpm**	**229 rpm**		**469 rpm**
conditions of screening experiment on which scale up is based	1 vvm, 300 rpm		1 vvm, 600 rpm	2.5 vvm, 300 rpm	2.5 vvm, 900 rpm
focus of scale up	High product yield		Compromise between STY and product yield		high STY
scale up criterion	ITS	k_La	ITS	k_La	k_La
k_La / h^{-1}	26.0 ± 0.2	39.8 ± 1.0	57.9 ± 1.7		162.5 ± 1.4
ITS / $m \cdot s^{-1}$	0.63	0.96	1.26		2.58
final concentration / $g \cdot l^{-1}$	21.36	16.66	16.56		15.37
final concentration in screening	26.3		17.1	22.9	19.6
deviation / %	*18.7*	*36.7*	*3.2*	*27.7*	*21.6*
Max. CDW	5.05	8.80	8.84		8.65
max. CDW in screening	5.75		9.34	9.40	9.16
deviation / %	*12.1*	*52.8*	*3.8*	*6.1*	*5.6*
product yield / $g_{ITA} \cdot g_{GLU}^{-1}$	0.43	0.33	0.32		0.32
product yield in screening	0.48		0.35	0.47	0.35
deviation / %	*10.4*	*31.3*	*3.0*	*31.9*	*8.6*
STY (40 h) / $g \cdot l^{-1} \cdot h^{-1}$	0.18	0.19	0.21		0.18
STY (40 h) in screening	0.17		0.24	0.21	0.27
deviation / %	*5.9*	*11.8*	*12.5*	*0.0*	*33.3*
Specific product formation rate / $g_{ITA} \cdot g_{CDW}^{-1} \cdot h^{-1}$	0.069	0.049	0.049		0.046
product yield per biomass / $g_{ITA} \cdot g_{CDW}^{-1}$	7.39	3.10	2.76		2.55

However, this correlation is especially close for impeller speeds above 400 rpm in the small scale; the previously performed experiments showed that below this value, the k_La in different media deviates

stronger. Therefore, it is possible that the oxygen transmission rates were not identical in the two different setups when choosing the k_La as scale up criterion. The deviations between the mass transfer coefficients could explain the higher deviations between the experiments at the different scales when scaling up based on the k_La value. Calculated according to Doran, impeller flooding occurs in the 30 L scale for impeller speeds below 200 rpm (Doran 2013). This might account for the differing k_La values. Generally, the results of the experiments in 30 L scale confirm the findings in the screening experiments where a higher product concentration is achieved with a lower oxygen availability.

The scale up using impeller tip speed (see Figure 3B) closely mirrored the pattern observed in the underlying screening experiment conducted at 1 vvm and 300 rpm (Figure 3A). Glucose depletion followed a linear trajectory over 96 h, during which biomass levels steadily increased, reaching an CDW of 5.05 $g \cdot L^{-1}$. Itaconic acid production began after 20 h, rising linearly to a final concentration of 21.4 $g \cdot L^{-1}$. A phase of low oxygen availability commenced at 12 h, persisting to 96 h until glucose was depleted, consistent with the screening fermentation. In contrast, the scale up based on k_La showed distinct dynamics (see Figure 3C). Glucose was depleted more rapidly, within 60 hours, accompanied by biomass growth to an CDW of 8.80 $g \cdot L^{-1}$ and an itaconic acid production reaching a final concentration of 16.7 $g \cdot L^{-1}$. Unlike the other fermentations, this process exhibited a clear transition in the growth phase after 24 h, shifting from exponential to linear growth as described by Klement et al. (Klement et al. 2012). The low oxygen availability phase in the k_La based scale up lasted only from 10 h until 46 h. Despite differences in fermentation profiles and final product concentrations, the STY remained similar across experiments: 0.17 $g \cdot L^{-1} \cdot h^{-1}$ for the screening experiment, 0.18 $g \cdot L^{-1} \cdot h^{-1}$ for the ITS-based scale up and 0.19 $g \cdot L^{-1} \cdot h^{-1}$ for the k_La-based scale up. Analysis of the product formation rate per biomass shows that the productivity of the biomass under low oxygen conditions is increased compared to the experiment with a higher k_La. The product yield per biomass of the experiment with the lowest k_La is 2.9 times higher than the product yield per biomass of the experiment with the highest k_La. The comparison of the fermentation courses of the two scale up experiments with the underlying screening experiment underscores the suitability of the ITS as scale up criterion to obtain reproducible results. However, this can only be stated for similar impeller configurations as it is the case in these experiments. Simultaneously, the comparison highlights the enhanced itaconic acid production during low oxygen availability. The relatively low k_La value of 26 h^{-1} of the experiment with 114 rpm induces to a phase of low oxygen availability, resulting in the highest product concentration among all scale up experiments. The data in Table 2 corroborate these findings, demonstrating that low k_La values are associated with higher itaconic acid concentrations, while higher k_La values correlate with a shorter phase of low oxygen availability and an increased biomass production.

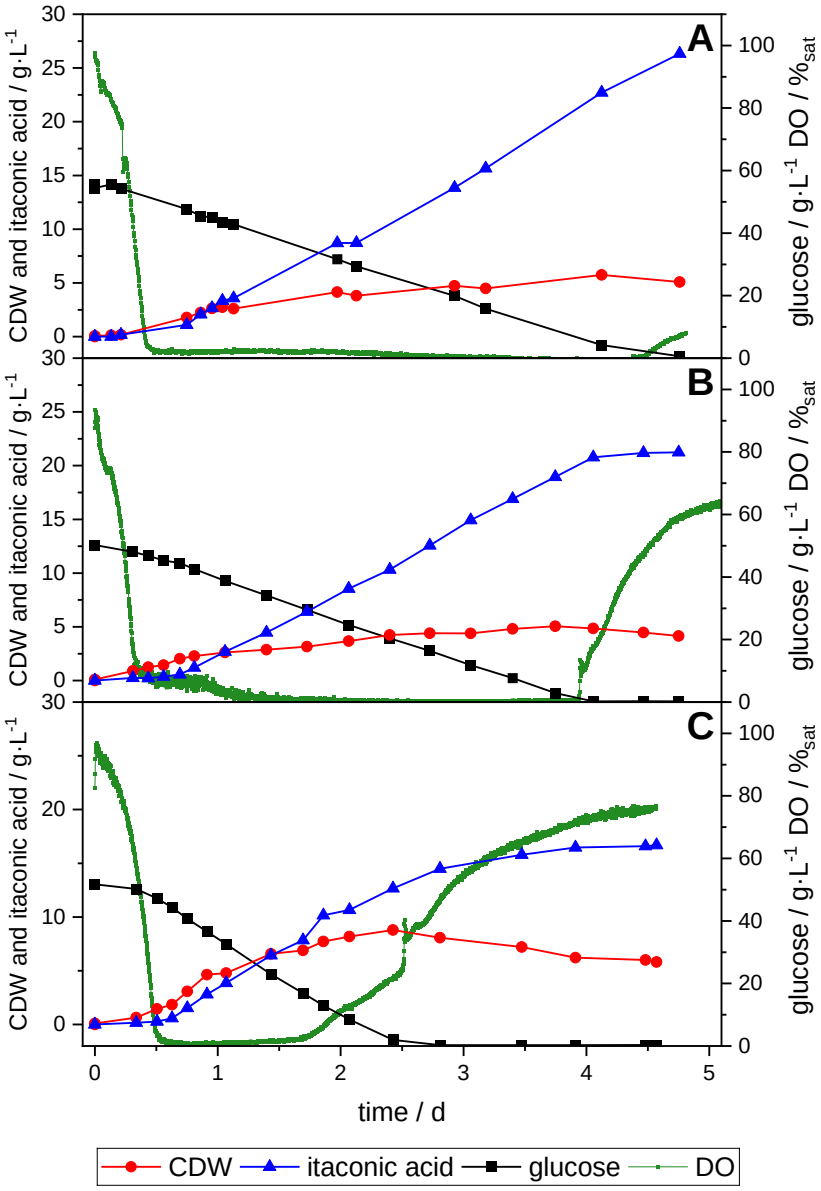

Figure 3: Course of fermentation for screening in 0.4 L at 300 rpm and 1 vvm (A), scale up to 30 L based on impeller tip speed with 114 rpm and 1 vvm (B), as well as scale up based on k_La value with 175 rpm and 1 vvm (C). For these fermentations CDW, DO, glucose and itaconic acid concentration are plotted against time.

VI.4.3 Influence of power input on productivity

The trend described in the previous chapter is also clear when analyzing the carbon mass balance at the time of glucose depletion, as shown in Figure 4. The flux of carbon into CDW, itaconic acid and CO_2 were compared for different agitation speeds in the large-scale bioreactor. For fermentations with agitation speeds of 175 rpm and higher, the end distribution of carbon was similar. A mean flux of 23.5% carbon to CDW and 30.7% carbon to itaconic acid was observed when applying agitation speeds above 175 rpm. For the fermentation performed at 114 rpm, carbon flux is differently distributed. Flux increased towards itaconic acid with 48.0% carbon and decreased towards biomass with only 13.8% carbon. Although, the dataset is insufficient for statistical analysis, the observations in the large scale agree with those in the small scale (see Table 1). Together the results show that under low oxygen

availability, there is a shift from using the carbon source for biomass accumulation towards itaconic acid production.

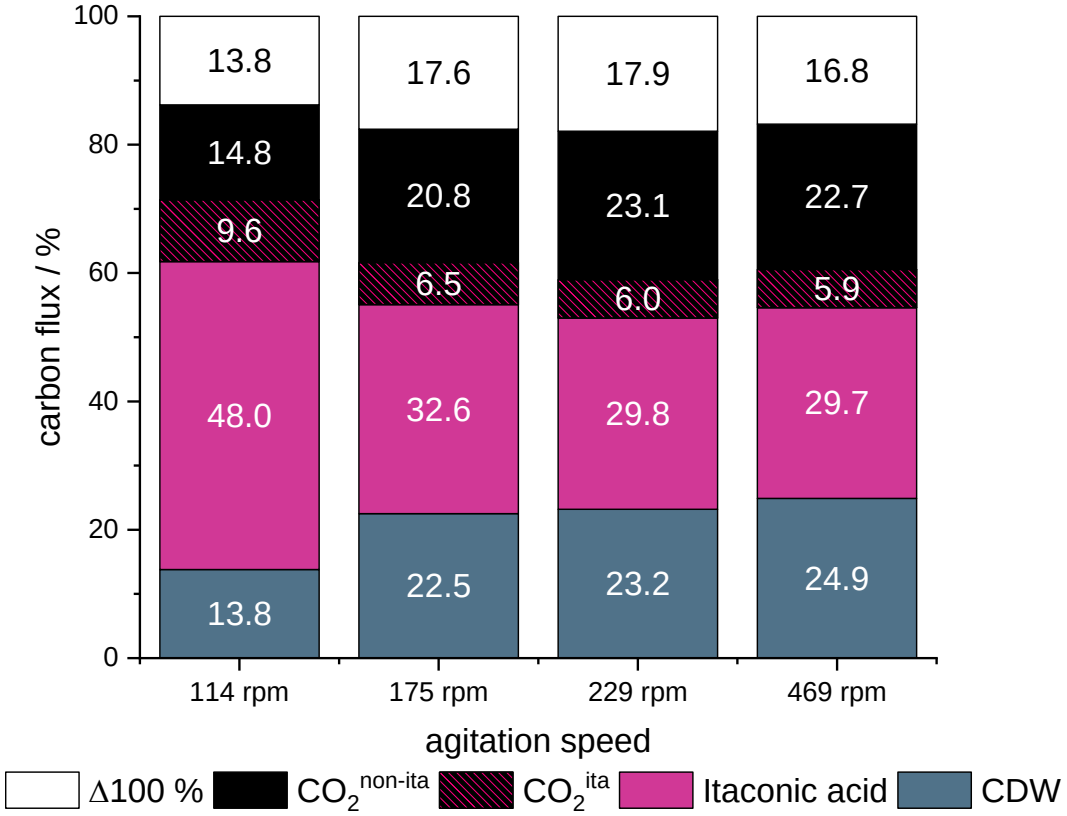

Figure 4: Carbon balance of the fermentations in 30 L scale, broken down by impeller speed. Difference to the total amount of carbon from glucose used (white), carbon in itaconic acid (violet), carbon bound in biomass (gray), CO_2 from itaconic acid production (violet-black) and non-itaconic CO_2 emission (black).

The total flux to CO_2 showed a mean of 27% over all agitation speeds. However, the decarboxylation of trans-aconitate to itaconic acid, also produces CO_2. Subtracting "itaconic" CO_2, the fermentation at 114 rpm had a flux of 14.8% carbon to CO_2, while the mean flux was 22% at higher agitation speeds. These "non-itaconic" CO_2 flux distributions show the same trend as the distribution of biomass flux. In fact, over all agitation speeds the quotient of "non itaconic" CO_2 to biomass is rather constant with 0.98 ± 0.07%. This indicates a coupling of CO_2 formation and biomass formation, with a generation of roughly 1 mol of CO_2 per 1 mol of biomass.

The increased flux towards itaconic acid under oxygen limited availability is not in agreement with common suggestions in the literature, where the importance of oxygen supply for the itaconic acid production of *U. maydis* is often highlighted (Hartmann et al. 2018; Guevarra and Tabuchi 1990; Klement et al. 2012; Tehrani et al. 2019). This is in part attributed to the finding on itaconic acid production with *A. terreus*. In the literature, similar oxygen metabolism is assumed between *A. terreus* and *U. maydis*. Shin et al. reported that *A. terreus* has a critical DO of 15%, below which a reduced production of itaconic acid took place (Shin et al. 2013). However, this has not been investigated in a

similar manner for *U. maydis* (Kuenz and Krull 2018). Most experiments in literature are focused on short interruptions of oxygen supply (Hartmann et al. 2018; Guevarra and Tabuchi 1990) or discuss oxygen consumption while changing other parameters as well (Klement et al. 2012; Hartmann et al. 2018). In other cases, the discussion is focused on inhomogeneous distribution of oxygen in the media (Tehrani et al. 2019). In the case presented here, due to the continuous gas flow of 1 vvm and the constant agitation, a long-term, constantly low oxygen availability was present at lower agitation speeds, see Figure 3. This clearly distinguishes the experiments shown here from the work in which the oxygen supply was switched off for a short time.

In general, introduction of low oxygen conditions to aerobic microorganisms are associated with high costs for the organism through the necessary metabolic adaptation, for example the energy metabolism (Ernst and Tielker 2009). Therefore, keeping the oxygen availability on a constantly low level might allow an adaptation with comparably high productivity as demonstrated here, while adapting to changing conditions might inhibit productivity. While dissolved oxygen was undetectable at times in the presented fermentations, the ongoing gas flow provided enough oxygen for the organism to avoid complete oxygen depletion. This suggests that the conditions in this work were hypoxic rather than anoxic.

Responses to oxygen limited conditions have been researched in several fungi. As mentioned, comparisons are often made with itaconic acid production in *A. terreus*. However, the critical DO for itaconic acid production is higher than that for biomass formation in *A. terreus* (Shin et al. 2013). This is in contrast to the observations made with *U. maydis* in this work. Therefore, a more general comparison to similar observations might give an insight into the adaptation of *U. maydis* to oxygen limitation.

As anaerobic growth so far has not been described for *U. maydis*, a comparison can be made to obligatory aerobic fungi, such as *Trichoderma reesei*, *Aspergillus nidulans* and *Aspergillus oryzae*. In the work of Bonaccorsi et al, *T. reesei* was subjected to hypoxia. In contrast to anoxia, which describes the total lack of oxygen, in hypoxic conditions oxygen is still present, but in insufficient amounts. Under hypoxia, among others, expression of genes for protein synthesis and cell division were downregulated in *T. reesei* (Bonaccorsi et al. 2006). A similar scenario might be observed here under low oxygen concentrations that do not foster growth. However, this does not explain the increased carbon flux towards itaconic acid.

Formation of itaconic acid leads to a net production of NADH, which needs to be reduced by oxygen. Assuming the theoretical route from glucose, 3 mol NADH are formed per mol of itaconic acid, see Figure 1. In comparison, 10 mol NADH and 2 mol $FADH_2$ are formed during cellular respiration of

glucose to CO_2. Cutting the TCA short by exporting cis-aconitate from the mitochondrion and converting it to itaconic acid, prevents the further production of reduced cofactors. This could be an avoidance strategy of NADH production similar to those observed in filamentous fungi like *A. nidulans* and *A. oryzae*, e.g. through the γ-aminobutyrate (GABA) shunt (Shimizu 2018). Upon entering hypoxic conditions, GABA shunt associated genes were upregulated in these organisms, which lead to a bypass of the NADH forming step from α-ketoglutarate to succinate in the TCA. This bypass avoids the production of 1 mol NADH, compared to the regular TCA reactions (Hillmann et al. 2015; Masuo et al. 2010; Terabayashi et al. 2012). The increased carbon flux towards itaconic acid under low oxygen availability in *U. maydis* may similarly suggest a use of the itaconic acid pathway as a mechanism to reduce the production of reduced cofactors, and thus the demand for oxygen in cofactor regeneration. To verify this thesis, further research concerning the metabolic processes in *U. maydis* under low oxygen conditions is necessary.

VI.5 Conclusion

The present study highlights the complex interplay of parameters impacting the cultivation of *Ustilago maydis* and its scale up for the production of itaconic acid. Lab scale experiments in 0.4 L scale identified optimal conditions for maximising product concentration, while also addressing the previously unclear influence of DO on itaconic acid production in *U. maydis*. A correlation between k_La and biomass growth, final itaconic acid concentration, and space time yield could be established. Notably, a higher k_La correlated with increased biomass production and space time yield after 40 h, but it did not enhance final product concentration. To meet the conflict of objectives between high product concentration and high space time yield for the scale up to 30 L scale, scale ups based on different operating points were performed. Using a constant impeller tip speed as scale up criterion provided greater consistency in replicating the screening results compared to a k_La -based scaling, which exhibited wider variability, possibly due to different positioning of the oxygen probe. These findings recommend the use of impeller tip speed as a more reliable criterion, enabling scalable and reproducible production of itaconic acid independent of the reactor geometry. The finding from the screening experiments that a lower k_La value correlates with an increased final product concentration was confirmed in the scale up experiments. This was also underlined by a carbon balance analysis. In experiments with a lower impeller speed, which is associated with a lower k_La, a higher carbon share based on the substrate glucose was directed into the product itaconic acid, while a higher impeller speed shifted the flux to a higher biomass concentration. The enhanced production of itaconic acid could be a mechanism to bypass NADH producing steps of the TCA under conditions with low oxygen availability. The results presented in this study stand in clear contradiction to previously published literature. Due to the consistent biological replicates, the results are nevertheless considered reliable.

Further research is needed to understand the metabolic processes in *U. maydis* under these conditions. Particularly, analyses of metabolic fluxes, cofactor balances, and gene regulation under limited oxygen availability are necessary to fully understand the underlying mechanisms. Another aspect that needs to be analysed to further explore the metabolic processes in *U. maydis* is the ratio between carbon and nitrogen sources under low oxygen conditions. Usually, a high carbon to nitrogen ratio is required for itaconic acid production. Especially when using complex media derived from renewable resources with only limited influence on the exact composition, the oxygen regime could be a valuable instrument to manipulate the metabolism of *U. maydis* towards increased itaconic acid production Oxygen availability might be an additional tool to influence the balance between the production of biomass and itaconic acid in favour of product formation for an increased product yield.

VI.6 Statements

VI.6.1 Data availability statement

The data that support the findings of this study are available from the corresponding author upon reasonable request.

VI.6.2 Funding

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VI.6.3 Conflict of interests

The authors declare no conflict of interests.

VI.6.4 Authorship

M.V.: conceptualization, data curation, formal analysis, investigation, methodology, visualization, Writing – Original Draft Preparation, Writing – Review & Editing; **W.L**.: conceptualization, data curation, formal analysis, investigation, methodology, visualization, Writing – Original Draft Preparation, Writing – Review & Editing; **F.B**.: data curation, formal analysis, investigation, methodology, visualization, Writing – Original Draft Preparation; **N.E.**: conceptualization, data curation, formal analysis, Writing – Original Draft Preparation, Writing – Review & Editing; **S.S.**: conceptualization, Writing – Original Draft Preparation, Writing – Review & Editing; **E.F.**: conceptualization, Writing – Original Draft Preparation, Writing – Review & Editing; **D.H.**: conceptualization, funding acquisition, project administration, supervision, Writing – Review & Editing;

L.M.B.: conceptualization, resources, supervision, Writing – Review & Editing; **R.U.**: conceptualization, funding acquisition, project administration, resources, supervision, Writing – Review & Editing

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VI.8 Supplementary material

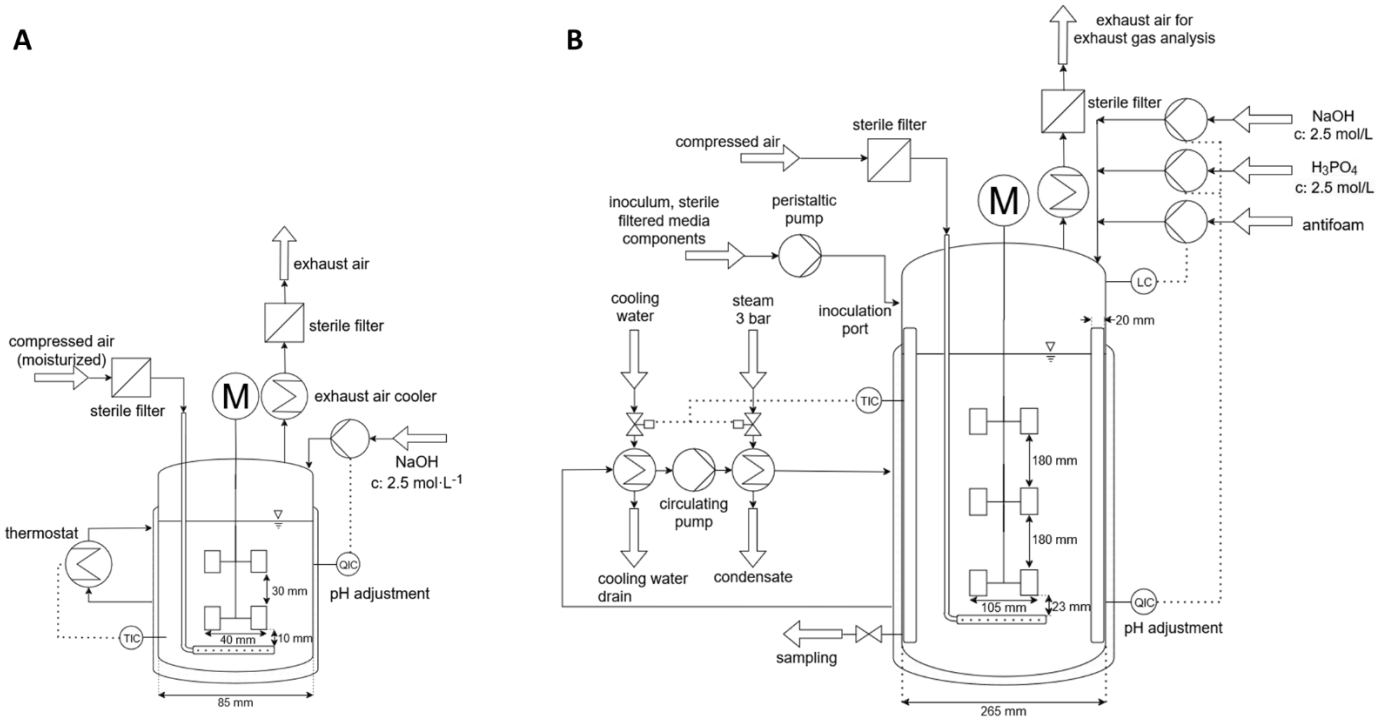

Figure S1: Sketch of the Biostat Q+ (A) and the Biostat C+ (B) test setups with the geometrical dimensions of the reactor internals.

VII. General Discussion

In this chapter, the main findings of this thesis are evaluated. The focus is on the goal of establishing municipal green waste as an alternative feedstock to produce chemicals for a circular bioeconomy. The results are critically discussed and compared with the most recent literature to identify relevant follow-up research questions.

VII.1 Pretreatment of Lignocellulosic Biomass

Lignocellulosic biomass is the most common alternative feedstock for a more sustainable industry. It consists of lignin, cellulose, and hemicellulose, which are of great importance as carbon sources. Due to the recalcitrant nature of lignocellulose, pretreatment of lignocellulosic biomass is necessary for an efficient biorefinery process. To obtain monosaccharides which can be metabolized by microorganisms, the lignocellulose macrostructure needs to be disrupted. The large number of different methods can be categorized according to the underlying principle into physical, chemical, physicochemical, and biological approaches. This is described in detail in chapter II.

Municipal green waste is defined in chapter II as low-lignin or herbaceous lignocellulosic biomass that occurs in urban areas such as private gardens, public parks, and roadside greeneries. The lower lignin content compared to for example mainly woody forestry waste allows a less harsh pretreatment and therefore reduced costs for the use as feedstock.

In chapter III, the benefits of the mechanical pretreatment of grass cuttings using a screw press on the subsequent enzymatic saccharification are demonstrated. Here, cellulose conversion is improved by 22 % compared to native grass cuttings, in contrast to a reduction of 18 % when the material is pretreated with a tincture press. Using the latter, the strong compression of the material obstructs the access for the enzymes, in contrast to enhanced access by the mechanical crushing of the material using a screw press. In total, 82 % cellulose conversion could be achieved using a screw press, compared to 67 % when applying a tincture press. The principle is similar to extrusion, which is discussed in literature as an additional possibility to pretreat herbaceous biomass by breaking up the lignocellulose structure via shear forces [1]. Karunanithy et al. observed a 50 % increase in surface area of switchgrass through single screw extrusion. This results in a combined sugar recovery of 48.4 %, 60 % of the conversion achieved in this work [2].

The beneficial effect of increasing the surface is also demonstrated by the comparison of milling or chopping mixed wood chips. Here, cellulose conversion can be increased by up to 19 % when the material is milled. Chemical and physicochemical processes also enhance the accessibility of carbohydrates. An organosolv pretreatment increases the enzymatic cellulose conversion of mixed wood chips by 77 % compared to material that is only pretreated physically. When combining physical

size reduction and organosolv pretreatment of wood chips, a total cellulose conversion rate of 91 % was reached. For material with a lower lignin content such as grass cuttings, the influence of physicochemical processes is not as pronounced. Here, an additional organosolv process increased the enzymatic cellulose conversion by 13 % compared to the physical pretreatment alone, reaching a final conversion rate of 93 %. This is in the range of values reported in literature. For example, Romaní et al. obtain around 95 % cellulose recovery from small eucalyptus wood chips through autohydrolysis followed by organosolv process [3] and Bekker et al. report up to 90 % monosaccharide yield from clover-grass press cake pretreated via liquid hot water [4].

The main cost factors in a biorefinery are the feedstock, the pretreatment, and the production [5]. Yang et al. state that the pretreatment accounts for 18 % of the total costs in the production of ethanol [6]. The literature reports energy consumption rates of up to 37.52 $kWh \cdot kg^{-1}$ sugar for organosolv pretreatment and 42.45 $kWh \cdot kg^{-1}$ sugar for alkaline pretreatment of sugarcane bagasse [7,8]. Physical size reduction is less energy consuming, with reported requirements from 1.54 $kWh \cdot kg^{-1}$ biomass for wheat straw using a knife mill to 9.1 $kWh \cdot kg^{-1}$ biomass using a rotary ball mill [8–10]. To realize an economically worthwhile biorefinery process it is essential to design the pretreatment step as cost-effective as possible. Zdeb et al. analyzed different methods of pretreating grass for subsequent methane production. Considering the cumulative energy demand, the global warming potential, a midpoint/damage life cycle assessment approach, the technical digestion time, and the direct costs, they determined the physical grinding of the biomass as the most cost-efficient process [11]. From an economic perspective, it is therefore favorable to omit physicochemical processes for biomass with low lignin and high moisture content and focus on physical and enzymatic pretreatments. As demonstrated above and in chapter III, mechanical processing in form of pressing using a screw press is sufficient as a pretreatment for herbaceous biomass.

Due to the prices for enzymes, the biocatalysis step is another cost factor. Hence, it is advisable to optimize the enzymatic saccharification of biomass [12]. One possibility is the immobilization of enzymes for multiple uses [13]. Lin et al. demonstrated an increase in the storage stability of laccase for the pretreatment of wheat straw of 120 % by immobilizing the enzymes [14]. A further approach is the selection or design of enzymes suitable for the substrates used. Suryanarayanan et al. identified enzymes originating from endophytic and plant litter fungi which demonstrated resistance to otherwise toxic inhibitors such as phenols and tannic acid [15]. Studying the kinetics of the applied enzymes can support the design of efficient saccharification processes by adapting the enzyme loading or modifying the reaction parameters. In this work, the maximum velocity achieved at the maximum substrate concentration v_{max} and Michaelis Menten constant K_M of the applied enzyme mixture were determined to be 0.6 $mmol \cdot min^{-1}$ and 93.4 $mmol \cdot L^{-1}$. While literature reports K_M in the same order of

magnitude [16–20], the results presented here are still slightly higher. This represents a lower enzyme substrate affinity, indicating potential for optimization of the process. Possible process adjustments are for example the mixing intensity, surfactants, and synergistic effects between enzymes. Xu et al. analyzed the effects of mixing on the enzymatic hydrolysis of dilute-acid pretreated corncobs depending on the solid loading. An increasing solid loading led to lower cellulose conversion. This could partly be compensated through higher mixing intensities as the rate constant increased under these conditions [21]. Adding surfactants such as Tween 60 or Trition to high solid enzymatic hydrolyses increases the mass transfer efficiency and therefore improves hydrolysis efficiency [22,23]. Kinetics can also unveil the synergistic effects of enzymes. Storani et al. observed synergies between a cellobiohydrolase and an endoglucanase from *Ruminococcus albus* which could be increased further by adding a hemicellulose, thereby enhancing lignocellulose degradation [24].

VII.2 Direct Valorization of Municipal Green Waste

Lignocellulosic biomass is commonly fractionated into the three main components lignin, hemicellulose, and cellulose, which are subsequently valorized in a biorefinery context. Yet, it is possible to directly extract components from the biomass and immediately generate added value without requiring additional processing steps. Of course, all three main building blocks can be extracted and exploited. Applications of lignin, hemicellulose, and cellulose are discussed in chapter I. Besides lignocellulose, municipal green waste contains further components that can be extracted and valorized directly, which is described in chapter II. Examples hereof are proteins [25,26], organic acids [27], as well as polyphenols and vitamins [28]. Additionally, municipal green waste can be employed as a starting material for functional materials. One common functionalization is carbonization. This so-called biochar can serve as an energy source, but it can also be applied for wastewater detoxification or to ameliorate soil [29]. Chapter II describes the hydrothermal carbonization of biomass. This HTC carbon has a high potential as an absorber for heavy metals, organic pollutants, or greenhouse gases and can also serve as anode material [30]. A special application of carbonized grass clippings is cost-effective electrodes, which could be applied in bio-electrochemical systems [31].

VII.3 Municipal Green Waste as Feedstock for Microbial Fermentations

The focus of the current literature concerning the valorization of lignocellulosic biomass and also of this thesis is on the utilization as feedstock for microbial fermentations. The biotechnological conversion of the carbohydrates contained enables higher added value than the extraction of components.

Chapter V demonstrates the successful use of municipal green waste as feedstock for microbial fermentations to produce platform chemicals. Both grass material and mixed wood cuttings were used as feedstock. As discussed in chapter III and VII, harsh physicochemical pretreatment methods are not

worthwhile for low lignin biomass from an economic point of view. Therefore, different processing routes were applied depending on the lignin and moisture content of the biomass. High lignin and low moisture biomass underwent an organosolv procedure, followed by enzymatic saccharification. The resulting hydrolysate was applied as a medium supplement in concentrations ranging from 20 to 40 %, diluting the standard media of the examined organisms and thereby reducing the need for commercial media components. Applying the fungus *Ustilago maydis*, up to 20 $g \cdot L^{-1}$ itaconic acid could be produced, increasing the yield by up to 35 % referencing cultivations in standard medium. This is an increase in itaconic acid concentration of 135 % compared to reports from literature. Klement et al. obtained 8.5 $g \cdot L^{-1}$ itaconic acid with *U. maydis* MB215 on a hydrolyzed hemicellulose fraction from beech wood with added glucose [32]. Cultivating *Clostridium acetobutylicum* on hydrolysate, an ABE concentration of 15.5 $g \cdot L^{-1}$, corresponding to a yield of 0.31 $g_{ABE} \cdot g_{glucose}^{-1}$, was obtained in this work, which is similar to product concentration and product yield of cultivations in reference media. However, the literature reports a solvent yield of 0.17 $g_{ABE} \cdot g_{total\ sugars}^{1}$ for *C. acetobutylicum* grown on a mixture of detoxified wood hydrolysate and alfalfa juice supplemented with buffering agents, vitamins, and minerals [33]. The yield achieved in chapter V is not only 83 % higher, but it was also obtained without time and cost intensive detoxification of the hydrolysate.

There is extensive coverage in the literature on using enzymatic hydrolysates of lignocellulosic biomass as a fermentation medium in general to produce different platform chemicals. For example, Deng et al. used steam-exploded corn stover to produce L-lactic acid in a simultaneous saccharification and fermentation process with a productivity of 1.83 $g \cdot L^{-1} \cdot h^{-1}$ [34]. Rudnyckyj et al. applied the organic fraction of municipal solid waste as a buffer for the hydrolysis of organosolv beechwood cellulose and obtained 30 $g \cdot L^{-1}$ ethanol after fermentation using *S. cerevisiae* [35]. However, this thesis is the first work to demonstrate specifically the use of municipal green waste to produce fermentation media.

As mentioned above, pretreatment is a complex and expensive step in biomass valorization. Another way to obtain a fermentation medium or medium supplement is by pressing the biomass and directly utilizing the resulting press juice. While this is not possible for biomass with high lignin or low moisture content, this is the most elegant way to obtain a fermentation medium from herbaceous biomass with a higher amount of moisture.

There are few examples in the literature of using press juices of lignocellulosic biomass as a fermentation medium. Alexandri et al. added press juice from lucerne to the hydrolysate of bread waste hydrolysate to create a fermentation medium for *Bacillus coagulans* and produced 62.2 $g \cdot L^{-1}$ L(+)-lactic acid in a continuous cultivation system. The beneficial effect of lucerne press juice was slightly higher than that of yeast extract, demonstrating that commercial media components could be replaced by supplements from renewable resources [36]. Magarelli et al. obtained a press juice of

Opuntia ficus-indica pruning and successfully used it to cultivate bacteria that promote plant growth to apply them as fertilizer. Compared to the standard medium, the survival rates of the microorganisms were significantly higher when grown in press juice medium [37]. Focusing on grass biomass, most publications first ensile the biomass before pressing it. Ensiling leads to the production of organic acids such as acetic, propionic, butyric, and lactic acid as well as ethanol [38]. An innovative approach to compensate for the produced acids and increase the pH of silage press juice is presented by Hohagen et al. They produced acetoin via immobilized enzymes from lactate, thereby obtaining a relevant product while simultaneously deacidifying the press juice for further applications [39]. Steinbrenner et al. modified the ensiling conditions to shift the production of lactic to butyric acid. By adding water and carbonated lime, 42.0 $g \cdot kg^{-1}$ butyric acid and 5.1 $g \cdot kg^{-1}$ lactic acid were produced after 90 days at 37 °C, compared to only 0.2 $g \cdot kg^{-1}$ butyric acid and 67.7 $g \cdot kg^{-1}$ lactic acid without any additives [40]. Besides fermentation during the ensiling process, silage press juice can be used for further fermentation processes. Cerrone et al. used press juice from grass silage as the sole carbon source for the production of polyhydroxyalkanoates and obtained a product content of 23.3 % with the organism *Burgholderia sacchari* IPT101 [41]. Schwarz et al. obtained a maximum of 8.5 $g \cdot L^{-1}$ poly-3-hydroxybutyrate in fed-batch cultivation of *C. necator* with grass silage press juice as feed [42].

In this work, press juice was generated using a screw press from fresh herbaceous biomass without a time-consuming ensiling process, saving up to 90 days compared to for example the work of Steinbrenner et al. [40]. The resulting juice was sterilized either by autoclaving or sterile filtration and immediately used as a fermentation medium or medium supplement. Both *Saccharomyces cerevisiae* and *Lactobacillus delbrueckii* subsp. *lactis* were able to grow in 100 % grass press juice without any additional components and produce 9.4 $g \cdot L^{-1}$ ethanol and 16.9 $g \cdot L^{-1}$ lactic acid, respectively. These product concentrations and yields were in the same ranges which were achieved using standard cultivation media. The lactic acid concentrations reported in literature based on herbaceous press juice media range from 11.91 $g \cdot L^{-1}$ [43] to 22 $g \cdot L^{-1}$ [44]. The higher titers originate in higher initial sugar concentrations of the media, indicating a possibility to increase the product concentration. Another possibility is adding components from standard cultivation media to the press juice. This further increased the product yield in this work by 4 % for ethanol production and 46 % in case of lactic acid production. *Ustilago maydis* MB215 ΔCyp3 P_{etef} Ria1 was cultivated in a mineral salt medium supplemented with 70 % grass press juice, the maximum possible proportion due to the solubility of the standard medium buffer. Here, a final itaconic acid concentration of 19.2 $g \cdot L^{-1}$ could be achieved, which corresponds to a 51 % increase in yield compared to reference cultivations in a standard medium. This is a 125 % increase compared to the results of Klement et al. [32]. *Clostridium acetobutylicum* was able to grow in medium diluted by 30 % grass press juice with a butyric acid yield of 0.3 $g \cdot g_{glucose}^{-1}$. Only marginal amounts of ABE products could be detected, indicating that the

metabolic switch from acidogenesis to solventogenesis did not occur. This is likely due to the reduced buffering capacity of the press juice, and it should be possible to remedy this with a pH-controlled setup.

Although most publications focus on the use of other agricultural waste material, there are a few publications that also describe the use of fresh grass press juice as a fermentation medium. Varriale et al. cultivated *L. delbrueckii* subsp. *lactis* in 75 % press juice from perennial ryegrass, obtaining 25.6 $g \cdot L^{-1}$ lactic acid [45]. By applying a short-term adaptation, they increased ethanol production of *S. cerevisiae* on press juice from perennial ryegrass from 11 to 18 $g \cdot L^{-1}$ [46]. Langsdorf et al. homogenized fresh grass clippings in a blender and utilized the resulting juice as a fermentation medium for *Cupriavidus necator* pKR-hum, successfully producing α-humulene with a concentration of 2 $mg \cdot L^{-1}$ [47].

This thesis demonstrates the suitability of different fractions of municipal green waste as feedstock to produce cultivation media for a broad range of microorganisms. Depending on the lignin and moisture content, media are either obtained through a physicochemical organosolv process followed by enzymatic saccharification or through pressing. Although both processing routes result in suitable media supplements, using press juices is a more promising approach due to drastically reduced pretreatment costs. The results show that press juice of herbaceous biomass contains sufficient nutrients to cultivate microorganisms and to produce platform chemicals, thereby making commercial media components obsolete. There are only few publications applying press juice as a fermentation medium so far. Using a hitherto unused material stream to produce new resources is an important step towards a circular bioeconomy.

VII.4 Improving Cultivation Strategies for a Circular Economy

Using municipal green waste as feedstock to produce platform chemicals requires reliable and robust cultivation processes. To ensure economic efficiency, they also need to take place at industrially relevant scales. It is therefore essential to understand and improve the cultivation processes on an industrial scale to realize a circular economy.

VII.4.1 Cultivation of Clostridia

Clostridia are the organism of choice to produce biobutanol from lignocellulosic biomass [48]. However, due to their biphasic metabolism, which is not fully understood yet, their cultivation proves to be difficult. In chapter IV, the influence of the pre-culture on solvent production was examined to establish a protocol for reliable and reproducible butanol production in the main culture. For cryo-conservation, cells from a late stationary phase grown in MP2opt medium should be selected. Compared to cells from a late exponential phase, butanol and total solvent concentration in a subsequent main culture increased by 16 % and 19 %. Sandoval-Espinola et al. observed the same

effect for *C. beijerinckii* cultivations [49]. Using MP2opt medium further increased the product concentrations by 19 % and 18 % compared to PY+X medium to a final butanol concentration of 11.22 $g \cdot L^{-1}$ and an ABE concentration of 16.08 $g \cdot L^{-1}$. The initial pH value of the pre-culture should be in the range of 5.0 to 6.2, otherwise no cell growth is possible. This also affects solvent production. Pre-cultures grown within the specific pH range produced 11.46 ±1.01 $g \cdot L^{-1}$ of butanol, approaching the theoretical maximum concentration of 14 $g \cdot L^{-1}$ [50]. Prolonging the age of the pre-culture prior to the inoculation of the main culture from 26 h to 48 h increased both butanol and total ABE concentration by more than 200 %. This underlines the importance of the pre-culture cells reaching a stationary growth phase before inoculating the main culture. Following this protocol enables more robust cultivation of *C. acetobutylicum* for reliable and increased solvent production. Oehlenschläger et al. discuss further current trends concerning the cultivation of *C. acetobutylicum* [51].

VII.4.2 Scaling up the Cultivation of *Ustilago maydis*

To ensure economic efficiency, industrially relevant scales are necessary for cultivation processes. In chapter VI, the influence of oxygen supply in the cultivation of *U. maydis* was analyzed, as this is an important factor in scaling up bioprocesses. Screening experiments on a 0.4 L scale indicated an inverse relation between itaconic acid production and k_La as the parameter for oxygen supply. Experiments with a lower k_La and therefore lower oxygen availability correlated with higher final product concentration and product yield. However, according to the OD_{600}, biomass growth was favored at higher k_La values. This was shown for k_La values between 40 and 77 h^{-1}, achieved by either changing the impeller tip speed or the gas rate. Further increasing the k_La did not influence cell growth or itaconic acid production. These findings were confirmed in a 30 L scale. Screening experiments with high product yield, high space-time yield, and a compromise between the two variables were selected for scale up. To achieve comparable conditions as in the screening scale, the scale up was either performed via impeller tip speed or k_La, two commonly used scale up criteria for aerobic fermentation processes [52]. Impeller tip speed hereby proved to be the more suitable scale up criterion, which could be explained by differences in the reactor geometry of the two systems. At the same time, the scale-up experiments also showed the correlation between lower oxygen availability and higher product concentration. Analyzing the carbon mass balance also confirmed this trend. In a fermentation with an impeller speed of 114 rpm, equaling a k_La of 26 h^{-1}, 48.0 % of carbon was incorporated into itaconic acid, while 24.4 % was emitted as CO_2 and only 13.8 % was used for biomass production. Increasing the impeller tip speed increased carbon flux to biomass and CO_2 while decreasing the flux to itaconic acid. At an impeller tip speed of 469 rpm, which corresponds to a k_La of 162.5 h^{-1}, 24.9 % and 28.6 % of carbon were used for biomass production and CO_2, respectively, while only 29.7 % went into the product itaconic acid. Considering the formation of CO_2 through decarboxylation of trans-aconitate in the production of itaconic acid and therefore subtracting the “itaconic CO_2” from the

exhaust gas, the shift of carbon flux from biomass to itaconic acid at lower agitation speeds becomes even more obvious. This trend has not been described in the literature so far. Quite the opposite, most publications emphasize the necessity of oxygen for successful itaconic acid production [32,53–55]. However, most references refer to *Aspergillus terreus* instead of *U. maydis* [56] or focus on complete interruption of oxygen supply for a short period of time [53,54]. In the case demonstrated in chapter VI, constant aeration and agitation ensured the continuous presence of oxygen in the fermentation broth, although at a low level. This might allow for a metabolic adaptation to these conditions without compromising productivity. A possible explanation for the increased itaconic acid production could be the avoidance of NADH accumulation. Compared to 10 mol NADH and 2 mol $FADH_2$, which are produced during cellular respiration of glucose to CO_2, only 3 mol NADH are formed per mol of itaconic acid. Under low oxygen conditions, cutting the TCA cycle short by producing itaconic acid from cis-aconitate decreases the number of reduced cofactors that need to be reduced by oxygen. Similar strategies to avoid NADH production are already reported for other filamentous fungi such as *A. nidulans* and *A. oryzae* which use the γ-aminobutyrate shunt [57]. This hypothesis needs to be verified via metabolic analysis. To ensure an economically worthwhile production, it is necessary to improve cultivation processes on all levels possible. The observed increased itaconic acid productivity under low oxygen conditions demonstrates that the oxygen regime constitutes a valuable tool to regulate itaconic acid production in *U. maydis*.

VII.5 Future Challenges

The present work demonstrates the suitability of municipal green waste as a feedstock to produce chemicals and, thereby, contributes to the development of a new carbon source for the chemical industry, supporting the imperative transformation to a more circular bioeconomy. However, there are still many challenges ahead. Products derived from renewable resources must compete with their petrochemical counterparts, necessitating the optimization of processes to achieve cost-effective production. Furthermore, comprehensive analyses are required to ensure both economic viability and environmental benefits compared to conventional petrochemical processes. Although this evaluation was beyond the scope of this work, it remains an essential next step for the processes presented. In general, conduction life cycle assessments in parallel with research projects is strongly recommended.

Key cost factors when utilizing lignocellulosic biomass as feedstock include biomass pretreatment, fermentative production, and downstream product purification. To reduce energy consumption, it makes sense to focus on biomass that requires less intensive pretreatment methods such as herbaceous biomass. In such cases, a mechanical pressing step is sufficient to obtain a fermentation medium. Additionally, and especially for biomass that needs harsher pretreatment methods, the energy should come from renewable and carbon-neutral sources such as wind or solar. To reach

industrially relevant fermentation processes, scale up processes are essential. However, the transfer of microbial cultivations to a larger scale is inherently complex, as changes in scale impact both biological parameters of the microbial systems and physical parameters of the fermentation broth. Therefore, a thorough understanding of the microbial metabolic pathways is essential. Fermentation media derived from lignocellulosic biomass represent complex matrices with numerous components, complicating downstream processing. Therefore, robust and efficient purification methods need to be developed.

Another significant challenge lies in the logistics and supply chain. The bulky nature of municipal green waste results in high transportation costs, making decentralized valorization facilities more practical. To prevent uncontrolled fermentation during storage, the feedstock should be kept at low temperatures. Pressing the biomass to produce a juice and a solid fraction can alleviate logistical issues, allowing for separate storage and transport of the resulting fractions. Seasonality further complicates the supply chain, as both the composition and the quantity of municipal green waste vary throughout the year. For process efficiency, a consistent supply of material should be ensured. One possibility could be to dry the press juice to obtain a shelf-stable green waste extract comparable to commercially available yeast extract. This could be produced during peak seasons and stored for use during periods of low biomass availability.

Legal and regulatory frameworks present additional obstacles. In Germany, the current Circular Economy Act imposes stringent regulations on the collection, storage, and processing of materials classified as waste (for example § 25, Kreislaufwirtschaftsgesetz). These regulations not only entail a significant administrative burden but also limit the flexibility of waste valorization. Incorporation provisions for real laboratories and experimentation clauses in the Circular Economy Act could significantly facilitate the transfer from research into industrial practice.

To ensure economic feasiblity, a cascade-like use of the biomass must be employed, prioritizing applications that deliver the highest added value. Residual materials from one processing step should be directed to subsequent value chains, with only the final, non-recyclable fraction subjected to thermal utilization.

To realize decarbonization and the transformation from a linear to a sustainable, circular bioeconomy, many challenges lie ahead, which require plenty of different steps, processes, and solutions. Utilizing municipal green waste as feedstock is one piece of the puzzle to solve the transition.

VII.6 References

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VIII. Supplementary Material

VIII.1 Supervised Student Theses

Master Theses

Katharina Oehlenschläger: Kultivierung von Clostridien (2022)

Dennis Back: Fermentative Herstellung organischer Säuren (2023)

Felix Bartzack: Scale-up der Kultivierung von *Ustilago maydis* zur Produktion von Itaconsäure auf Basis nachwachsender Rohstoffe (2024)

Master Study Projects

Anna Schwartzkopff: Kultivierung von *Clostridium acetobutylicum* mit Zusätzen von Wiesenschnitt-Presssaft (2021)

Martin Weisbrodt: Kultivierung von *Ustilago maydis* auf hydrothermal und enzymatisch aufgeschlossenem Grünschnitt (2023)

Samira Bauschatz: Produktion von Lösemitteln und organischen Säuren auf Basis nachwachsender Rohstoffe (2024)

Linnea Schliewe: Grünschnitt als nachwachsender Rohstoff für Feststofffermentationen mit *Ustilago maydis* (2024)

Hai Long Le: Nutzung von industriellen Nebenströmen als Rohstoff für Fermentationen (2024)

Bachelor Theses

William Ly: Hydrothermale und enzymatische Vorbehandlung von kommunalem Grünschnitt als Basis für Fermentationen (2021)

Martin Weisbrodt: Kultivierung von *Ustilago maydis* auf Grünschnitt-Fraktionen (2021)

Hatice Bektas: Fermentative Produktion von organischen Säuren (2022)

Elias Bentz: Elektro-unterstütze Fermentation von Clostridien (2022)

Daniel Wolf: Enzymatische Hydrolyse von nachwachsenden Rohstoffen zur Verwendung in Fermentationen (2022)

Samira Bauschatz: Einfluss gemischter Substrate in Bioraffinerien (2022)

Peter Feise: Der Einfluss verschiedener Parameter auf die Kultivierung von Clostridien (2023)

Other Student Projects

Elias Bentz: Elektro-unterstützte Fermentation von *Clostridium acetobutylicum* (2021), bachelor study project

Josiah Cherian: Electro-fermentation on the basis of renewable resources (2021), RISE internship

Jonathan Bohlender: Kultivierung von Clostridien mit Medienzusätzen aus nachwachsenden Rohstoffen (2022), study project

Moise Ndayambaje: Heterogene Katalyse (2022), study project

Aashna Momin: Electro-fermentation on the basis of municipal green waste (2023), RISE internship

VIII.2 List of Publications

Peer-reviewed Publications

A. Langsdorf, **M. Volkmar**, D. Holtmann, R. Ulber; Material utilization of green waste: a review on potential valorization methods; Bioresources and Bioprocessing (2021) 8:19; https://doi.org/10.1186/s40643-021-00367-5

L. Varriale, **M. Volkmar**, J. Weiermüller, R. Ulber; Effects of Pretreatment on the Biocatalysis of Renewable Resources; Chemie Ingenieur Technik (2022), 94(11), 1818-1826; https://doi.org/10.1002/cite.202200137

A. Langsdorf, A.-L. Drommershausen, **M. Volkmar**, R. Ulber, D. Holtmann; Fermentative α-humulene production from grass clippings as growth medium; Molecules, (2022); https://doi.org/10.3390/molecules27248684

A. Langsdorf, **M. Volkmar**, R. Ulber, F. Hollmann, D. Holtmann; Peroxidases from grass clippings for the removal of phenolic compounds from wastewater; Bioresource Technology Reports (2023); https://doi.org/10.1016/j.biteb.2023.101471

M. Volkmar, A.-L. Maus, M. Weisbrodt, J. Bohlender, A. Langsdorf, D. Holtmann, R. Ulber; Municipal green waste as carbon source for the fermentative production of platform chemicals; Bioresources and Bioprocessing (2023) 10:43; https://doi.org/10.1186/s40643-023-00663-2

K. Oehlenschläger, **M. Volkmar**, J. Stiefelmaier, A. Langsdorf, D. Holtmann, N. Tippkötter, R. Ulber; New insights into the influence of the pre-culture on the solvent production of *C. acetobutylicum*; Applied Microbiology and Biotechnology (2024) 108:143; https://doi.org/10.1007/s00253-023-12981-8

A. Langsdorf, M. Halim, **M. Volkmar**, M. Stöckl, R. Harnisch, P. Hahn, R. Ulber; Electrodes from carbonized grass clippings for bioelectrochemical systems; Sustainable Materials and Technologies (2024); https://doi.org/10.1016/j.clce.2024.100118

K. Oehlenschläger, J.-N. Hengsbach, **M. Volkmar**, R. Ulber; From pre-culture to solvent: current trends in *Clostridium acetobutylicum* cultivation; Applied Microbiology and Biotechnology (2025) 109:47; https://doi.org/10.1007/s00253-025-13428-y

M. Volkmar, W. Laudensack, F. Bartzack, N. Erdmann, S. Schönrock, E. Fuderer, D. Holtmann, L. M. Blank, R. Ulber; Low oxygen oxygen availability increases itaconate production by *Ustilago maydis*; Biotechnology and Bioengineering (2025) 122:3007–3017; https://doi.org/10.1002/bit.70035

Presentations at National and International Conferences

M. Volkmar, E. Bentz, A.-K. Schwartzkopff, J. Stiefelmaier, M. Engel, D. Holtmann, R. Ulber, Electro-assisted fermentation of *Clostridium acetobutylicum* with municipal green waste as feedstock, 13th European Congress of Chemical Engineering and 6th European Congress of Applied Biotechnology (2021), digital

M. Volkmar, E. Bentz, A.-K. Schwartzkopff, J. Stiefelmaier, M. Engel, D. Holtmann, R. Ulber, Municipal Green Waste as Feedstock for conventional and electro-assisted Fermentations, 17th International Conference on Renewable Resources & Biorefineries (2021), Aveiro, Portugal

M. Volkmar, A.-K. Schwartzkopff, M. Weisbrodt, A.-L. Maus, R. Ulber, Press juice of municipal grass clippings as medium for microbial fermentations, Grassification Webinar "Municipal Green Waste as Feedstock for conventional and electro-assisted Fermentations", Biorefine Cluster Europe (2021), digital

M. Volkmar, E. Bentz, A. Langsdorf, D. Holtmann, R. Ulber; Investigation of alternative feedstocks based on municipal green waste for conventional and electro-assisted fermentations; International Conference on Renewable Resources & Biorefineries (2022); Bruges, Belgium

M. Volkmar, M. Weisbrodt, A. Langsdorf, D. Holtmann, R. Ulber; Alternative feedstocks for microbial fermentations based on municipal green waste; (Bio)Process Engineering – a Key to Sustainable Development (2022); Aachen, Germany

M. Volkmar, M. Weisbrodt, L.M. Blank, R. Ulber; Production of itaconic acid using *Ustilago maydis* based on municipal green waste; International Conference on Renewable Resources & Biorefineries (2023); Riga, Latvia; 2. Green Chemistry Presentation Prize

M. Volkmar, M. Weisbrodt, L. M. Blank, R. Ulber; Municipal green waste as feedstock for the fermentative production of itaconic acid using *Ustilago maydis*; 14th European Congress of Chemical Engineering and 7th European Congress of Applied Biotechnology (2023), Berlin, Germany

M. Volkmar, A. Langsdorf, D. Holtmann, R. Ulber; The potential of municipal green waste as sustainable feedstock for fermentations; 2nd Scotland-Rheinland-Pfalz Life Sciences and Biotechnology Conference (2024), digital

D. Holtmann, **M. Volkmar**; Process integration and scale-up of a green waste biorefinery considering system robustness – GreenProScale; Innovative solutions for the defossilisation of chemical process industry – A bioeconomy workshop, ACHEMA (2024), Frankfurt am Main, Germany

M. Volkmar, F. Bartzack, L. M. Blank, R. Ulber; Production of itaconic acid using *Ustilago maydis* based on renewable feedstocks, DECHEMA Forum (2024), Friedrichshafen, Germany

M. Volkmar, W. Laudensack, R. Ulber, S. Schönrock, E. Fuderer, D. Holtmann, P. Hahn; Process integration and scale-up of a green waste biorefinery considering system robustness – GreenProScale, Jahrestreffen BioBall (2024), Frankfurt am Main, Germany

M. Volkmar, R. Ulber; Renewable resources for a green and sustainable chemistry, 19ème Congrès Français de Génie des Procédés (2024), Deauville, France

Poster contributions at national and international conferences

M. Volkmar, J. Weiermüller, J. Stiefelmaier, A. Akermann, A.-L. Maus, A.-K. Schwartzkopff, W. Ly, R. Ulber; Municipal green waste as feedstock for microbial fermentations; Himmelfahrtstagung on Bioprocess Engineering (2021) – New Bioprocesses, New Products (2021), digital; poster price

M. Volkmar, J. Weiermüller, A. Langsdorf, D. Holtmann, N. Feizy, R. Hommel, R. Ulber, Kommunaler Grünschnitt als Rohstoff für Fermentationen, Jahrestreffen BioBall (2021), Frankfurt am Main, Germany

M. Volkmar, E. Bentz, A. Langsdorf, D. Holtmann, R. Ulber; Analysis of municipal green waste as feedstock for electro-assisted fermentations with *Clostridium acetobutylicum*; Himmelfahrtstagung on Bioprocess Engineering (2022); Mainz, Germany

M. Volkmar, A. Langsdorf, D. Holtmann, R. Harnisch, P. Hahn, R. Ulber; Kommunaler Grünschnitt als Rohstoff für Fermentationen, Jahrestreffen BioBall (2022), Frankfurt am Main, Germany

M. Volkmar, Municipal green waste as feedstock for fermentations; RPTU Verfahrenstechnik Doktorandenseminar (2023), Annweiler, Germany

M. Volkmar, A. Langsdorf, D. Holtmann, R. Harnisch, P. Hahn, R. Ulber; Kommunaler Grünschnitt als Rohstoff für Fermentationen, Jahrestreffen BioBall (2023), Frankfurt am Main, Germany

M. Volkmar, F. Bartzack, L. M. Blank, R. Ulber; Cultivation of *Ustilago maydis* based on municipal green waste for the fermentative production of itaconic acid, International Conference on Renewable Resources & Biorefineries (2024); Brussels, Belgium

VIII.3 Curriculum Vitae

Personal information

Name	Marianne Volkmar
Place of birth	Munich, Germany
Nationality	German

Education

Since 03/2020	**PhD candidate**
	Chair of Bioprocess Engineering, University of Kaiserslautern-Landau
10/2015-09/2019	**Biochemistry (Master of Science)**
	Technical University of Munich
10/2011-09/2015	**Biochemistry (Bachelor of Science)**
	Technical University of Munich
05/2011	**General University Entrance Qualification (“Abitur”)**
	Theodolinden-Gymnasium Munich