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Abstract

In this work, material recycling methods for green waste in (electro)biotechnology and the bioelectrochemical use of CO_2 from flue gas were investigated. The overall aim was to find alternatives to conventional recycling of green waste and ways to reduce CO_2 emissions. Initially, it was investigated whether grass clippings, a main component of green waste, can serve as a substrate for fermentation. For this purpose, grass clippings were homogenized and the solids separated. The juice was examined as a medium additive and as the sole medium for the cultivation of *Cupriavidus necator* pKR-hum. Different sterilization methods for the grass juice and different concentrations as an additive in the fermentation medium were tested. *Cupriavidus necator* pKR-hum could be successfully cultivated with autoclaved grass juice as the sole growth medium without further additives. A growth rate of 0.43 h^{-1} was achieved on the grass medium compared to 0.50 h^{-1} on a conventional LB medium. In addition, 2 mg L^{-1} of the terpene α-humulene could be produced on the grass medium. As a further utilization method, the extraction of industrially relevant enzymes from green waste was investigated. For this purpose, the common lawn grasses *Lolium perenne* and *Festuca arundinacea* were cultivated in the laboratory and a purification scheme for peroxidases was developed. The purified peroxidases were characterized with regard to reaction optima and kinetics and used for the oxidation of the phenolic substances phenol, *m*-cresol, and 2,4-dichlorophenol for wastewater treatment. The peroxidases of both grasses showed efficient conversion of the substrate 2,4-dichlorophenol. Subsequently, the experiments were repeated with crude extracts of the individual grasses and with a crude extract of real grass clippings. With the crude extract of the real grass clippings, more than 95% of 0.5 mM 2,4-dichlorophenol could be converted within 20 min. Finally, the use of green waste as starting material for electrodes for bioelectrochemical systems was investigated. Grass clippings were carbonized via hydrothermal carbonization and pyrolysis. Electrodes were manufactured from the resulting biochar using a binder and a metallic carrier. Biochar and electrodes were characterized in terms of materials science and electrochemistry. The electrodes were then used in microbial electrosynthesis (MES) with *Cupriavidus necator* H16 PHB^{-}4 and in a microbial fuel cell (MFC) with *Geobacter sulfurreducens* and compared with conventional electrodes. As expected, the manufactured electrodes were less suitable for water electrolysis in the MES than conventional metal electrodes. However, the manufactured electrodes showed a similar performance to commercial graphite electrodes in the MFC. In addition, long-term operation of the MFC for more than six weeks could be realized. With regard to real-life application in an industrial environment, the MES was carried out in a cogeneration plant to investigate flue gas as a possible source of CO_2. For this purpose, *Cupriavidus necator* H16 was used to produce the bioplastic polyhydroxybutyrate (PHB) in the MES. The MES was carried out and compared in the laboratory with a clean gas mixture and in the cogeneration plant with real flue gas. The use of flue gas had no detectable effect on either microbial growth or PHB production in comparison to the experiments in the laboratory. In the cogeneration plant, 333 ± 44 mg L^{-1} PHB could be produced with the MES at a proportion of 43 ± 3% of the dry cell mass. The results demonstrate alternative recycling methods for green waste and CO_2 from flue gas, which can contribute to a reduction in the use of fossil resources and CO_2 emissions. Overall, this work can support a shift towards a bioeconomy and a circular economy.

Zusammenfassung

In der vorliegenden Arbeit wurden stoffliche Verwertungsmethoden für Grünschnitt in der (Elektro-)Biotechnologie sowie die bioelektrochemische Nutzung von CO_2 aus Rauchgas untersucht. Das übergeordnete Ziel war Alternativen zum konventionellen Recycling von Grünschnitt und Möglichkeiten zur Senkung von CO_2-Emissionen zu finden. Im Rahmen der Arbeiten wurde zunächst untersucht, ob Grasschnitt, ein Hauptbestandteil von Grünschnitt, als Substrat für die Fermentation dienen kann. Dafür wurde Grasschnitt homogenisiert und die Feststoffe abgetrennt. Der Saft wurde als Medium-Zusatz und als alleiniges Medium für die Kultivierung von *Cupriavidus necator* pKR-hum untersucht. Es wurden zunächst verschiedene Sterilisationsmethoden für den Grassaft und unterschiedliche Konzentrationen als Zusatz im Fermentationsmedium getestet. *Cupriavidus necator* pKR-hum konnte mit autoklaviertem Grassaft als alleiniges Wachstumsmedium ohne weitere Zusätze erfolgreich kultiviert werden. Dabei ergab sich auf dem Gras-Medium eine Wachstumsrate von 0,43 h^{-1} im Vergleich zu 0,50 h^{-1} auf einem konventionellen LB-Medium. Außerdem konnten auf dem Gras-Medium 2 mg L^{-1} des Terpens α-Humulen produziert werden. Als weitere Verwertungsmethode wurde die Extraktion von industriell relevanten Enzymen aus Grünschnitt untersucht. Dafür wurden die häufigen Rasengräser *Lolium perenne* und *Festuca arundinacea* im Labor kultiviert und ein Aufreinigungsschema für Peroxidasen entwickelt. Die aufgereinigten Peroxidasen wurden hinsichtlich Reaktionsoptima und -kinetik charakterisiert und für die Oxidation der phenolischen Substanzen Phenol, *m*-Cresol und 2,4-Dichlorophenol zur Abwasserreinigung eingesetzt. Die Peroxidasen beider Gräser zeigten eine effiziente Umwandlung des Substrats 2,4-Dichlorophenol. Im Anschluss wurden die Experimente mit Rohextrakten der einzelnen Gräser und mit einem Rohextrakt eines realen Grasschnitts wiederholt. Mit dem Rohextrakt des realen Grasschnitts konnten über 95% von 0,5 mM 2,4-Dichlorophenol innerhalb von 20 min umgewandelt werden. Schließlich wurde die Nutzung von Grünschnitt als Ausgangsmaterial für Elektroden für bioelektrochemische Systeme untersucht. Grasschnitt wurde mittels hydrothermaler Karbonisierung und Pyrolyse karbonisiert. Aus der resultierende Biokohle wurden mithilfe eines Bindemittels und eines metallischen Trägers Elektroden hergestellt. Biokohle und Elektroden wurden materialwissenschaftlich und elektrochemisch charakterisiert. Im Anschluss wurden die Elektroden in der mikrobiellen Elektrosynthese (MES) mit *Cupriavidus necator* H16 PHB^{-}4 und in der mikrobiellen Brennstoffzelle (MFC) mit *Geobacter sulfurreducens* eingesetzt und mit konventionellen Elektroden verglichen. Für die MES mit Wasserelektrolyse waren die Elektroden erwartungsgemäß weniger gut geeignet als die metallischen Vergleichselektroden. Jedoch zeigten die hergestellten Elektroden in der MFC eine ähnliche Performance wie kommerzielle Grafit-Elektroden. Außerdem konnte ein Langzeitbetrieb der MFC über mehr als sechs Wochen realisiert werden. Hinsichtlich einer realen Anwendung im industriellen Umfeld wurde die MES in einem Heizkraftwerk durchgeführt, um Rauchgas als mögliche CO_2-Quelle zu untersuchen. Dafür wurde mittels *Cupriavidus necator* H16 der Bioplastik Polyhydroxybutyrat (PHB) in der MES produziert. Die MES wurde im Labor mit einem reinen Gasgemisch und im Heizkraftwerk mit realem Rauchgas durchgeführt und verglichen. Die Nutzung von Rauchgas hat im Vergleich zu den Experimenten im Labor weder das mikrobielle Wachstum noch die PHB-Produktion nachweisbar beeinflusst. Im Heizkraftwerk konnten mit der MES 333 ± 44 mg L^{-1} PHB bei einem Anteil von 43 ± 3% an der Zelltrockenmasse produziert werden. Die Ergebnisse demonstrieren alternative Verwertungsmethoden von Grünschnitt und CO_2 aus Rauchgas, wodurch zu einer Reduzierung der Nutzung fossiler Rohstoffe und der CO_2-Emissionen

beigetragen werden kann. Insgesamt können diese Arbeiten einen Wandel hin zu einer Bioökonomie und einer Kreislaufwirtschaft unterstützen.

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List of abbreviations

Abbreviation	Definition
DET	Direct extracellular electron transfer
DoE	Design of experiments
EPA	(United States) Environmental Protection Agency
EU	European Union
HRP	Horseradish peroxidase
HTC	Hydrothermal carbonization
IET	Indirect extracellular electron transfer
K_m (value)	Michaelis constant
LB (medium)	Lysogeny broth
MES	Microbial electrosynthesis
MET	Mediated extracellular electron transfer
MFC	Microbial fuel cell
MRS (broth/medium)	Medium by deMan, Rogosa, and Sharpe
PEG	Polyethylene glycol
PHB	Polyhydroxybutyrate
PLA	Polylactic acid
PTFE	Polytetrafluoroethylene
PVDF	Polyvinylidene fluoride
SDG	UN Sustainable Development Goal
UN	United Nations

1 Introduction

Given the growing interest in bioeconomy and circular economy, there is an urgent need for the profitable and sustainable recycling of biogenic waste materials. Some of the most pressing challenges of this time are the growing world population combined with decreasing arable land, the depletion of fossil resources, and probably the most threatening issue of climate change. All of these challenges can be tackled through the increased and more efficient use of renewable raw materials. Currently, a major part of the chemical industry is based on fossil-based raw materials. The aim should be to unlock biogenic residues and waste materials as industrial carbon sources and consequently close material cycles to switch to a biobased chemical production. In metropolitan regions, in particular, a large amount of underutilized residual and waste materials accumulate. The European Commission's updated bioeconomy strategy advocates for EU cities to become pivotal hubs for circular bioeconomy, emphasizing the exploitation of urban biowaste (European Commission, 2018). Lignocellulosic biomass is gaining prominence as a versatile feedstock, extending its utility beyond the energy sector to encompass the chemical industry. While there are numerous publications on the use of agricultural residues, there is still a lack of research on recycling methods for biogenic residues that arise in metropolitan regions. In urban areas, large quantities of green waste are generated from parks, gardens, and roadside greenery. Due to increasing urbanization around the world, the number of urban green spaces is increasing and with it the amount of green waste that burdens both the environment and the city development (Zhang et al., 2013). In 2020, over 5.7 million tons of biodegradable garden and park waste were generated in Germany (German Federal Statistical Office, 2022). Ten years earlier, there were still less than 5 million tons of these waste materials. Presently, the primary method for handling green waste involves recycling through composting, followed by its use as fertilizer or utilization as raw material for energy production, such as in biogas plants. The collection and recycling of green waste using current methods generates significantly more costs than value. With regard to alternative processes to use plant biomass, a lot of research has been carried out concerning the energetic use in the form of bioenergy or biofuels. In general, research into plant biomass has focused heavily on energy crops such as *Miscanthus*, *Arundo donax*, switchgrass, *Populus nigra*, or *Eucalyptus* (Ventorino et al., 2017). However, energy crops compete with food crops for arable land (Johansson and Azar, 2007). As the world's population continues to grow, competition between food and fuel is becoming an increasingly serious issue. When using biomass as a raw material, it is advantageous to use waste materials, as these do not have to be specially cultivated and therefore do not compete with the food industry. Limited research has been conducted on the material utilization of green waste, complicating efforts to develop more sustainable and efficient recycling methods. A major challenge in processing green waste is its collection, as it does not arise at specific locations but is spread across a wide area. Heterogeneity increases sharply when green waste is collected from a variety of sources. Fossil raw materials display a high density and a rather consistent composition in contrast to such biological waste materials. The great heterogeneity of green waste complicates the development of alternative recycling methods. Nevertheless, green waste holds potential as an underutilized resource for the chemical and biotechnological industry. By recycling green waste in a more ecologically sensible way and simultaneously reducing the use of fossil raw materials, CO_2 emissions can also be reduced.

The reduction of anthropogenic greenhouse gas emissions is one of the most urgent challenges of this decade and beyond. A report by the Global Carbon Project shows that global

CO_2 emissions from fossil fuels rose by 1.1% to 36.8 gigatons in 2023 (Friedlingstein et al., 2023). Although there have been reductions in emissions in the EU and the USA, emissions have continued to rise, particularly in India and China. According to the UN Environment Programme's Emissions Gap Report 2023, the 2 °C or 1.5 °C target of the Paris Agreement for global warming cannot be met by 2030 with the measures currently in place (United Nations Environment Programme, 2023). However, it is encouraging that when the Paris Agreement was signed in 2015, the increase in greenhouse gases was estimated at 16% by 2030, while current estimates put the increase at just 3%. Nevertheless, emissions must be reduced further, as a warming of 2.9 °C is currently predicted. Factory farming and the transportation sector due to the use of fossil fuels are particularly strong contributors to greenhouse gas emissions. In both cases, more sustainable alternatives must be found, as the emissions cannot be captured and recycled. In the case of power plants, for example, the CO_2 emissions from exhaust gases can be recycled before they are released into the atmosphere. At the same time, yet another raw material for the process that uses the CO_2 is replaced and thus saved. All in all, this approach can help to move towards a circular economy. Biotechnology offers the potential to make the chemical industry more sustainable through the biotransformation of waste materials such as green waste and off-gas CO_2 to produce chemicals. This will enable the industry to move away from depleting fossil resources, which leave a large carbon footprint. Furthermore, novel bioelectrochemical processes offer particular potential due to a wide range of possibilities for using CO_2 as a raw material. A major advantage of these waste materials is that they are renewable and are not going to be exhausted at some point. One way or another, processes based on fossil raw materials will have to be switched to alternative raw materials anyway.

This thesis will focus on investigating novel material utilization methods for green waste as well as off-gas CO_2. The use of green waste as a substrate for biotechnological conversions into basic or fine chemicals, the carbonization of green waste and its subsequent use as electrode material in electro-biotechnological applications, and the extraction of valuable products from green waste like industrially relevant enzymes will be investigated. The processing of green waste into value-added products might help not only to arrange the disposal of green waste profitably but also to close material cycles and reduce the climate impact and the dependence on fossil resources. The bioelectrochemical process of microbial electrosynthesis is used to investigate the use of CO_2 from flue gas of a cogeneration plant as a substrate for the production of a biodegradable polymer. The aim is to present an option for reducing CO_2 emissions while simultaneously demonstrating an alternative raw material for the production of bioplastic.

2 Theoretical background

2.1 Green waste – waste material or untapped resource?

Green waste is a biogenic waste material arising in populated areas that is currently not sufficiently utilized. Previously, green waste was defined as grass and leaves collected from public parks, and 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 (Langsdorf et al., 2021). This also includes green waste from cemeteries. It is difficult to quantify the amount of green waste produced in a uniform way (Liu et al., 2023). The city of Berlin alone produces around 120,000 tons of green waste every year (Medick et al., 2017). It consists of 72,000 tons of leaves from yards and roads and 48,000 tons of grass cuttings from lawns, roadside greenery, and biotope areas. Globally, an average of 47 kg of green waste is generated per person each year (Liu et al., 2023). Public waste disposal companies, park authorities, and private gardening and landscaping companies are responsible for the collection and recycling of green waste in urban areas, while larger quantities of green waste produced in rural areas can be disposed of at public collection points (Medick et al., 2017). In Germany, the majority of green waste is composted, resulting in fertilizer for agriculture (Medick et al., 2017). In 2021, around 2.24 million tons of green waste compost were produced in Germany (German Federal Environment Agency, 2023). In terms of energetic usage, low-lignin green waste is utilized in biogas plants while predominantly woody waste is thermally utilized. Mainly wet biowaste and food waste are particularly suitable for fermentation in biogas plants (German Federal Environment Agency, 2023). Most of the resulting compost and almost all digestate from biogas plants is used as fertilizer and humus in agriculture (German Federal Environment Agency, 2023). Globally, a significant volume of the lignocellulosic material is disposed of through incineration, an approach accompanied by detrimental emissions impacting air quality, especially in urban environments (Shao et al., 2019; Sivertsen, 2006). In 2020, there were 1102 composting and biogas plants in Germany, comprising 218 biowaste composting plants, 599 green waste composting plants, 227 biogas plants, and 58 combined biogas and composting plants (German Federal Ministry for the Environment, Nature Conservation, Nuclear Safety and Consumer Protection, 2022). Composting and subsequent reuse as compost is described as the most suitable recycling method for lignin- and cellulose-rich plant material in Germany (German Federal Environment Agency, 2023). Although composting is generally considered the optimal method for green waste recycling regarding economic and environmental considerations (Zhang et al., 2013; Zhang and Sun, 2014), the present costs of green waste removal surpass the accrued value.

The great heterogeneity of green waste is the major obstacle to recycling the waste material. A study has shown that 618 different plants can be found in green spaces in Beijing city (Zhao et al., 2010). On a global scale, green waste also differs extremely. In the city of Singapore, large quantities of dead eucalyptus leaves arise (Liu et al., 2013), which do not arise in European cities, for example. Apart from local differences, the composition of green waste varies over the course of the year (Boldrin and Christensen, 2010; Hanc et al., 2011). While more waste is generated from grasses and shrubs in the warmer seasons, there is a lot of foliage in the fall and mainly woody waste in the winter. Thus, there are strong seasonal variations in green waste. Biowaste from private households is even more heterogeneous as it usually contains a mixture of other biogenic waste materials such as food scraps and green waste. Green waste from public sources like parks or roadsides can be collected much cleaner.

Biowaste also differs greatly between urban and rural areas, as garden waste has a much greater impact on biowaste in rural areas (Hanc et al., 2011). The amount of garden waste produced is significantly higher in the summer months than in the winter months (Boldrin and Christensen, 2010). Furthermore, the varying composition of the individual plants leads to an increase in heterogeneity. The chemical composition of an individual plant depends primarily on its species (Mohapatra et al., 2017). In addition, the individual plants of a species show differences in their chemical composition depending on the growing conditions (Kim et al., 2015; Mohapatra et al., 2017) and the stage of maturity of the plant (Herrmann et al., 2014; Mohapatra et al., 2017). The influence of growing conditions was insightfully demonstrated through the differences in grass composition during two different years (Kim et al., 2015). Grasses differ in sugar content, protein content, fiber, fat, and dry matter depending on the place and time of harvest (Herrmann et al., 2014). Furthermore, the composition of lignocellulose varies depending on the time of harvest (Mashingo et al., 2008). Lignocellulose is the main component of plant biomass, which is made up of the polymers cellulose, hemicellulose, and lignin (Pérez et al., 2002). Lignin is an aromatic polymer, while hemicellulose and cellulose are composed of various sugars (Pérez et al., 2002). Lignin consists primarily of the phenolic syringyl and guaiacyl units as well as ferulic acid and *p*-coumaric acid (Timell et al., 1992). The main functions of lignin in the cell wall are structural stability, protection against microorganisms and oxidative stress, and it acts as a permeability barrier (Pérez et al., 2002). The polymer cellulose consists of glucose units that are connected via ß-1,4-glycosidic bonds (Peterson et al., 2008). Cellobiose molecules combine through hydrogen bonds and Van der Waals forces to form fibrils (Pérez et al., 2002), which are resistant to swelling in water or enzymatic attacks (Peterson et al., 2008). The ß-1,4-glycosidic bonds of the cellulose can be hydrolyzed, for example by using hydrothermal extraction, to obtain the glucose monomers (Peterson et al., 2008). Hemicellulose possesses a random structure of various sugars (Peterson et al., 2008). Due to its more disordered and branched structure, hemicellulose is not as stable as cellulose (Pérez et al., 2002). The chemical structures of the lignocellulose components have been visualized previously in a review article (Langsdorf et al., 2021). The wide range of the proportions of cellulose, hemicellulose, and lignin from grasses has been demonstrated in different publications (Juneja et al., 2011; Premjet et al., 2012). An analysis of the lignocellulose composition of lawn grass showed a share of 41.7% cellulose, 35.8% hemicellulose, and 8.0% lignin (Guo et al., 2015). The ratio of cellulose to lignin in grass can be almost ten times higher than in leaves or branches (Komilis and Ham, 2003). However, leaves from the Java plum or bamboo can also have a high lignin content of over 25% (Das et al., 2013), while it has been shown that pruning from an olive tree contains only 16.8% lignin (Raspolli Galletti et al., 2012). The composition of the lignocellulose of numerous plants with a low lignin content has been summarized in a review article. Accordingly, the values of possible plants in green waste range between 20.6 - 57.0% cellulose, 11.7 - 54.8% hemicellulose, and 3.1 - 24.7% lignin (Langsdorf et al., 2021). In a few cases, mixed green waste was also examined with regard to its lignocellulose components. Concentrations of 27% cellulose, 11% hemicellulose, and 24% lignin were reported for yard waste, wherein the high concentration of lignin was due to a high proportion of woody materials (Komilis and Ham, 2003). In another case, concentrations of 25.3% cellulose and 46.3% hemicellulose were found for green waste (Zhang and Sun, 2014). In addition to cellulose, hemicellulose, and lignin, plant biomass also contains proteins, amino acids, lipids, organic acids, salts, and minerals (Mohapatra et al., 2017). Up to 18.5% of the dry matter of plants in green waste can be proteins (Langsdorf et al., 2021). The concentration of lipids is about 2.5% of the dry matter for yard waste as well as grasses and leaves (Komilis and Ham, 2003). The

proportion of extractives of grasses varies and amounts to, e.g., 13.6% for switchgrass or 6.9% for *Miscanthus* (Reza et al., 2013).

In general, green waste has been insufficiently examined with regard to alternative recycling methods in comparison to woody lignocellulosic biomass or agricultural residues. Especially grass-like biomass with low lignin content lacks research. The majority of research into the utilization of such lignocellulosic biomass focuses on energy crops such as *Miscanthus* or switchgrass. From an economic point of view, the production of bioethanol is currently one of the most promising alternative ways of utilizing low-lignin lignocellulosic biomass (Pérez et al., 2002). Accordingly, there are a large number of publications on the subject of various biomasses including grasses. Different methods for the production of bioethanol have been described for switchgrass, Napier grass, Coastal Bermuda grass, and *Miscanthus* (Mohapatra et al., 2017). The lack of alternative ways to materially recycle green waste is primarily due to its great heterogeneity. Possibilities for the material utilization of green waste have previously been highlighted (Langsdorf et al., 2021). Basic chemicals can be obtained through the direct chemical conversion or via fermentation of lignocellulosic biomass. Previously, a total of 12 potential sugar-derived building block chemicals that can be obtained from biomass and generate added value were identified (Werpy and Petersen, 2004). These include levulinic acid, succinic acid, and xylitol, all of which have already been produced from green waste-like biomass. Levulinic acid is mainly produced chemically by an acid-catalyzed reaction, while succinic acid and xylitol are produced through fermentation. An example is shown by Girisuta et al. with the production of levulinic acid by acidic hydrolysis from water hyacinth (Girisuta et al., 2008). The fermentative production of succinic acid has been demonstrated from *Miscanthus* (Dąbkowska et al., 2019; Kuglarz and Rom, 2019), *Arundo donax* (Ventorino et al., 2017), and industrial hemp (Gunnarsson et al., 2015). The fermentative use of green waste is described in more detail in the following chapter. Another interesting chemical conversion process was demonstrated by Cao et al. through the production of cellulose acetate from pretreated green landscaping waste (Cao et al., 2018). Rivas et al. have shown the production of furfural and 5-hydroxymethylfurfural from *Miscanthus* by a combination of enzymatic and chemical conversion (Rivas et al., 2019). Promising extraction products from green waste are proteins, aromas, or lignin as a by-product from pretreatment methods (Langsdorf et al., 2021). Presumably, only a combination of different recycling methods can help to generate more value from the green waste. A biorefinery aims to fully utilize biomass by combining various process steps. Biorefineries can be classified for example as green, cereal, oilseed, forest, or lignocellulosic biorefineries depending on their feedstock (Vinoth Kumar et al., 2016). In this case, a green biorefinery is defined by grasses and green plants as feedstock, which is why the processing of green waste fits into this class. Vinoth Kumar et al. show a comprehensive overview of possible biorefinery products ranging from C1 compounds such as methanol up to C6 compounds like sorbitol and Cn compounds such as polyhydroxyalkanoates (Vinoth Kumar et al., 2016). In order to achieve the highest possible added value, it is important to select the most valuable end products possible. A more in-depth description of potential material recycling methods for green waste and the possible products was shown previously (Langsdorf et al., 2021). In the following, the material utilization of green waste as a fermentation substrate, source of industrially relevant enzymes, and raw material for the production of electrodes for bioelectrochemical systems are described.

2.2 Green waste as a fermentation substrate

Biomass can serve as feedstock for microorganisms in biotechnology. In particular, agricultural waste has often been used as a substrate for fermentation. The use of waste biomass can reduce the feedstock costs of fermentation processes. As described above, plant material consists largely of the sugar polymers hemicellulose and cellulose. With a carbohydrate content of up to 60% (Álvarez et al., 2016), lignocellulosic biomass is therefore an appealing carbon and energy source for fermentation. As a result, chemicals can be produced biotechnologically from plant waste and at the same time higher added value can be generated for the prior waste material. Typically, the cellulose and hemicellulose fractions of lignocellulose are converted into fermentable sugars, e.g. monosaccharides, by hydrolysis. The proportions of cellulose, hemicellulose, and lignin in the plant biomass are crucial, as they determine the type and concentration of the various sugars contained. However, the composition can vary greatly depending on the species and origin of the plant as previously described (Langsdorf et al., 2021). Typically, when biomass is used as a carbon source in fermentation, a pretreatment is applied to the lignocellulose. In such a pretreatment, the lignocellulosic biomass is usually shredded followed by delignification and/or structural conversion of hemicellulose and cellulose. The overall aim of pretreatment is to maximize the accessible surface area in order to increase the efficiency of hydrolysis to generate as many fermentable sugars as possible (Dasari and Eric Berson, 2007; Hendriks and Zeeman, 2009). Pretreatment methods can be categorized into physical, chemical, physicochemical, and biological methods (Langsdorf et al., 2021). Although, the mode of action is often a combination of different categories. Physical pretreatment mainly includes mechanical methods such as grinding, milling, cutting, or shredding. Chemical pretreatment can be done using acidic or basic solutions, organic solvents, ionic liquids, or advanced oxidation processes. Physicochemical pretreatment methods combine physical forces with chemicals and primarily include hydrothermal methods such as liquid hot water, steam explosion, or supercritical water. Enzymes or microorganisms can be used as biological pretreatment for delignification or saccharification. As in nature, mainly fungi and their enzymes are used for these processes. To keep the costs as well as the environmental impact of pretreatment to a minimum, it is important to use as little energy and chemicals as possible (Yang and Wyman, 2008). Various pretreatment methods have been previously described in detail (Langsdorf et al., 2021). A typical procedure involves shredding the green waste, followed by pretreatment using liquid hot water to separate hemicellulose and lignin, a washing step, detoxification, and enzymatic hydrolysis. Enzymatic hydrolysis produces sugar monomers such as glucose and xylose as the main components of cellulose and hemicellulose. The digestibility of biomass in enzymatic conversion is affected by the proportions of cellulose, hemicellulose, and lignin, ash content, acetate content, surface area, particle size, pore volume, and cellulose crystallinity (Chang and Holtzapple, 2000). In addition to sugar monomers, the hydrolysis of lignocellulose can also produce various by-products, which can have inhibitory effects on microorganisms, such as phenolic substances, furan derivatives, or weak acids (Palmqvist and Hahn-Hägerdal, 2000). Phenolic substances are formed by the degradation of lignin, which can intercalate the cell membrane due to their hydrophobic structure. Furfural and the related substance 5-hydroxymethylfurfural are formed from pentoses and hexoses, respectively. They can affect cell growth negatively (Palmqvist and Hahn-Hägerdal, 2000) and lead to cell death in high concentrations (Palmqvist et al., 1999). These detrimental substances can be detoxified using different physical, chemical, or biological methods (Jönsson et al., 2013). The hydrolysis process should result in a ready-to-use liquid hydrolysate that contains a high concentration of usable sugars and a minimal amount of inhibiting substances. Different pretreatment methods have been previously

examined for the biocatalysis of green waste materials (Varriale et al., 2022). Due to the varying composition of the biomass, the sugar mixture after enzymatic hydrolysis can fluctuate greatly, e.g. 30 - 50% glucose or 10 - 25% xylose as a proportion of the dry matter (Unrean, 2016). The varying substrate composition can strongly influence fermentation, which is why a robust cultivation system must be selected. The evaluation of the robustness of a microorganism was previously demonstrated with *Clostridium saccharoperbutylacetonicum*, wherein the utilization of lignocellulosic sugars and the effect of lignocellulosic inhibitors on fermentation were investigated (Yao et al., 2017).

Various fermentation processes using hydrolysates from green waste for the production of chemicals have already been demonstrated. Such a process has already been shown, for example, for xylitol production with a hydrolysate from switchgrass with *Scheffersomyces stipitis* (Neeru et al., 2013) or with a hydrolysate from North American perennial prairie grass with *Candida* species (West, 2009). Another example is the succinic acid production with a hydrolysate from *Miscanthus x giganteus* by *Actinobacillus succinogenes* 130Z (Dąbkowska et al., 2019; Kuglarz and Rom, 2019). Furthermore, the fermentative production of polyhydroxyalkanoate by *Pseudomonas* species was shown using a grass hydrolysate as substrate (Davis et al., 2013). The production of fatty acids from *Miscanthus* hydrolysates was demonstrated using the yeast *Rhodotorula glutinis* (Mast et al., 2014). A mixed culture of the yeast *Yarrowia lipolytica* and the microalgae *Chlorella pyrenoidosa* was used to produce microbial lipids from pretreated garden waste (Yu et al., 2019). The additional step of enzymatic hydrolysis can be avoided if hydrolysis and fermentation are combined in a consolidated bioprocess. For this purpose, the producing microorganism must be able to express the necessary enzymes itself or a co-cultivation with a second microorganism must be realized. By introducing the expression pathway of hemicellulases and the relevant product pathways through genetic engineering, the production of biodiesel from hemicellulose was made possible with an *Escherichia coli* strain (Steen et al., 2010). Later, the same working group demonstrated 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 from pretreated switchgrass as the main carbon source with their engineered *E. coli* strain (Bokinsky et al., 2011). Additionally, the cultivation of the bacterium was shown on *Eucalyptus globulus* and yard waste. The promising aspect of these results is that the organism could be used for a range of pretreated lignocellulosic biomasses. Such an organism would therefore be suitable for a heterogeneous raw material such as green waste.

Since plants usually have higher nutrient requirements than microorganisms, there is a high probability that plants contain all the nutrients necessary for microorganisms. In addition to the sugars contained, the protein content of up to 18.5% of the dry matter of plants (Langsdorf et al., 2021) can also be of value, particularly as a source of nitrogen. Therefore, the lignocellulosic biomass could also be used as a growth medium supplement or as the only feedstock for a complete growth medium. Boakye-Boaten et al. have demonstrated the use of press juice from *Miscanthus* as a medium supplement for the cultivation of *Saccharomyces cerevisiae* and lactic acid bacteria (Boakye-Boaten et al., 2016). Higher press juice concentrations of up to 90% resulted in increased microbial growth and product concentrations compared to cultivation on the usual YM medium alone (Boakye-Boaten et al., 2016). Press juice from silage containing valuable organic acids has also been used as a fermentation substrate. Cerrone et al. demonstrated the cultivation of *Pseudomonas chlororaphis* and *Burkholderia sacchari* for polyhydroxyalkanoate production with press juice from ensiled perennial ryegrass mainly as a carbon source (Cerrone et al., 2015). The press juice from Italian ryegrass (*Lolium multiflorum*), white clover (*Trifolium repens*), and alfalfa (*Medicago*

sativa) was used as a complete growth medium for lactobacilli (Andersen and Kiel, 2000). For this purpose, the press juice was depleted of proteins, and the glucose concentration was adjusted to the reference growth medium MRS broth. Compared to MRS broth, a higher growth of lactobacilli was observed with the press juice as a growth medium. Furthermore, the resulting fermented broth was shown to be a suitable alternative to peptone, yeast, or hydrolyzed soy protein for the fermentation of bacteria. Alfalfa press juice was also used for the production of L-lysine-L-lactate with the help of lactobacilli (Leiß et al., 2010). After adjusting the sugar content of the press juice, production rates similar to those in an MRS medium were achieved. Another example was shown for lactic acid production with *Bacillus coagulans* on alfalfa press juice with the addition of glucose (Papendiek, 2014). Finally, the cultivation of microalgae on agricultural grass juice was demonstrated for feed protein production (Schoeters et al., 2023). For this purpose, a mixed culture of *Chlorella sorokiniana* and *Acutodesmus obliquus* was cultivated on different dilutions and after different pretreatments of grass juice.

In this work, the cultivation of the microorganism *Cupriavidus necator* on green waste is investigated. *Cupriavidus necator* H16 is a Gram-negative litho-autotrophic β-proteobacterium (Pohlmann et al., 2006). The organism is able to use a variety of carbon sources, with growth rates ranging from 0.14 to 0.46 h^{-1} (Friedrich et al., 1981). Atypically, *C. necator* cannot naturally utilize glucose (Makkar and Casida, 1987). Fructose is often used as the primary sugar for the cultivation of *C. necator*. The organism is also able to use various amino acids like L-leucine, L-aspartate, or L-glutamate (Makkar and Casida, 1987). The great variability in the substrate spectrum is a great strength of the organism, as it allows it to use a variety of complex substrates. Complex substrates that have been used as a carbon source for the cultivation of *C. necator* are chicory roots (Haas, 2015), wheat bran (Annamalai and Sivakumar, 2016), sunflower meal (Kachrimanidou et al., 2015), rice paddy straw (Saratale and Oh, 2015), by-products from the biodiesel industry (García et al., 2013), olive mill wastewater (Agustín Martinez et al., 2015), beer brewery wastewater (Amini et al., 2020), fermented food waste liquid (Hafuka et al., 2011), digestate liquors (Passanha et al., 2013), waste glycerol (Cavalheiro et al., 2009), soybean oil (Kahar et al., 2004; Mifune et al., 2010), jatropha oil (Batcha et al., 2014; Ng et al., 2010), and used cooking oil (Martino et al., 2014; Verlinden et al., 2011). The ability to use a wide variety of waste materials as a substrate makes *C. necator* a promising organism in terms of processes for a circular economy (Bellini et al., 2022). In terms of low-lignin lignocellulosic biomass, cultivation of *C. necator* was demonstrated using a hydrolysate from alligator weed (*Alternanthera philoxeroides*) as a carbon source for the production of polyhydroxybutyrate (PHB) (Zhang et al., 2020). Furthermore, Koller et al. have previously shown that the addition of fresh grass juice or silage juice to a minimal medium can be growth-promoting for *C. necator* and can result in higher PHB concentrations (Koller et al., 2005). Schwarz et al. demonstrated a two-stage fed-batch cultivation of *C. necator* by also using grass silage for the production of PHB (Schwarz et al., 2018). While press juice without any supplements was used for microbial growth in the first stage, the press cake was hydrolyzed and used in seperate lactic acid fermentation. The products of lactic acid fermentation were fed in the second stage for PHB production. *C. necator* H16 produces polyhydroxybutyrate as a carbon storage substance inside granules (Steinbüchel and Füchtenbusch, 1998). PHB is produced when there is sufficient carbon, but other macronutrients such as oxygen, bound nitrogen, or phosphate are growth-limiting (Pohlmann et al., 2006). By using recombinant *C. necator* strains, isopropanol (Garrigues et al., 2020), alkanes/alkenes (Crépin et al., 2016), the biopolymer cyanophycin (Lütte et al., 2012), 2-hydroxyisobutyric acid (Przybylski et al., 2015), and methyl ketones (Müller et al.,

2013) have been produced in the past. In order to achieve the highest possible added value from the lignocellulosic feedstock, a product of maximum value must be selected. The strain *Cupriavidus necator* pKR-hum was previously engineered for the production of the terpene α-humulene by introducing the α-humulene synthase of the shampoo ginger plant *Zingiber zerumbet* and the mevalonate pathway (Krieg et al., 2018). Terpenes are naturally produced by plants and can be extracted from them in the form of essential oils (Schwab et al., 2013). Although several challenges need to be overcome, microbial synthesis can help to produce valuable terpenes more efficiently (Kirby and Keasling, 2009). Apart from the fact that terpenes can be used as aromatic substances, α-humulene also exhibits promising pharmaceutical properties (Mendes de Lacerda Leite et al., 2021). The compound shows anti-tumor (Chen et al., 2019; Legault and Pichette, 2007), anti-inflammatory (Fernandes et al., 2007; Rogerio et al., 2009), and anti-bacterial (Jang et al., 2020) properties. Furthermore, α-humulene can also serve as a precursor for the synthesis of zerumbone, which is a highly potent anticancerogenic agent (Alemdar et al., 2017). By utilizing the same metabolic pathway, the strain can also be used to synthesize other terpenes in the future (Oldfield and Lin, 2012). Accordingly, the production of the terpene β-farnesene with a recombinant *C. necator* strain was also demonstrated (Milker and Holtmann, 2021). Compared to extraction from plants or chemical production, biotechnological production can meet the increasing demand for terpenes and circumvent the disadvantages of other production processes (Sonntag et al., 2015).

2.3 Extraction and technological application of plant peroxidases

Peroxidases (EC 1.11.1.7) are a promising choice for an extraction target from green waste as they are present in relatively high concentrations in any plant (Deepa and Arumughan, 2002) and are used in a large number of technological applications such as analytics, diagnostics, polymer synthesis, or wastewater treatment (Pandey et al., 2017; Sellami et al., 2022). Peroxidases belong to the group of oxidoreductases and catalyze the oxidation of various substances by the reduction of H_2O_2 (Pandey et al., 2017). The use of H_2O_2 as a co-substrate is a major advantage of peroxidases as they do not need any other costly co-factors (Burek et al., 2019). Peroxidases from plants are heme-dependent enzymes that are part of the class III peroxidases of the peroxidase catalase superfamily (Pandey et al., 2017). In plants, peroxidases fulfill a multitude of functions including cell wall metabolism, lignification, defense against pathogens, germination, fruit ripening, and stress tolerance (Pandey et al., 2017). The horseradish peroxidase (HRP) is the established enzyme of choice for technological applications. However, new sources of useful peroxidases are constantly being sought. For example, the purification and characterization of peroxidases from papaya fruit (Pandey et al., 2012), turnip (Duarte-Vázquez et al., 2000), the tree vegetable *Leucaena leucocephala* (Pandey and Dwivedi, 2011), or the fungus *Coprinus* (Ikehata et al., 2005) was previously demonstrated. The use of green waste or grass clippings as a source of peroxidases for technological applications has not yet been investigated. However, various grasses such as wheat grass (Lai et al., 2006), Guinea grass (Centeno et al., 2017), or *Miscanthus* (Dragana et al., 2017; Scebba et al., 2006) have been investigated for the isolation of peroxidases. Gulsen et al. (Gulsen et al., 2010) as well as Du et al. (Du et al., 2009) examined the peroxidase activity of different grasses cultivated under defined conditions. The use of waste materials as a source of peroxidases has the advantage that the raw material does not have to be cultivated specifically for this purpose and the waste material can still be processed in a useful way. Additionally, this results in higher added value from the production of the biomass and reduces

the costs of the products involved. Moreover, in the case of plant material, biomass does not compete with food for agricultural land. The extraction of peroxidases from plant material often follows a typical purification scheme (Lavery et al., 2010). First, the plant material is broken down mechanically or via sonication followed by the removal of the solids. The proteins are then fractionated using ammonium sulfate precipitation. After precipitation, hydrophobic interaction chromatography can be used for further purification due to the high salt concentration present. Otherwise, a buffer exchange would have to be performed for subsequent purification. Further chromatography steps in the form of ion exchange chromatography or size exclusion chromatography as well as a concentration step can follow before a final formulation of the purified enzyme ensues. An assay with the phenolic substrate guaiacol and H_2O_2 can be used for activity determination and thus, in combination with a determination of the total protein content, also for assessing the purification of peroxidases from plants (Dragana et al., 2017; Du et al., 2009; Gulsen et al., 2010; Scebba et al., 2006).

A major field of application for peroxidases is wastewater treatment. In this process, the peroxidases are used for the depletion of phenolic substances. Phenolic substances can be toxic, harmful to the environment, and even carcinogenic, which is why their release into the environment is highly regulated. Furthermore, these substances can accumulate in the food chain. Phenolic substances occur in wastewater from the textile and leather industry, coal and petroleum refining, as well as pulp and paper mills, amongst others (Kumaran and Paruchuri, 1997; Nicell et al., 1993). In Bitterfeld in Saxony-Anhalt, the consequences of water contamination by phenolic substances from the chemical industry became apparent. In the past, high concentrations of chlorophenols were released into the groundwater through contaminated waste from chemical plants (Vieth, 2002), which have not been fully remediated to this day. Chlorophenols are considered to be particularly harmful. Due to the chlorine functional groups, chlorinated phenols such as 2,4-dichlorophenol are difficult to biodegrade (Xu et al., 2016). The molecules phenol and 2,4-dichlorophenol are part of the Priority Pollutant List of the United States Environmental Protection Agency (EPA) (United States Environmental Protection Agency, 2015). According to the EPA, the exposure limits for phenol and 2,4-dichlorophenol, which have no adverse effect on human health when consumed in water and organisms, are 4000 µg L^{-1} and 10 µg L^{-1}, respectively (United States Environmental Protection Agency, 2015). Within the European Union, a Water Framework Directive was published in 2000, which sets out a framework for the protection of water bodies (European Union, 2000). The European Union recommends limit values for phenols in wastewater emissions of 0.5 mg L^{-1} for surface waters and 1 mg L^{-1} for sewage systems (Busca et al., 2008). There are a number of possible methods for removing these phenolic substances. These include adsorption, membrane processes, distillation, extraction, chemical or electrochemical oxidation, advanced oxidation processes as well as biological methods (Mohamad Said et al., 2021; Mohd, 2022; Villegas et al., 2016). The problems with most methods are that they do not achieve complete removal of all target substances and that they are time-consuming and cost-intensive (Torres-Duarte and Vazquez-Duhalt, 2010). Biological methods for removing phenolic substances include treatment with microorganisms or enzymes. The use of enzymes was proposed initially, as enzymes are less susceptible to pH value and temperature, as well as substrate concentration or toxic contaminants in comparison to microorganisms (Klibanov et al., 1983). Furthermore, enzymes are generally characterized by a high selectivity and a high conversion rate (Mohamad Said et al., 2021). However, selectivity can also be a disadvantage when it comes to separating a large variety of phenolic substances. Also, the high conversion rate applies only under appropriate reaction conditions, which is why enzymes are still highly dependent on environmental conditions compared to

physical wastewater treatment methods. For example, plant peroxidases possess an temperature optimum of about 40 °C and a pH value optimum between 5 and 6 (Pandey et al., 2017).

During wastewater treatment, peroxidases form free radicals from phenolic substances, which leads to their polymerization and the formation of aggregates (Klibanov et al., 1983). The resulting products are less harmful and are also often biodegradable (Torres-Duarte and Vazquez-Duhalt, 2010). Due to the aggregate formation, the insoluble polymers can be stripped from the wastewater. The principle of polymerization of phenolic substrates by peroxidases has been illustrated by Steevensz et al. (Steevensz et al., 2014). Quantifying the product of polymerization is difficult when converting phenolic substances due to the variety of polymers and aggregates formed. Thus, the phenolic substrate of the enzymatic reaction is quantified in order to be able to assess the efficiency of the reaction (Wu et al., 1997). Klibanov et al. have demonstrated the use of peroxidases for the elimination of phenolic substances from wastewater for the first time (Klibanov et al., 1980). Later, the conversion of phenol in coal-conversion wastewater with HRP was used to demonstrate the robustness of the method with regard to varying pH values and substrate concentrations (Klibanov et al., 1983). In addition to the use of enzymatic conversion for the treatment of coal-conversion wastewater, the successful application of peroxidases could also be demonstrated for wastewater from the leather industry (Diao et al., 2011) or a petroleum refinery (Wagner and Nicell, 2001). During the conversion of phenolic substances, a gradual inactivation of the peroxidases can be detected, which is caused either by the phenoxy radicals interacting with the active site of the enzyme (Klibanov et al., 1983) or by the adsorption of the enzyme onto the formed polymer, blocking the active site (Nakamoto and Machida, 1992). Through the addition of additives such as polyethylene glycol (PEG), inactivation can be counteracted increasing phenol conversion (Nakamoto and Machida, 1992). The polymeric product adsorbs preferentially to PEG rather than to the enzyme, thereby reducing the impact on enzyme activity (Kinsley and Nicell, 2000). It has been shown that generally a higher molecular weight of the PEG is advantageous (Kinsley and Nicell, 2000; Nakamoto and Machida, 1992) and that a concentration of around 100 mg L^{-1} is optimal (Quintanilla-Guerrero et al., 2008; Wu et al., 1997). To further optimize the reaction, the enzymes can be immobilized. This can reduce the amount of enzyme required by increasing enzyme stability and enabling the enzymes to be reused. However, the challenge with immobilization is not to significantly impair enzyme activity. The immobilization of HRP for the conversion of phenolic substances has been demonstrated in various ways (Dalal and Gupta, 2007; Wang et al., 2016; Wang et al., 2015).

The two currently most promising alternative sources of peroxidases for the application in wastewater treatment seem to be turnips (Duarte-Vázquez et al., 2003) and soybeans (Bódalo et al., 2006). Caza et al. demonstrated the conversion of the three substrates phenol, *m*-cresol, and 2,4-dichlorophenol with the help of soybean peroxidase and the addition of PEG (Caza et al., 1999). Using an immobilized turnip peroxidase, Quintanilla-Guerrero et al. were able to convert more than 95% of up to 1.2 mM phenol while simultaneously showing reduced reaction time and improved operational stability of the turnip peroxidase through the addition of PEG (Quintanilla-Guerrero et al., 2008). In 1994, it was first shown that crude plant material (minced horseradish, potatoes, and white radish) can be used to convert phenolic substances such as 2,4-dichlorophenol and other chlorinated phenols in industrial wastewater (Dec and Bollag, 1994). The use of crude plant material resulted in a similar conversion of the phenolic substances in comparison to isolated peroxidases. Similar results were shown to Cooper and Nicell, who demonstrated that more than 95% of a 3.5 mM phenolic mixture from foundry

wastewater could be converted with a purified HRP and a crude HRP extract both with the addition of PEG at approximately the same time (Cooper and Nicell, 1996). With a crude extract of turnip and the addition of PEG, more than 95% of 0.5 mM phenol, *m*-cresol, and 2,4-dichlorophenol could be converted within 10 min (Duarte-Vázquez et al., 2003). Another interesting example of the use of peroxidase crude extracts was demonstrated in the conversion of phenolic substances using gourd fruit juice as an enzyme source (Yadav et al., 2017). By eliminating the need to purify the enzymes from plant material, a large proportion of the costs arising can be saved. Previously, a method for immobilization of peroxidases from a crude extract of mechanically homogenized horseradish was demonstrated by immobilizing the HRP onto nano-spray-dried ethyl cellulose particles (Dahili et al., 2015). However, the immobilization of peroxidases from plant waste materials for wastewater treatment does not appear to be very practical, as this represents an additional step that probably does not justify the benefits gained. The use of peroxidases in crude plant material can have the advantage that the enzymes are already naturally immobilized and therefore show greater stability (Dec and Bollag, 1994).

2.4 Electrodes from biomass for bioelectrochemical systems

2.4.1 Bioelectrochemical systems

Biotechnological processes can be intensified through the integration of electrochemical processes (Stöckl et al., 2022b). Electrobiotechnology combines biotechnology and electrochemistry into novel processes, which possess the potential to contribute to a circular economy and to meeting the UN sustainability goals (Gizewski et al., 2023). In microbial bioelectrochemical systems, electroactive microorganisms are used, which have the ability to accept or release electrons extracellularly, allowing microbiology to be coupled with electrochemistry (Logan et al., 2019). The different extracellular electron transport mechanisms are described in detail by Stöckl et al. (Stöckl et al., 2022b). In general, a distinction is made between direct (DET), mediated (MET), and indirect (IET) extracellular electron transfer. DET requires direct contact between cell and electrode, usually through biofilm formation, using conductive pili, nanowires, or cytochromes for the electron transfer. In MET, the cells are typically submerged with redox-active mediators, which are secreted by cells or can be added to the medium, transporting electrons between the cell and the electrode. In IET, intermediates are produced electrochemically which are not recycled but consumed by the microorganism, in contrast to MET. Processes based on direct extracellular electron transfer through biofilm formation enable simple continuous process control, as no cell retention is required. The advantages of MET and IET are high possible space-time yields, as no direct electrode contact is necessary, which also results in lower diffusion limitations. Furthermore, in processes based on the IET, the electrochemical and microbial processes can be decoupled in separate reactors and can therefore be optimized independently. Technologies based on the IET have the highest technology readiness level compared to the other extracellular electron transport mechanisms (Stöckl et al., 2022b). In bioelectrochemical cells, there is generally a reduction reaction at the cathode and an oxidation reaction at the anode. The two bioelectrochemical systems covered in this thesis are microbial electrosynthesis and the microbial fuel cell, which are based on opposite reaction directions.

Bioelectrosyntheses combine biotechnological syntheses with electrochemical processes. The great advantage of biotechnology over chemical production is the possibility of producing complex molecules with high stereoselectivity and regioselectivity. Electrochemistry is characterized by high energy and atomic efficiencies. Bioelectrosynthesis combines the advantages of both technologies. In enzymatic electrosynthesis, enzymes are used for biological catalysis. In this work, however, only microbial electrosynthesis, which uses whole-cell catalysis, is covered. The synthesis of substances from electrical energy with the help of microorganisms is the aim of microbial electrosynthesis (MES). In MES, electrons are transferred from a cathode to microorganisms, which use the electrons for reductive synthesis reactions (Krieg et al., 2014). By using electrical energy directly for the biocatalysis of chemicals, the energy industry and the chemical industry can be linked. For example, electroactive methanogens can be used to produce methane using electricity (Enzmann et al., 2019). This offers a new option for energy storage, especially when surplus energy is generated by photovoltaics. The most advanced variants of microbial electrosynthesis are secondary microbial electrochemical technologies as well as hybrids of different technologies (Fruehauf et al., 2020). In the case of secondary microbial electrochemical technologies, microbial biotransformation is integrated into electrosynthesis. Water electrolysis can be used to realize the IET via hydrogen as an electron donor. For example, the microorganism *C. necator* can use hydrogen as an electron donor in combination with oxygen as a terminal electron acceptor and carbon dioxide as a carbon source for autotrophic growth. Ideally, renewable energy is used for the MES. Additionally, CO_2 from off-gas can be used as a carbon source to create an overall highly sustainable process. The microbial fuel cell (MFC) can be used to generate electrical energy from organic substances. During this process, electroactive microorganisms oxidize organic carbon from wastewater to CO_2 and transfer the electrons released to the anode (Krieg et al., 2014). Therefore, a typical area of application for the MFC is in wastewater treatment plants, where residual materials are turned into electrical energy. The first microbial fuel cell was described in 1911 by M. C. Potter (Potter, 1911). Typically, mixed microbial cultures are present in real MFC applications. An important member of such mixed cultures is the microorganism *Geobacter sulfurreducens* (Sun et al., 2016). Due to its predominant contribution to the performance of microbial fuel cells, *G. sulfurreducens* is usually used within experiments in the laboratory. *G. sulfurreducens* is able to oxidize acetate and transfer the resulting electrons to graphite electrodes (Bond and Lovley, 2003). For this purpose, it forms a biofilm on the electrode with the help of pili (Reguera et al., 2007). It was shown that the pili are not only necessary for biofilm formation but are also used by the organism as nanowires for the direct electron transfer to the electrode (Reguera et al., 2005). The properties of the anode are decisive for biofilm formation and therefore also for the electron transfer. The commercialization of the two bioelectrochemical systems MFC and MES is primarily prevented by high operating and investment costs. Various strategies are being researched to minimize these costs and improve process efficiency. Costly electrode materials, which have been developed originally for other areas of application, are a major obstacle hindering the commercialization. Krieg et al. have previously described reactor concepts for the efficient design of bioelectrochemical systems including the electrodes (Krieg et al., 2014). Cheap biomass-derived carbon electrodes may facilitate the commercialization of these innovative technologies. The manufacture, technical requirements and application in bioelectrochemical systems of these biomass-derived electrodes are described below.

2.4.2 Electrodes from carbonized biomass

As an alternative to expensive precious metal electrodes, graphite is often used as an electrode in electrochemical processes. Graphite is typically obtained from fossil resources. However, the mining and production of graphite is a costly, energy-intensive, and time-consuming process (Jara et al., 2019). Graphite does not have to come from fossil resources but can also be produced from biomass through the process of carbonization. Carbonization of biomass produces a solid biochar, which can have a high concentration of electrically conductive graphite depending on the carbonization process (Zhang et al., 2021). The carbonization of lignocellulosic biomass into a carbonaceous material allows it to be used as a raw material for electrodes. The carbonization and subsequent use as electrode material could contribute to the profitable recycling of green waste. Carbon-based electrodes are already being used in bioelectrochemical systems such as microbial fuel cells. Graphite is often the electrode material of choice in the MFC. The use of carbonized biomass as an electrode material can help to reduce the costs of MFCs and make them more environmentally friendly (Yang and Chen, 2020). Electrical conductivity, surface area and structure, biocompatibility, and stability are some of the most important properties for electrobiotechnological applications. However, for the different extracellular electron transfer mechanisms of electroactive microorganisms, different electrode properties are important. For example, pore structure is a decisive characteristic as it influences mass transport within the electrode and the biofilm formation on the electrode surface (Yang and Chen, 2020). Fortunately, biochar shows several advantaged properties such as high electrical conductivity, low costs, naturally occurring pores, and therefore a high surface area (Yang and Chen, 2020). Besides the properties of the raw material, the biochar characteristics are primarily dependent on the pretreatment and the carbonization process of the biomass. The carbonization of different lignocellulosic biomass has already been investigated pretty extensively regarding the production of coal products for energy purposes, which are attractive fuel sources in comparison to raw biomass (Sadaka et al., 2014). For example, the carbonization of *Miscanthus* and the use of the biochar as an energy source were investigated (Kambo and Dutta, 2015, 2014). Carbonized biomass has the advantage that it is easier to store and transport and, compared to fresh biomass, causes fewer problems during combustion due to a lower content of moisture and ash (Sadaka et al., 2014).

Lignocellulosic biomass is often processed into biochar through hydrothermal carbonization (HTC), in which the raw material is converted by heated compressed water (Guo et al., 2015; Medick et al., 2017). The process of hydrothermal carbonization usually takes place at 200 to 275 °C and applies pressure above the saturation point of water to keep the water liquid (Reza et al., 2013). The HTC is typically followed by recovery of the biochar by filtration before the product is dried (Eibisch et al., 2013; Guo et al., 2015; Liu et al., 2013; Reza et al., 2013). The temperature, duration, and other reaction conditions determine the structural transformation of the individual lignocellulose components and thus the properties of the resulting biochar (Sadaka et al., 2014). Hemicellulose is completely degraded at temperatures above 200 °C (Reza et al., 2013). Higher temperatures are required for the full degradation of cellulose. Cellulose is broken down slowly at 240 °C (Guo et al., 2015), with the cellulose content generally decreasing with increasing temperature (Reza et al., 2013). Much higher temperatures of around 300 °C are required to break down lignin (Liu et al., 2013). When carbonizing biomass, it is not only the properties of the product that are relevant but also the solid mass yield (Guo et al., 2015). The mass yield of biochar from hydrothermal carbonization decreases sharply with increasing carbonization temperature (Guo et al., 2015; Liu et al., 2013;

Reza et al., 2013). However, with increasing carbonization temperature, an increase in the carbon content and a decrease in the oxygen content can be observed (Eibisch et al., 2013; Liu et al., 2013). The HTC of different grasses has already been demonstrated using lawn grass cuttings (Eibisch et al., 2013; Guo et al., 2015), switchgrass (Reza et al., 2013; Sadaka et al., 2014), and *Miscanthus* (Reza et al., 2013). The production of biochar from green waste by HTC for use as an energy source was previously evaluated technically, economically, and environmentally (Zeymer et al., 2017). The HTC was also tested on further potential components of green waste such as *Eucalyptus* foliage (Liu et al., 2013) or mixed green waste consisting of dead wood and foliage (Shao et al., 2019). The problem with HTC is that the low temperatures typically lead to insufficient graphite formation for use as electrodes (Yang and Chen, 2020). The structure of the carbon is decisive for the electrical conductivity of the electrodes and therefore also their electrical performance. If the biochar has a high carbon content but does not have an ordered structure in the form of graphite, the material shows a high electrical resistance. Since higher temperatures of around 300 °C are needed for the degradation of lignin (Liu et al., 2013), the process of pyrolysis is applied for the complete conversion of lignocellulose components. During pyrolysis, much higher temperatures are used in the absence of oxygen, resulting in a high degree of graphitization. While not usually present after hydrothermal carbonization, electrical conductivity can be measured after pyrolysis (Hoffmann et al., 2019a). The electrical conductivity of different biochars could only be measured from carbonization temperatures of 600 °C (Kwon et al., 2013) or 800 °C (Gehring et al., 2019). The combination of HTC and pyrolysis for carbonization achieves the highest carbon content and electrical conductivity for biochar, as the HTC causes leaching of the inorganic material beforehand (Hoffmann et al., 2019a). For various lignocellulosic biomasses such as maple wood (Zhang et al., 2014), palm leaves (Ferreira et al., 2018), corncobs (Hoffmann et al., 2019b), corn straw (Qiu et al., 2018), vineyard residues (Hoffmann et al., 2019a), peanut shells (Purkait et al., 2017; Zhan et al., 2021), brewers' spent grain (Cancelliere et al., 2019), fungi (Zhu et al., 2011), and *Cotinus coggygria* flowers (Li et al., 2020), pyrolysis has already been demonstrated. Although numerous publications exist on the carbonization of lignocellulosic biomass, there is a lack of publications exploring the use of carbonized materials as electrode material in electrobiotechnological applications. The most common area of application for biochars from pyrolyzed lignocellulosic biomass is supercapacitors. Accordingly, the focus lies primarily on the electrical properties of the biochar. For example, Jin et al. demonstrated the pyrolysis of big bluestem grass in order to use the activated biochar as an electrode in supercapacitors (Jin et al., 2014). Another similar pyrolysis process was demonstrated by Kabir et al. who pyrolyzed real green waste (Kabir et al., 2015). However, their aim was to produce biofuel in the form of bio-oil, biochar, and syngas. An example for the application in bioelectrochemical systems was shown with the carbonization of alfalfa leaves and the use of the resulting biochar as a cathode catalyst in a microbial fuel cell (Deng et al., 2017). The biochar exhibited higher current density and long-term stability, along with performance characteristics similar to those of a Pt/C cathode catalyst. The current status and future perspectives of electrodes made from carbonized biomass for microbial fuel cells have been summarized in detail in review articles (Chakraborty et al., 2020; Yang and Chen, 2020). Neither mixed green waste nor more specifically grass clippings have yet been carbonized to investigate the biochar as electrodes for bioelectrochemical systems.

To manufacture electrodes from the biochar, various methods have previously been demonstrated. These include the pressing of monoliths (Farma et al., 2013), carbon paste electrodes (Kalinke et al., 2017), the packing and mechanical immobilization of biochar (Huggins et al., 2014), or drop casting of biochar slurries (Cancelliere et al., 2019). The

application of a slurry of biochar and a binder to a metallic current collector for electrode manufacturing has already been carried out several times, using polyvinylidene fluoride (PVDF) (Ding et al., 2020) or polytetrafluoroethylene (PTFE) (Jin et al., 2014) as the binding agent. The polymers PVDF or PTFE are often chosen for the manufacture of electrodes due to their inert properties. Further advantageous properties of PVDF for electrode production are great electrochemical stability, efficient bonding between current collectors and biochar as well as good wettability with the electrolyte (Wang et al., 2017). The effectiveness of electrodes hinges on a range of factors such as conductivity, surface area, stability, and structure. When used in bioelectrochemical systems, the requirements of the microorganisms must also be taken into account. Above all, the electrodes should be biocompatible, i.e. they should neither have a (negative) influence on the microorganisms nor should the microorganisms damage the electrodes. These factors should be taken into account when manufacturing the electrodes and choosing the binding agent. Several advantages of biochar electrodes that can be attributed to the biological material include an enlarged surface area conducive to microbial growth, pore structures facilitating the transport of ions and oxygen, excellent electrical conductivity enabling rapid electron transfer, and minimal resistance, all contributing to their cost-effectiveness (Yang and Chen, 2020). Plant material has a naturally occurring three-dimensional structure (Harussani and Sapuan, 2022; Kwon et al., 2013) that can be advantageous for use as an anode in the MFC, for example by providing a large surface area and favoring biofilm formation. An example has been demonstrated using carbonized *Hibiscus cannabinus* showing a three-dimensionally ordered microporous structure as an electrode for microbial bioelectrochemical systems (Chen et al., 2012). For the unhindered adherent growth of bacteria, pores must be hundreds of micrometers to millimeters in size (Yang and Chen, 2020). Conversely, when pore sizes are smaller, the thickness of the biofilm diminishes due to restricted mass transport within the electrode. A higher internal surface area also results in improved ion transport. The surface properties also play an important role in the growth of the bacteria (Yang and Chen, 2020). For example, high hydrophilicity of the biochar is advantageous for microbial growth. All of the above-mentioned attributes are crucial prerequisites for the application of biochar electrodes in commercial and large-scale settings.

2.4.3 CO_2 as a substrate for biopolymer production by microbial electrosynthesis

Third-generation biorefineries aim to combine microbial production with renewable energy and CO_2 to produce chemicals and fuels (Liu et al., 2020). For this purpose, microbial electrosynthesis can be used, in which CO_2 is utilized as a carbon source for bioelectrochemical production. This allows CO_2 to be used for the production of chemicals while potentially reducing CO_2 emissions. However, efficient synthesis of large and energy-rich molecules from CO_2, besides C1 and C2 products, is a major challenge. Recently, an interesting bioelectrochemical approach was demonstrated by Zheng et al., in which spatially decoupled CO_2 electrolysis to acetic acid and subsequent biocatalysis of the acetic acid with genetically modified *S. cerevisiae* to glucose was shown (Zheng et al., 2022). Attractive products for the production from biorefineries are polyhydroxyalkanoates. Polyhydroxyalkanoates are considered promising substitutes for conventional plastics since they are biodegradable and highly biocompatible (Akaraonye et al., 2010). Research into biodegradable plastics is of great importance, as environmental pollution caused by non-recycled plastics is becoming an increasingly serious issue. Plastic pollution of the environment is considered difficult to reverse (MacLeod et al., 2021). The consequences of

plastic pollution include changes to carbon and nutrient cycles, habitat alterations of various ecosystems, biological impact on various species, ecotoxicity, and related social impacts (MacLeod et al., 2021). Weathering processes cause plastics to decompose in the environment, increasing their complexity and mobility and allowing their integration into natural organic material (MacLeod et al., 2021). As a result, it is practically impossible to remove plastic residues from the environment. Microplastics can already be detected in the human body and have been shown to cause adverse health effects (Marfella et al., 2024). In comparison, polyhydroxyalkanoates are biodegradable in environments such as fresh and seawater, soil, household and industrial compost, and by anaerobic digestion. The production of biodegradable polyhydroxyalkanoates might reduce the production of petroleum-based polymers. One of these polyhydroxyalkanoates is polyhydroxybutyrate (PHB). PHB is a short-chain polyhydroxyalkanoate (Akaraonye et al., 2010) and has similar properties to the petroleum-based polymers polypropylene and polyethylene. It is stiff and brittle and has low thermal stability and a high degree of crystallinity (McAdam et al., 2020). PHB is primarily compared with the thermoplastic polypropylene, which is used as a packaging material, in the furniture and construction industry, in the textile industry, in the automotive industry, and many other areas. PHB shows even improved water vapor barrier properties than polypropylene and improved oxygen barrier properties than both polypropylene and polyethylene terephthalate, which is why it is considered suitable as a packaging material for food (Hankermeyer and Tjeerdema, 1999). As with all innovations, the costs of the alternative play the most important role in the market introduction. As the usual polymers produced in large quantities are significantly cheaper to manufacture, biodegradable polymers have a hard time. Feedstock accounts for a large proportion of the costs of PHB production, which is why the use of waste materials can help to reduce production costs and increase the sustainability of the process, making such alternative polymers more attractive. Castilho et al. provide an overview of the fermentative production of polyhydroxyalkanoates from various waste materials (Castilho et al., 2009). The specific case of PHB production from CO_2 has been extensively reviewed by Lee et al. (Lee et al., 2021). Two important strategies described for fermentative PHB production are the use of a two-stage bioprocess, divided into a microbial growth phase and a PHB production phase, and the use of bioelectrochemical systems, i.e. the combination of electrochemical processes with microbial production as in microbial electrosynthesis (Lee et al., 2021).

The bacteria that can produce PHB using CO_2 as a carbon source include mainly proteobacteria, but also cyanobacteria and purple non-sulfur bacteria (Lee et al., 2021). *C. necator*, whose advantageous characteristics have already been described in Chapter 2.2, is one such microorganism. *C. necator* produces PHB naturally as a storage substance when sufficient carbon sources are available but essential macroelements are limited (Schlegel et al., 1961). Several other carbon sources such as formate (Stöckl et al., 2020), acetic acid (Wang and Yu, 2001), oleic acid (Eggink et al., 1992), lactic acid (Linko et al., 1993), or glycerol (Bormann and Roth, 1999) have already been used to produce PHB with *C. necator*. Previously, 61.9 g L^{-1} PHB could be produced in autotrophic processes (Tanaka et al., 1995), while up to 125 g L^{-1} PHB could be produced in a heterotrophic fed-batch process (Mozumder et al., 2014) with *C. necator*. Furthermore, a maximum PHB content of up to 83% of the dry cell mass was reported (Lim et al., 2023). Due to its autotrophic metabolism, *C. necator* is able to grow with hydrogen as electron donor, oxygen as terminal electron acceptor, and carbon dioxide as carbon source. In microbial electrosynthesis, hydrogen and oxygen can be generated *in situ* through water electrolysis. Indirect extracellular electron transport using hydrogen is particularly attractive due to the possibility of efficient production using water

electrolysis and the low reduction potential of hydrogen (Claassens et al., 2018). Ideally, the electrical energy for water electrolysis comes from renewable energy. Exhaust gas can be used as a source of CO_2 for the organism, resulting in a sustainable process overall. The autotrophic cultivation of *C. necator* has already been studied extensively, but only rarely has real exhaust gas been investigated as a CO_2 source. While CO is often used as a substrate in the form of syngas, sulfur and nitrogen oxides in exhaust gases in particular could harm the growth of microorganisms. Using a mixed microbial culture from an anaerobic digester, it was shown that real exhaust gas had no relevant influence on the bioelectrochemical conversion of CO_2 to acetate (Rovira-Alsina et al., 2022). The influence of real off-gases on autotrophic PHB production with *C. necator* was investigated by Garcia-Gonzalez and Wever showing that the gas impurities had no relevant influence on PHB production or PHB properties compared to pure CO_2 (Garcia-Gonzalez and Wever, 2017). However, the off-gases were only used in the second phase for PHB production, after an initial heterotrophic growth phase. In addition, the CO_2 was previously enriched, collected from the industrial plant, and fed into a bioreactor in the laboratory with pure O_2 and H_2. A first microbial electrosynthesis with real flue gas was demonstrated by Wu et al. for the production of lycopene using a genetically engineered *C. necator* strain (Wu et al., 2022). Again, the gas was collected from an industrial plant and fed into the reactor in the laboratory at regular intervals. The CO_2 in exhaust gases can also be used indirectly for fermentation by first converting the CO_2 electrochemically into another substrate for the microorganism. One such process was demonstrated by Stöckl et al. in which CO_2 was electrochemically reduced to formate, which was subsequently used as a carbon source and electron donor for *C. necator* to produce PHB (Stöckl et al., 2020). Later, using a similar process consisting of electrochemical reduction of CO_2 to formate with a Sn gas diffusion electrode and subsequent fermentation of formate with *C. necator*, 1.38 g PHB was produced using an electrode surface area of 4 cm^2 (Lim et al., 2023). By using renewable energy for electrochemical conversion, a sustainable process similar to microbial electrosynthesis can be realized. In addition, higher efficiencies can be achieved in the individual electrochemical and biotechnological processes, as no compromise has to be found for the experimental setup as in microbial electrosynthesis. The major disadvantage of the electrochemical conversion of CO_2 is that the process can never be as specific as the biotechnological transformation. For example, depending on the catalyst, other components of the gas such as oxygen can also be converted, thus significantly reducing the efficiency of CO_2 conversion. Despite possible toxic gas components, fermentation can therefore be the more robust process.

3 Thesis motivation and outline

The overarching goal of this work is the material utilization of biogenic waste materials arising in metropolitan areas for the production of value-added products to promote the transition to a circular bioeconomy. For this purpose, green waste was investigated as a raw material for fermentation to produce basic or fine chemicals, for the production of electrode materials and their subsequent use in electrobiotechnological applications, and for extraction of industrially relevant enzymes. These approaches should also indirectly reduce CO_2 emissions. Additionally, the direct use of CO_2 from off-gases as a carbon source for microbial electrosynthesis was being investigated. Initially, a review article was published that examines various material recycling methods for green waste. This includes a comprehensive review of the status quo of recycling, analysis of the characteristics of green waste, chemical and fermentative conversion methods including potential pretreatment methods, possible products from pressing and extraction, and the production of electrodes from carbonized green waste. The review article provides a solid basis for future efforts to promote the recycling of green waste. The first work package covers the fermentative production of valuable chemicals based on green waste fractions. The biomass should not only be used as a source of carbon but should rather be used as completely as possible. The broad substrate spectrum of the microorganism *C. necator* makes it a promising organism for bioeconomy and circular economy applications. Here, it was investigated whether juice from grass clippings can be used as a growth medium for the production of the terpene α-humulene with *C. necator* pKR-hum. The microbial growth and terpene production in the grass medium were compared with a conventional growth medium. The use of green waste as a substrate for fermentation with *C. necator* enables the utilization of green waste and expands the feedstock base of *C. necator*. Afterward, green waste was investigated as a potential source for the extraction of peroxidases since the enzymes can be found ubiquitously in plants of all kinds. Green waste could be a cheap alternative source of the industrially relevant enzymes. The aim of this work was the extraction of peroxidases from grass clippings and their application for wastewater treatment. Common lawn grasses were cultivated in the laboratory under defined conditions before peroxidases were extracted and characterized. The purified grass peroxidases as well as crude grass extracts were investigated for the conversion of common phenolic contaminants in industrial wastewaters. Next, the carbonization of green waste fractions and the subsequent production of biochar electrodes for use in bioelectrochemical systems was investigated. Biochar produced from grass clippings via hydrothermal carbonization and pyrolysis and the manufactured electrodes were characterized electrochemically and in terms of materials science before the electrodes were used in microbial electrosynthesis and microbial fuel cells. The manufactured electrodes were compared to established electrodes for the use in microbial electrosynthesis with *C. necator*. Additionally, the electrodes were compared to commercial graphite electrodes in a microbial fuel cell with *Geobacter sulfurreducens*. If successful, electrodes from green waste could help to improve the profitability of bioelectrochemical technologies. Finally, it was investigated whether the biodegradable polymer polyhydroxybutyrate could be produced using CO_2 from flue gas as a carbon source by means of microbial electrosynthesis with the microorganism *C. necator*. In particular, it was being investigated whether the use of a polluted gas mixture in the form of real flue gas influences fermentation with regard to microbial growth or product formation. The microbial electrosynthesis with flue gas was carried out at a coal-fired cogeneration plant of Mainova AG and compared with experiments in the laboratory with a similar clean gas mixture and pure CO_2. At the end of the thesis, a comprehensive discussion of the investigated material

utilization methods is carried out. Through the desired valorization of municipal green waste as well as off-gas CO_2, the work is expected to contribute to the material use of biomass, closing of material cycles, reduction of CO_2 emissions, shift from petrochemical to renewable raw materials in the chemical industry, coupling between chemical production and the energy industry and in particular increased added value from previous waste material.

4 Publications

4.1 Material utilization of green waste: a review on potential valorization methods

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Material utilization of green waste: a review on potential valorization methods

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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.

Keywords: Green waste, Biomass pretreatment, Biomass conversion, Valorization, Carbonization

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

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Dedicated to Prof. Thomas Scheper on the occasion of his 65th birthday.

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 bioproductive 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.

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 comprising *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 Fig. 1.

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 5–25% (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

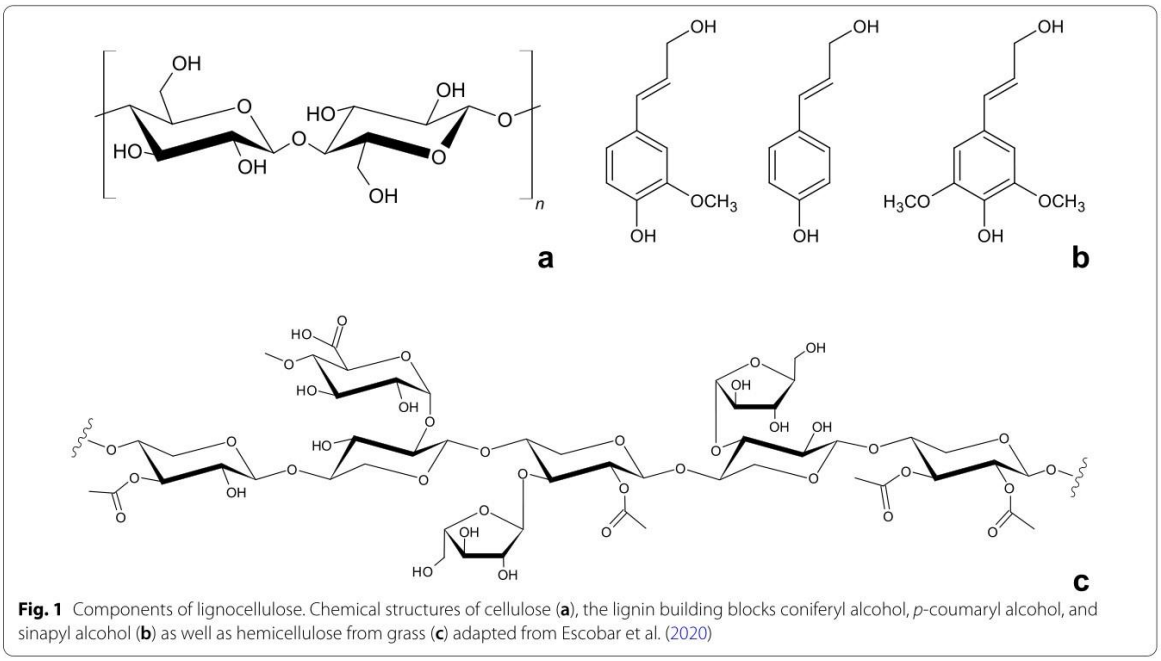

Fig. 1 Components of lignocellulose. Chemical structures of cellulose (**a**), the lignin building blocks coniferyl alcohol, *p*-coumaryl alcohol, and sinapyl alcohol (**b**) as well as hemicellulose from grass (**c**) adapted from Escobar et al. (2020)

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

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	Wongwatanapaiboon 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	Wongwatanapaiboon 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	Wongwatanapaiboon 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	Wongwatanapaiboon 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	Wongwatanapaiboon 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	Wongwatanapaiboon 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	Wongwatanapaiboon et al. (2012)
Vetiver grasses (*Chrysopogon zizanioides*)	Thailand	31.9–38.5	37.9–42.6	3.7–5.1	Wongwatanapaiboon 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)

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.

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 Fig. 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

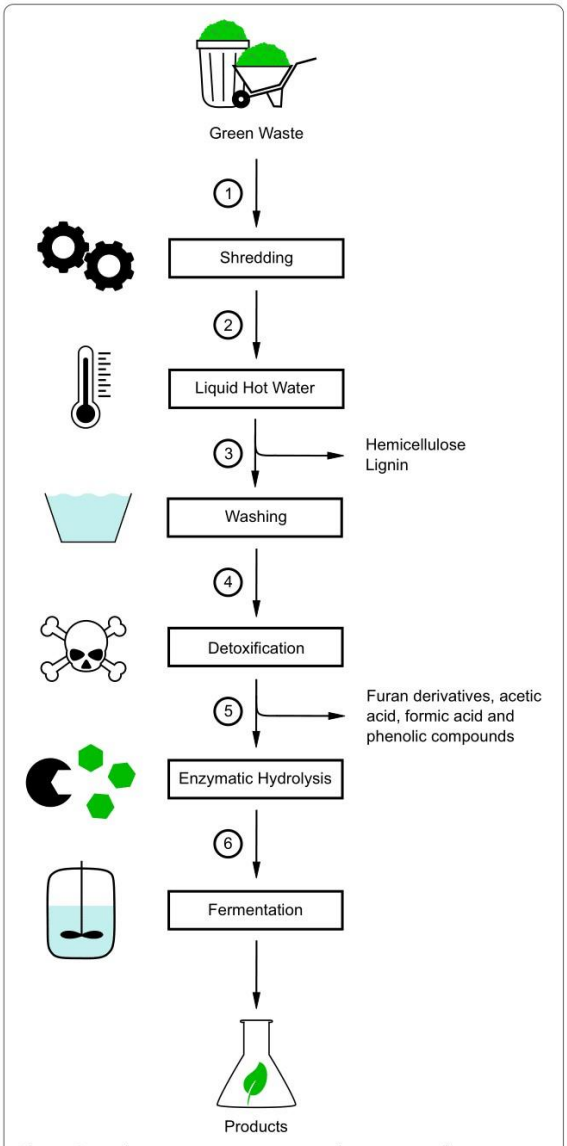

Fig. 2 Exemplary pretreatment process of green waste for fermentative utilization. Green waste is shredded (1) and pretreated using elevated temperature and pressure, removing hemicellulose and lignin (2). The resulting hydrolysate is washed (3) and detoxified, e.g., using activated carbon, to eliminate 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)

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. (2018), Mohapatra et al. (2017), Hendriks and Zeeman (2009), Yang and Wyman (2008), and Taherzadeh and Karimi (2008).

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. Vibroball 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.

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

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)
Physicochemical									
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)

Table 2 (continued)

	Size reduction	Fractionation	Reduction of cellulose crystallinity	Dissolution of lignin	Dissolution/ hydrolysis of hemicellulose	Dissolution/ hydrolysis of cellulose	Delignification	Removal of aromatic compounds	References
Enzymes					+	+	+	+	Bayer et al. (2004), Gutiérrez et al. (2012), Prawitwong et al. (2013), Rabemanolontsoa and Saka (2016)

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).

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).

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).

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).

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 Eq. 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):

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

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 Eq. 2:

$$\log CS = \log R_0 - pH. \quad (2)$$

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.

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.

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

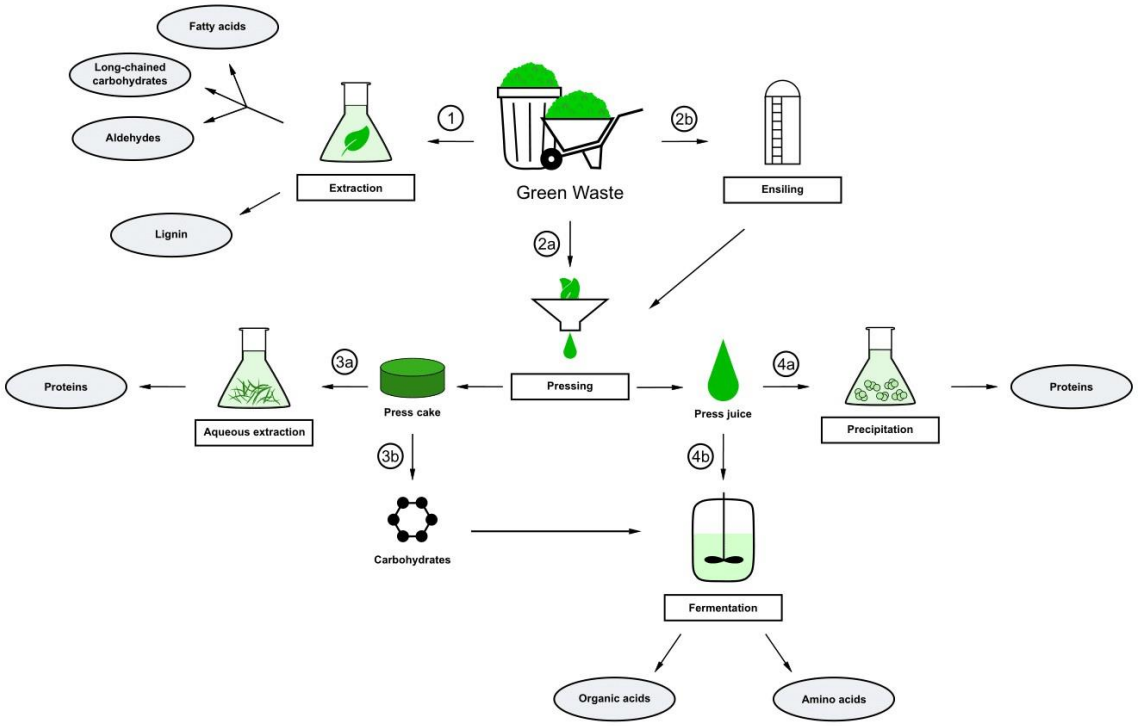

Fig. 3 Processes for the production of extracts from green waste. Products are obtained from green waste directly by extraction (1) or via pressing (2a), possibly after ensiling the biomass (2b). After pressing, proteins are obtained from aqueous extraction of the press cake (3a) and precipitation of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

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).

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 *Cupriavidus 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).

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).

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).

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.

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 Fig. 4.

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. (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

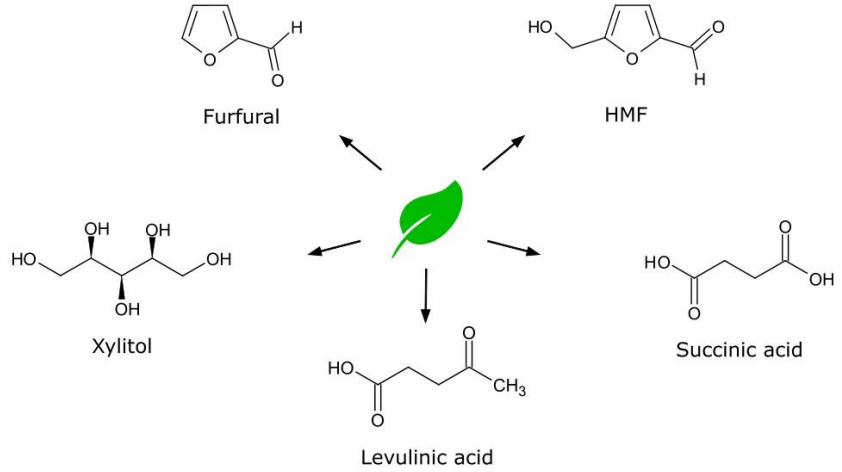

Fig. 4 Potential chemicals from herbaceous lignocellulosic materials

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.

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).

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 pyrenoidosa* 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).

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.

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 Fig. 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

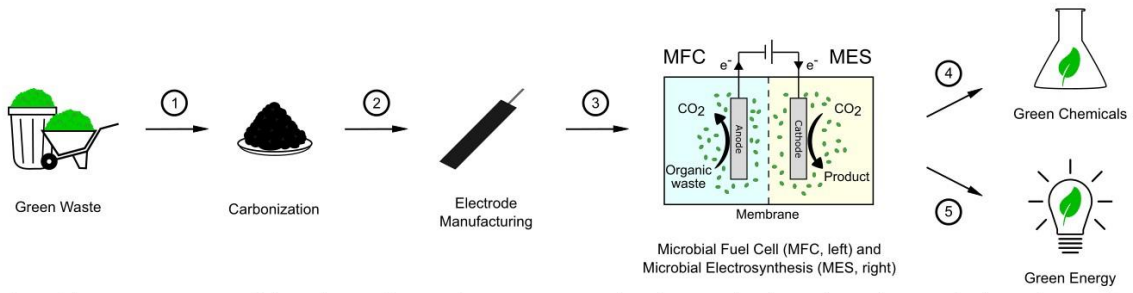

Fig. 5 Schematic presentation of electrode manufacturing from green waste and application in bioelectrochemical systems for the production of "green" chemicals or energy: green waste is carbonized and activated through hydrothermal carbonization and/or pyrolysis (1), formed into suitable electrodes (2)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

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.

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.

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.

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

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

Raw material	Temp. [°C]	LTSR (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	0.5–2 h	50–77	Shao et al. (2019)

LTSR Liquid-to-solid ratio

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.

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.

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4.2 Fermentative α-Humulene Production from Homogenized Grass Clippings as a Growth Medium

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Fermentative α-Humulene Production from Homogenized Grass Clippings as a Growth Medium

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Abstract: Green waste, e.g., grass clippings, is currently insufficiently recycled and has untapped potential as a valuable resource. Our aim was to use juice from grass clippings as a growth medium for microorganisms. Herein, we demonstrate the production of the sesquiterpene α-humulene with the versatile organism *Cupriavidus necator* pKR-hum on a growth medium from grass clippings. The medium was compared with established media in terms of microbial growth and terpene production. *C. necator* pKR-hum shows a maximum growth rate of 0.43 h^{-1} in the grass medium and 0.50 h^{-1} in a lysogeny broth (LB) medium. With the grass medium, 2 mg/L of α-humulene were produced compared to 10 mg/L with the LB medium. By concentrating the grass medium and using a controlled bioreactor in combination with an optimized in situ product removal, comparable product concentrations could likely be achieved. To the best of our knowledge, this is the first time that juice from grass clippings has been used as a growth medium without any further additives for microbial product synthesis. This use of green waste as a material represents a new bioeconomic utilization option of waste materials and could contribute to improving the economics of grass biorefineries.

Keywords: green waste; *Cupriavidus necator*; α-humulene; terpenoid; biorefinery; bioeconomy

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1. Introduction

Biotransformation offers promising approaches to convert the chemical industry into a greener and more sustainable industry. One approach is using waste or residual products from other industries to produce basic chemicals. Especially given limited fossil resources, biomass is an increasingly attractive renewable resource that often also comes along with a lower CO_2 footprint. In the case of lignocellulosic biomass, it is advantageous to use waste material, as it does not compete with food for arable land. While the use of many biomass wastes, especially from agriculture, has been widely studied, the use of green waste has not been comprehensively investigated yet. Green waste is defined as garden and park waste, as well as cuttings from roadside greenery, consisting predominantly of grass clippings and other plant biomass with low lignin content. In major cities, large quantities of potential substrates occur, e.g., in Berlin (3.7 million residents) more than 120,000 tons of herbaceous fresh matter are generated annually [1]. The waste material is mostly composted or applied in biogas plants, although these current methods are not particularly profitable. The mostly herbaceous material consists of carbohydrate-rich polymers such as cellulose and hemicellulose, proteins, and minerals [2], all of which could be put to much better use. However, the composition of green waste is extremely heterologous and varies depending on the season and location, among other factors [2]. The strong heterogeneity is the major obstacle to biomass valorization.

Recently, we have already reviewed potential material utilization methods for green waste [2]. The waste material shows particular potential in biotechnology, where it can

serve as a feedstock for microorganisms. Up to 60% of the biomass dry weight is carbohydrates, which can be used as a carbon source [3]. Above all, the composition of the lignocellulose of the plants, consisting of cellulose, hemicellulose, and lignin is important, as it determines the amount and type of sugars in the plants. Cellulose and hemicellulose are macromolecules consisting of monosaccharides, while lignin is an aromatic polymer [4]. The composition of lignocellulose in herbaceous biomass can vary widely depending on the plant species and origin [2]. Furthermore, these plants contain up to 18.5% (dry weight) proteins that can be used by microorganisms [2]. Typically, biomass is hydrolyzed to use the resulting monosaccharides as a carbon source. Fermentative production of platform chemicals from herbaceous biomass hydrolysates has already been demonstrated, e.g., the production of succinic acid from *Miscanthus* × *giganteus* hydrolysate with *Actinobacillus succinogenes* 130Z [5,6] or the production of xylitol from hydrolysates of North American perennial prairie grass [7] or switchgrass [8] with yeasts.

In this work, we use grass clippings as a substrate for the organism *Cupriavidus necator*, which is a Gram-negative facultative chemolithoautotrophic bacterium that is classified as a β-proteobacterium. The organism is known for its versatile metabolism and can utilize a wide range of carbon sources with maximum specific growth rates between 0.14 and 0.46 h^{-1} [9]. While it can use fructose as well as various amino acids as a carbon source, *C. necator* is not able to use glucose [10]. Previous studies reported the use of many complex substrates like jatropha oil [11,12], vegetable oil [13], soybean oil [14], used cooking oil [15,16], sunflower meal [17], beer brewery wastewater [18], fermented food waste liquid [19], digestate liquors [20], by-products from the biodiesel industry [21], waste glycerol [22], rice paddy straw [23], chicory roots [24], olive mill wastewater [25], and wheat bran [26] as a carbon feedstock for *C. necator*. Naturally, the organism has the ability to produce the bioplastic polyhydroxybutyrate (PHB) [15]. However, metabolic engineering enables the production of various products like alkanes and alkenes [27], alcohols [28,29], methyl ketones [30], 2-hydroxyisobutyric acid [31], or cyanophycin [32]. The strain *Cupriavidus necator* pKR-hum used in this work has the property of producing the terpene α-humulene due to the mevalonate pathway and α-humulene synthase from the shampoo ginger plant *Zingiber zerumbet*, which were introduced by genetic engineering [33]. A variety of other terpenes are formed via the same intermediates according to a sort of modular system [34] as shown by Milker et al. for the terpene β-farnesene [35], allowing the strain to serve as a basis for further terpene syntheses. Terpenoids can be isolated from a variety of plants in the form of essential oils [36]. However, valuable isoprenoids are increasingly produced microbiologically due to improvements in bioengineering [37]. As described by Sonntag et al., the extraction of α-humulene from plants as well as the chemical production of α-humulene is associated with many disadvantages and cannot meet the increasing demand, which is why biotechnological production is preferred [38]. Besides *C. necator*, biotechnological production of α-humulene has already been demonstrated with, e.g., *E. coli* [39], *Methylotuvimicrobium alcaliphilum* [40], or *Methylorubrum extorquens* [38]. α-Humulene shows anti-inflammatory properties [41–43], antitumor activity [44–48], and antibacterial and antibiofilm properties [49]. Furthermore, α-humulene is a precursor of the highly potent anticancerogenic agent zerumbone [39]. Therefore, it is a promising substance for pharmaceutical or cosmetic applications.

Juice from herbaceous biomass, especially from grass clippings, has been used for fermentation and the production of chemicals on a limited number of occasions before. The idea to use green grass juice or silage juice for supplementing the cultivation medium of *C. necator* first came up in 2005 in order to minimize the costs of the production of PHB. Koller et al. were able to show that an addition of 5% (v/v) silage juice increased PHB production by *C. necator* compared to the pure minimal medium [50]. However, the addition of green grass juice did not lead to any improvement in productivity. Unlike Koller et al., Boakye-Boaten et al. could confirm that increasing concentration of grass juice results in higher growth levels and higher product concentrations with *Saccharomyces cerevisiae* and *Lactobacillus brevis* [51]. Andersen et al. [52] and Leiß et al. [53] demonstrated

the use of press juice from alfalfa as a fermentation medium for lactobacilli after adjustment of sugar levels. In 2015, Cerrone et al. used press juice from ensiled perennial ryegrass as a carbon source for the production of polyhydroxyalkanoates with *Burkholderia sacchari* and *Pseudomonas chlororaphis* [54]. In 2018, a two-step fed-batch process for the production of PHB with *C. necator* was published, in which untreated nutrient-rich grass press juice was used for cell growth and saccharides deriving from lactic acid fermentation of the press cake for PHB accumulation [55].

Herein, a process will be presented, where juice from grass clippings is used as a growth medium to produce α-humulene with *Cupriavidus necator*. Instead of using biomass merely as a source of carbon, we are striving for a holistic use of biomass. Grass clippings, which make up a large portion of green waste, are used as a model substrate. The growth of *C. necator* as well as terpene production in the grass medium are compared to an established lysogeny broth (LB) medium. The experiments are performed in shaking flasks and are monitored regarding optical density (OD), carbon concentration, nitrogen concentration, pH, and α-humulene yield.

2. Materials and Methods

2.1. Production of Growth Medium from Grass Clippings

The grass clippings for the experiments were taken from a semi-shaded meadow in the city of Giessen, Germany (50°35′24″ N, 8°40′55″ E) during summer and autumn in 2021. If not used immediately after harvest, the grass clippings were stored at −80 °C until use. The growth medium from grass was prepared via two methods. Firstly, the grass juice was made with a juice extractor. For this purpose the grass was washed with ddH_2O, dried with a towel, and cut into 1 cm long pieces. The grass fragments were run multiple times through the extractor until the remaining grass was dry. The grass juice from the juice extractor was centrifuged at 16,000× *g* for 20 min at room temperature and the supernatant was collected. Since the production with the juice extractor is very labor-intensive and time-consuming, we switched to the production with a blender after the screening studies. For the second method, 100 g of grass clippings were homogenized with 500 mL of ddH_2O (200 g/L) for 2 min. The mass was then passed through a cotton cloth and wrung out.

If indicated, the grass juice was either sterile-filtered or autoclaved. When the juice was autoclaved, it was centrifuged afterward at 3000× *g* and 4 °C for 30 min to separate any suspended solids produced during autoclaving. To ensure comparability, experiments being compared are conducted using the same batches of grass juice.

2.2. Cultivation of Cupriavidus Necator pKR-hum

The organism *Cupriavidus necator* pKR-hum was constructed in a previous work [33]. Precultures were set up in LB medium with 15 µg/mL tetracycline from cryo-stocks of *C. necator* pKR-hum. The LB medium used in this work consisted of 10 g/L tryptone, 5 g/L NaCl, and 5 g/L yeast extract dissolved in ddH_2O. The precultures were incubated at 30 °C and 180 rpm until the late exponential phase or early stationary phase. All cultivations were performed in 100 mL shake flasks unless otherwise described. To each culture, 15 µg/mL of tetracycline was added and every culture was inoculated to an OD of 0.1 from the LB preculture. All cultivations were incubated at 30 °C and 180 rpm. In the preliminary screening experiments, the grass juice from the juice extractor was diluted with the minimal medium MMasy. The minimal medium MMasy was previously developed by Sydow et al. [56] and consists of 2.895 g/L Na_2HPO_4, 2.707 g/L $NaH_2PO_4 \cdot H_2O$, 0.17 g/L K_2SO_4, 0.097 g/L $CaSO_4 \cdot 2H_2O$, 0.8 g/L $MgSO_4 \cdot 7H_2O$, 0.943 g/L $(NH_4)_2SO_4$, and trace elements (1:20,000). The trace elements stock was composed of 15 g/L $FeSO_4 \cdot 7H_2O$, 2.4 g/L $MnSO_4 \cdot H_2O$, 2.4 g/L $ZnSO_4 \cdot 7H_2O$, 0.48 g/L $CuSO_4 \cdot 5H_2O$, 1.8 g/L $Na_2MoO_4 \cdot 2H_2O$, 1.5 g/L $Ni_2SO_4 \cdot 6H_2O$, and 0.04 g/L $CoSO_4 \cdot 7H_2O$ in 0.1 M HCl.

The α-humulene production was induced by adding 0.2% (*w*/*v*) L-rhamnose to the cultivation broth at an OD of 0.5 to 0.7. At the same time, 20% (*v*/*v*) n-dodecane was added to the culture for in situ product removal. n-Dodecane was previously described as a suitable organic phase for the removal of α-humulene during the cultivation of the strain *C. necator* pKR-hum [33]. Samples of 1 mL were taken from the media to measure OD, pH, or TOC and N content and were stored at −20 °C until analysis. OD was measured at 600 nm against the respective medium as blank.

For the continuous recording of growth curves, the Cell Growth Quantifier (Scientific Bioprocessing, Inc., Pittsburgh, PA, USA) was used. The Cell Growth Quantifier (CGQ) is a non-invasive online monitoring system for biomass accumulation in shaking flasks by measuring backscatter intensity. The backscatter intensity is converted to OD using a calibration curve. All measurements in the CGQ were performed in 250 mL shake flasks.

2.3. Carbon and Nitrogen Analysis

For the analysis of carbon and nitrogen in the medium, the samples from the aqueous medium were sterile-filtered, centrifuged at 16,000× *g* for 3 min, and diluted with ddH_2O if necessary. Since the analysis of the usable carbon, like the different sugars via HPLC, is difficult, sum parameters like total organic carbon (TOC) or total nitrogen were used to analyze the consumption of carbon or nitrogen. TOC was determined with a Total Organic Carbon Analyzer TOC-L (Shimadzu Corp., Kyoto, Japan). Cuvette tests were used to determine nitrogen content and consumption. Nitrogen was quantified with Laton Total Nitrogen cuvette tests (Hach Lange GmbH, D-40549 Düsseldorf, Germany) and Vario Total Nitrogen HR cuvette tests (Tintometer GmbH, D-44287 Dortmund, Germany). The cuvettes were analyzed in a DR6000 UV-Vis spectrophotometer (Hach Lange GmbH, D-40549 Düsseldorf, Germany) or an XD 7000 (Vis) spectrophotometer (Tintometer GmbH, D-44287 Dortmund, Germany), respectively.

2.4. Quantification of α-Humulene

To quantify α-humulene production, 200 µL samples were taken from the n-dodecane phase of the cultivation. Samples are centrifuged at 16,000× *g* for 3 min and stored at −20 °C until analysis. Prior to analysis, the samples were diluted with acetone 1:10 including 50 mg/L zerumbone as an internal standard. A standard curve was made with α-humulene diluted with n-dodecane. Prior to analysis, the standards were diluted 1:10 with acetone including zerumbone like the samples. 1 µL of samples and standards were applied to a GC-MS system with an HP-5MS GC column (Agilent, Santa Clara, CA, USA).

3. Results and Discussion

3.1. Screening Studies

In order to identify the optimal grass juice (GJ) concentration and to investigate possible inhibitory effects, different concentrations of grass juice from the juice extractor diluted in a minimal medium (MMasy) without a carbon source were used for the cultivation of *C. necator* pKR-hum. The antibiotic tetracycline was added to all cultures. In combination with the minimal medium, the grass juice serves mainly as a carbon source, since the medium contains all other elements/compounds that *C. necator* needs to grow. Analysis of the grass juice from the juice extractor shows a total organic carbon (TOC) content of 20.0 g/L and a total nitrogen (N) content of 1.2 g/L. Other waste materials used as carbon source for cultivation of *C. necator* show similar TOC concentrations with, for example, 19.7 g/L for rice straw hydrolysate [57] or 23.6 g/L for tuna condensate [58]. The grass juice has an advantageous C/N ratio for microbial growth of about 17. However, it must be mentioned that not all of this carbon and nitrogen can be utilized by the organism. The C/N ratio in the grass juice describes the total C and N concentrations and not what is available for the microorganism. The optimal C/N ratio for the growth of *C. necator* depends on the substrates. For example, an optimal C/N ratio of 6 was determined for

food waste-based volatile fatty acids [59], but an optimal ratio of 12 or 24 was determined for various vegetable oils [60].

Figure 1a shows the optical density after 77.5 h of cultivation as a function of the grass juice concentration. The very first thing that can be highlighted is that *C. necator* does grow on the minimal medium supplemented with grass juice. The maximum OD of 10.9 was reached with the medium made of 80% GJ. An OD of 6.2 was reached with 60% GJ, 4.4 with 40% GJ, and 2.5 with 20% GJ. No growth could be detected in the culture that was made of 100% minimal medium without a carbon source. The final optical density increases proportionally to the grass juice concentration. This finding is consistent with previously described results [51]. The data points form a linear function with an $R^2 = 0.976$. The slope is 0.13 units of OD per percent grass juice. No evidence of growth inhibition could be detected with increasing concentrations of grass juice. It has previously been demonstrated that the addition of grass juice can be growth-promoting. Koller et al. have shown that the addition of 5% green grass juice or silage juice to the growth medium can promote the growth of *C. necator* [50]. In that case, however, the grass juice was used as an additive and not primarily as a carbon source, although silage juice in particular has a high concentration of sugars. In addition to the increasing final OD, it can be observed that the final pH is higher with increasing grass juice concentration. While the pH in the pure minimal medium without growth is 7.4, 40% GJ shows a final pH of 8.0 and 80% GJ shows a final pH of 8.9. The increasing pH is observed in complex media when e.g., amino acids are consumed by the organism as a carbon source when the cells need more carbon than nitrogen. In this process, ammonia is split off from the amino acid, which forms an ammonium ion in the aqueous medium. As a result, the medium becomes alkaline.

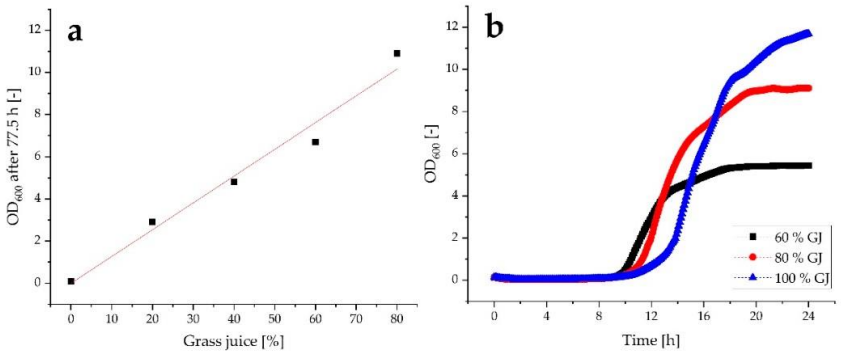

Figure 1. (**a**) Final optical density of *C. necator* pKR-hum culture after 77.5 h with 0%, 20%, 40%, 60%, and 80% of raw grass juice from the juice extractor diluted in MMasy (linear fit with $R^2 = 0.976$; $n = 1$); (**b**) growth curves of *C. necator* pKR-hum in 60%, 80%, and 100% raw grass juice (GJ) from the juice extractor diluted in MMasy (recorded with the CGQ; $n = 1$).

To further investigate the growth, *C. necator* pKR-hum was cultivated in shake flasks with 60%, 80%, and 100% GJ from the juice extractor diluted in MMasy and was monitored online for 24 h by using the Cell Growth Quantifier (Scientific Bioprocessing, Inc., Pittsburgh, PA, USA). The resulting growth curves are presented in Figure 1b. The highest OD could be reached with the medium made of 100% GJ. The culture reached an optical density of 11.6 after 24 h of incubation. A maximum growth rate of 0.65 h^{-1} could be achieved during the exponential phase. Therefore, it can be concluded that *C. necator* is able to grow on grass juice without the addition of minimal medium. Similar results were previously shown by Schwarz et al. who used the diluted and neutralized press juice of grass silage as a growth medium for *C. necator* without any further additives [55]. The culture with 80% GJ grew to an OD of 9 with a maximum growth rate of 0.65 h^{-1} and the culture with 60% GJ grew to an OD of 5 with a maximum growth rate of 0.69 h^{-1}. The maximum growth rate during the exponential phase is almost equal for all three concentrations, which again

indicates that a higher concentration of grass juice in the medium does not inhibit the growth of *C. necator* pKR-hum. Interestingly, the 100% GJ culture showed the longest lag phase, indicating that the cells needed a little longer to adapt to the grass medium after being cultured in the LB medium. From both experiments, it is evident that higher final OD values are obtained with increasing grass juice concentrations.

After using raw grass juice for fermentation, the next step was to investigate two methods for sterilizing the grass medium. For this purpose, sterile filtration and autoclaving were investigated for their feasibility and influence on the growth behavior of the microorganism. While sterile filtration may not affect the ingredients of the grass medium, it is very tedious to perform. However, autoclaving is expected to change the juice and consequently the microbial growth. In the literature, the (ensiled) grass juice was either autoclaved [54], sterile-filtered [50] or not sterilized at all [55]. The amount of sugar in the grass juice varies greatly from sample to sample depending on the time of harvest or grass composition [61]. Autoclaving will probably render most of these sugars unusable due to the Maillard reaction. In addition to sugars, vitamins or trace elements might be affected. We observed that the grass juice turns into a brown cloudy liquid during autoclaving. Hence, possible substrates are no longer available to the organism. In the following experiment, *C. necator* pKR-hum was cultivated in three flasks with 30% raw, 30% sterile-filtered, or 30% autoclaved grass juice from the juice extractor diluted in MMasy for 6 days and was analyzed for the OD. Again, the antibiotic tetracycline was added to all cultures. The resulting growth curves are shown in Figure 2. The first thing to note is that *C. necator* is growing on all three variations of the grass juice. By comparing the growth curves of the different pretreated grass juices, it can be observed that the untreated and sterile-filtered cultures basically follow the same pattern. Both cultures reach a maximum OD of 1.8 after 50 h, experience a peak at 30 h, and are leveling off in a stationary phase to an OD of 1.8 after 48 h. This indicates that the untreated grass juice contains no microorganisms, which can grow in the tetracycline-supplemented medium and therefore influence the growth in the screening studies. This assumption is confirmed by control cultures without inoculation and microscopic controls of the cultures. The culture grown in the autoclaved medium reached a maximum OD of 1.6 after 56 h of incubation. In comparison to the other two cultures, the growth curve flattens after 30 h, resulting in a slightly lower final OD.

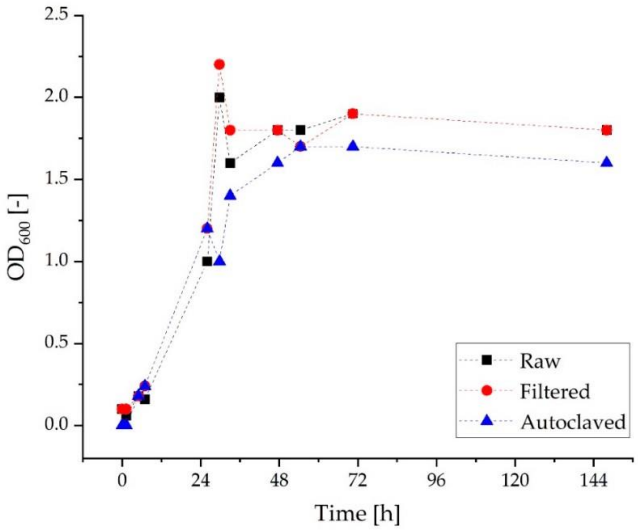

Figure 2. Growth curves of *C. necator* pKR-hum in 30% raw, 30% sterile-filtered, and 30% autoclaved grass juice from the juice extractor diluted in MMasy (*n* = 1, dashed *line* serves as a *guide* to the *eye*).

The screening studies have shown that the final optical density increases linearly with higher grass juice concentrations. Accordingly, no inhibitory substance appears to be present in grass juice to any relevant extent. Furthermore, autoclaving the grass juice resulted in similar maximum OD as the raw grass juice, although the growth curve is slightly different. Comparable results were obtained with raw and sterile-filtered grass juice. However, sterile filtration of the particulate grass juice is difficult and it cannot be excluded that contaminations are introduced using raw grass juice. Therefore, all experiments hereinafter were performed with autoclaved grass juice. Furthermore, we switched to the much simpler production of grass juice with a blender at the expense of high substrate concentrations (see Section 2.1). Since it was shown that no supplementation with additional salts and trace elements was needed, only water was used in the production of grass juice, resulting in a growth medium consisting only of homogenized grass clippings and water.

3.2. Comparison of Growth and α-Humulene Production in Grass Medium and LB Medium

After demonstrating the successful growth of *C. necator* pKR-hum in the grass juice, the next step focused on the question of whether or not it is possible to produce α-humulene from a fully grass juice-based medium. In addition, the grass medium was compared with the established LB medium. For this purpose, experiments with the autoclaved grass medium and the LB medium were conducted. At the time of induction, an organic dodecane phase was added for in situ product removal of α-humulene. During the cultivation, OD, TOC, nitrogen, pH, and α-humulene concentration were analyzed.

Figure 3 shows the OD and the α-humulene concentration in the organic phase throughout cultivation in the LB medium and the grass medium. After 6 h, product formation was induced with L-rhamnose (indicated by the arrow). The first thing that can be observed is the higher final OD of the grass medium in comparison to the LB medium. A maximum OD of over 8 is reached in the grass medium after 48 h. In the LB medium, the maximum OD of 3.9 is already reached after 13 h. Although the preculture was grown in the LB medium, the culture in the grass medium did not show any adaptation difficulties in the form of longer lag phases compared to the main culture in the LB medium. α-Humulene can be detected in the organic phase of the grass medium after 23 h and thus 13 h later than in the LB medium. In the grass medium, 2 mg/L of α-humulene can be detected at 42 h after induction. In comparison, 10 mg/L of α-humulene can be detected in the LB medium after the same time. However, the production of α-humulene is growth-coupled and thus should be higher in the grass medium than in the LB medium according to the OD values. PHB formation cannot affect OD and α-humulene production because the strain used, *C. necator* pKR-hum, is PHB-deficient. For this reason, we examined the cultures more closely with the microscope. We were able to quickly rule out contamination. But, under the microscope, vesicles can be seen in the grass culture which looks like an oil-in-water (O/W) emulsion (see Figure S1). It can therefore be assumed that an emulsion of dodecane forms in the aqueous grass medium. This emulsion causes the aqueous phase to become turbid and subsequently increases the OD. The formation of vesicles was not observed in the LB medium. The growth of *C. necator* pKR-hum in the grass medium without the addition of dodecane is shown in Figure S2. According to the growth curve without dodecane, the true maximum OD by the microorganisms in the grass medium is about 2.3. Additionally, the maximum growth rate resulting from the growth curve in the grass medium without dodecane is 0.43 h^{-1}. In comparison, the maximum growth rate in the LB medium is not much higher, with a value of 0.50 h^{-1}. Obviously, there are some until now unknown interactions between the components in the media and the organic phase. Some of the product is presumably trapped in the aqueous phase by the dodecane vesicles. Compared to the LB medium, the α-humulene concentration in the grass medium tends to follow a linear course. An explanation could be that the α-humulene is previously collected in the emulsified dodecane vesicles in the aqueous phase. During another experiment with the same setup, an α-humulene concentration of 1.3 mg/L was achieved after 48 h and

a maximum OD of 12 suggesting that less α-humulene is found when there is a higher OD due to stronger emulsification. Treatment of samples from the grass medium with dodecane prior to analysis confirms this assumption. Sterile filtration leaves the suspension turbid, although the microorganisms should have separated. After centrifugation, a small organic phase can be detected on the clear aqueous phase. This emulsion forms only in the presence of microorganisms. In abiotic control cultures, OD did not increase due to the addition of dodecane. Thus, while the in situ product removal is suitable for conventional media, it poses a problem in the complex grass medium.

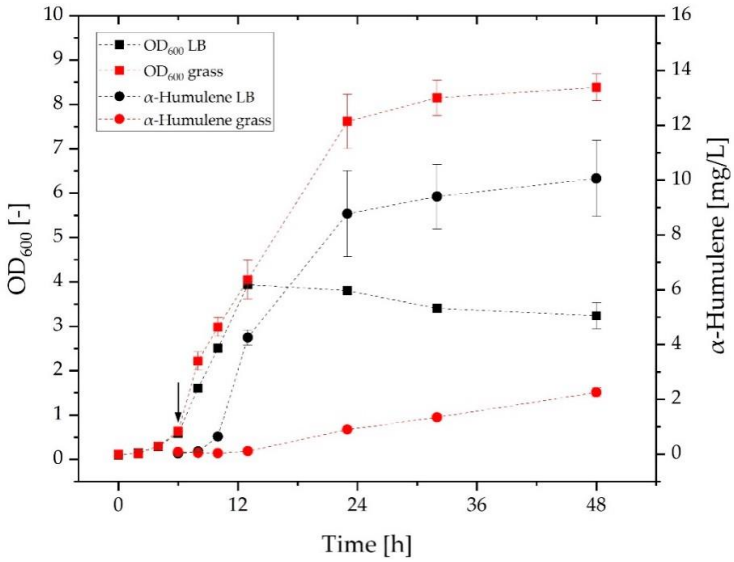

Figure 3. Growth curves of *C. necator* pKR-hum in grass medium and LB medium as well as the respective α-humulene concentration in the organic phase. The induction of α-humulene production after 6 h is indicated by the arrow (n = 3, dashed *line* serves as a *guide* to the *eye*).

Subsequently, pH as well as TOC and N concentrations of the cultivation in the grass medium and the LB medium were compared. Figure 4 shows the pH throughout the cultivation in the grass medium and the LB medium. In the LB medium, the pH rises continuously from 7.1 to a value of 9.3 over the cultivation period of 48 h. As previously described in the context of the screening studies, the pH in the grass medium also increases to alkaline. Here, the pH starts at 6.4 and rises to 8.9. However, after 13 h, a dip in the curve is noticeable. This dip occurred in all cultivations with grass medium and can also be observed in the cultivation without the addition of dodecane (see Figure S2). The discontinuous course of the pH value results from the complex composition of the grass medium. Overall, the pH curves are similar due to the shift to alkaline.

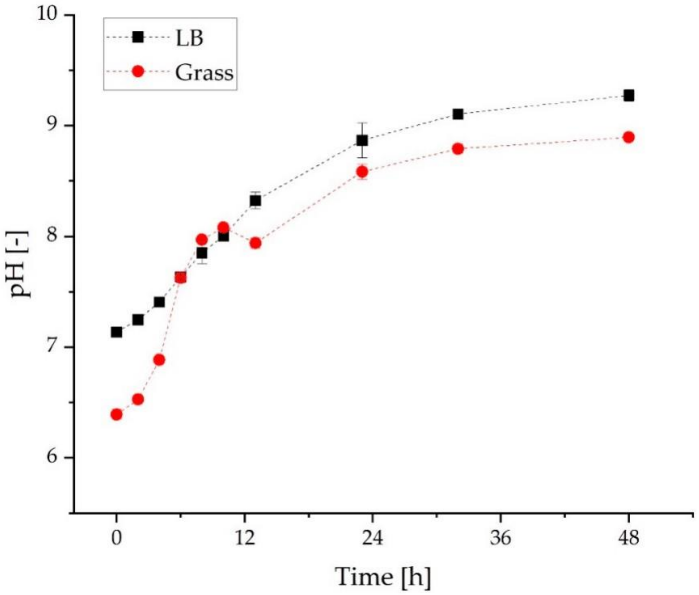

Figure 4. The course of pH of *C. necator* pKR-hum cultivation in LB medium and grass medium (n = 3, dashed *line* serves as a *guide* to the *eye*).

Finally, TOC and N were analyzed throughout the fermentation. The results are shown in Figure 5. As expected, TOC and N levels decrease with increasing OD in both cultures. Less organic carbon is available in the grass medium compared to the LB medium. At the beginning of cultivation, the TOC concentration of 6.3 g/L in the LB medium decreases to 4.2 g/L after 48 h. Less than half of the TOC concentration is present in the grass medium. It decreases from 2.7 g/L to 2.0 g/L. The lower carbon consumption is reflected in lower OD and lower productivity within the grass medium. The difference in initial TOC concentration may affect microbial growth, although it is a sum parameter and does not reflect available carbon. The results of the nitrogen analysis show about 1.7 g/L of nitrogen in the LB medium and only 0.2 g/L in the grass medium. Thus, the LB medium has about eight times as much nitrogen as the grass medium. However, the concentration decreases by only 0.2 g/L in the LB medium in comparison to 0.1 g/L in the grass medium. Therefore, the high N concentrations present are not required by the microorganism. The initial values result in a C/N ratio of 11 for the grass medium and a little less than 4 for the LB medium. This is an advantage for the grass medium if the absolute amount of carbon and nitrogen is higher. Since higher optical densities were achieved in the screening studies with more concentrated grass juice, the availability of substrates is likely the limiting factor for microbial growth. Boakye-Boaten et al. have shown that juice from *Miscanthus x giganteus* can have 37.8% elemental carbon and thus a higher carbon concentration than juice from lawn grass [51]. In terms of minerals, potassium is predominant, followed by magnesium and calcium. Components such as minerals in the grass juice and their influence on fermentation need to be analyzed in the future. In addition to an optimized production method, the grass juice can be concentrated by evaporation to obtain higher carbon concentrations as shown by Cerrone et al. [54]. The question remains whether the organism can utilize the substrates before the pH is too alkaline.

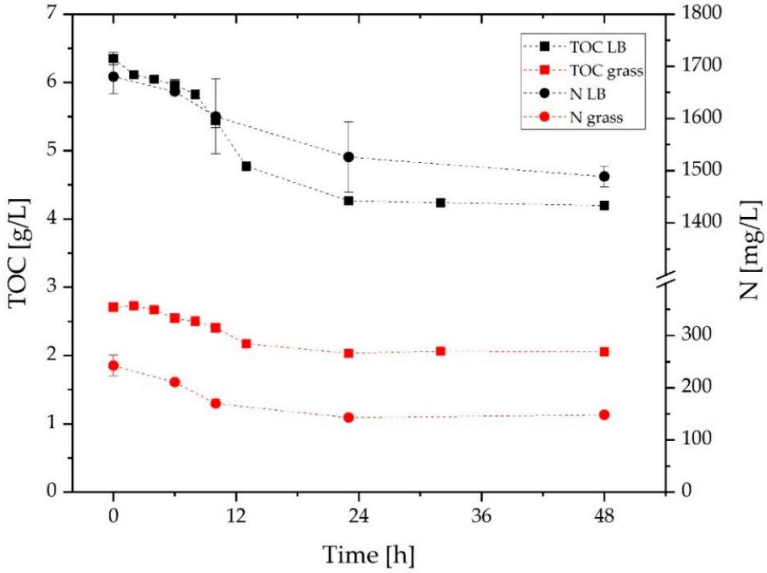

Figure 5. The concentration of TOC and N during the cultivation of *C. necator* pKR-hum in LB medium and grass medium ($n = 3$, dashed *line* serves as a *guide* to the *eye*).

All in all, it is difficult to compare the holistic use of grass juice as a growth medium with results from other publications, since in those cases the grass juice is used either only as a carbon source or as an additive. As described earlier, only the juice of grass silage was used as a growth medium for the cultivation of *C. necator* [55]. However, silage juice has a strongly different composition than green grass juice [50]. The main carbon sources are lactic and acetic acid and *C. necator* shows a much lower maximum growth rate of 0.06 h^{-1} in comparison to our experiments [55]. Nevertheless, ensiling can be used to store grass clippings and make them available as a resource throughout the year. However, the necessary storage capacity for ensiling must also be considered.

Milker et al. were able to produce 2 g/L of α-humulene with *C. necator* pKR-hum in a fed-batch process with fructose as a carbon source [62]. To achieve similar values with the grass juice as a growth medium, a similar fed-batch process with pH regulation in a bioreactor would have to be developed. Cerrone et al. have demonstrated a fed-batch process with the feeding of ensiled grass press juice with the organisms *Burkholderia sacchari* IPT101 and *Pseudomonas chlororaphis* IMD555 [54]. To design an efficient process with grass juice as the growth medium, different factors should be optimized. First of all, it must be investigated how the heterogeneity of the raw material affects the success or the course of the fermentation. Although "real" and unpurified grass clippings were used in our experiments, it also remains to be investigated how impurities affect growth and productivity. The raw material must at least be cleaned of pollution such as plastic waste. It is also possible that plants contain substances that have an inhibitory effect on the microorganism.

There is also still a great deal of potential for optimization in the biotransformation of green waste. Krieg et al. highlight that the unique physiological flexibility of *C. necator* allows it to utilize a wide variety of carbon and energy sources [33]. However, the use of substances in biomass can be improved. Identifying the substances that the organism can use for growth could help to optimize the process. There are certainly other microorganisms that can use the nutrients of the grass juice even more extensively. *Cupriavidus necator*, for example, is naturally unable to use glucose. Thus, an organism that can use a variety of sugars as a carbon source would be advantageous. Pretreatment of biomass is mainly

intended to make the sugars of cellulose and hemicellulose accessible and to delignify the biomass. However, pretreatment can be energy intensive or might use toxic chemicals, and must be judged to be worthwhile to justify undertaking. Consolidated bioprocessing can additionally help to fully utilize the carbohydrates of lignocellulose by combining enzymatic biomass hydrolysis with product generation in one system. One such consolidated bioprocess was demonstrated by Bokinsky et al. who engineered *E. coli* with the ability to utilize cellulose and hemicellulose from pretreated switchgrass to produce biofuels [63]. A process with an organism that combines these characteristics is also likely to be more robust when dealing with heterogeneous raw material. Nevertheless, only fermentation in a bioreactor is expected to result in significantly higher growth, especially due to pH regulation, and thus in increased α-humulene production. Finally, the method for grass juice production is crucial for the concentration and composition of the grass medium. At laboratory scale, we could already determine strong differences between methods. On a larger, industrial scale, a screw press would probably be most effective, as demonstrated by Cerrone et al. [54] or by Schwarz et al. [55].

The use of green waste juice for fermentation in combination with other novel valorization methods in the form of a biorefinery has great potential to make the reuse of green waste significantly more profitable. Due to the high seasonal variability of green waste, it is probably also beneficial to use different methods at different times of the year. Grass juice as a product is more promising in the summer months when grass clippings with high liquid content are abundant, while in the winter months, e.g., electrodes can be created from carbonized green waste where low moisture is beneficial.

4. Conclusions

In our study, we were able to show that homogenized grass clippings can be used as media for the microbial production of high-value chemicals. After demonstrating that *C. necator* grows on a mixture of minimal medium with grass juice as a carbon source, we could also show that *C. necator* grows solely on grass juice without any additives. Finally, we were able to demonstrate the production of α-humulene with *Cupriavidus necator* pKR-hum on a 100% green clippings-based medium. As far as we know, this is the first time that juice from grass clippings has been used as a growth medium without further additives for microorganisms to produce chemicals.

This enables a new value-added production of chemicals based on waste materials. Since various products can be produced with *C. necator*, the results shown here have a platform character. Given current challenges such as climate change, increasing depletion of resources, the growing world population, and a decline in usable agricultural land, new ways of utilizing waste as a material must be found. The results shown represent an option for a bioeconomy based on green waste. Furthermore, an improvement of the financial situation of grass-based biorefineries may also become possible. However, only a comprehensive utilization of green waste in the form of such a biorefinery will ensure an optimized valorization of the waste material.

Supplementary Materials: The following supporting information can be downloaded at: https://www.mdpi.com/article/10.3390/molecules27248684/s1, Figure S1: Microscopic image of a grass medium sample with dodecane after 48 h of cultivation (1000× magnification; squares have a size of 50 × 50 µm). The needle points to a vesicle of the oil-in-water emulsion of dodecane in the grass medium; Figure S2: Growth curve and pH during the cultivation of *C. necator* pKR-hum in grass medium without the addition of dodecane (n = 3, dashed *line* serves as a *guide* to the *eye*).

Author Contributions: Conceptualization, A.L., D.H. and R.U.; methodology, A.L. and M.V.; validation, A.L.; investigation, A.L. and A.-L.D.; resources, D.H.; writing—original draft preparation, A.L.; writing—review and editing, A.-L.D., M.V., R.U. and D.H.; visualization, A.L. and A.-L.D.; supervision, D.H.; project administration, D.H.; funding acquisition, D.H. and R.U. All authors have read and agreed to the published version of the manuscript.

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4.3 Peroxidases from grass clippings for the removal of phenolic compounds from wastewater

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Peroxidases from grass clippings for the removal of phenolic compounds from wastewater

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ABSTRACT

Green waste, especially of municipal origin, is currently used as a material only to a limited extent. However, the large material flows could also be used in a more economical way if they were integrated into biorefinery concepts. Besides the production of basic and fine chemicals, green waste could also be used as source of industrial relevant enzymes. Here, the purification and characterization of peroxidases from common lawn grass species *Lolium perenne* and *Festuca arundinacea* are reported. The purified peroxidase fractions as well as crude extracts were investigated for the removal of common wastewater pollutants such as phenol, *m*-cresol, and 2,4-dichlorophenol by oxidative polymerization. The grass-derived peroxidases showed the highest affinity towards 2,4-dichlorophenol, followed by *m*-cresol and phenol. A crude extract of real lawn grass was able to remove over 95 % of 0.5 mM 2,4-dichlorophenol within 20 min.

1. Introduction

In view of climate change, ongoing urbanization, and the depletion of fossil sources for fuels and chemicals, the use of bio-based resources is becoming more and more important. One approach is to optimize the (re-)use of organic waste as a raw material for different products. Especially in major cities, a large amount of organic waste is generated, which is still not recycled in an economical manner. A large share of organic waste that accumulates in urban areas is green waste. Herein, green waste is defined as plant material with a low lignin content, which accumulates primarily in urban areas from parks, gardens, and roadsides. It consists mainly of grass clippings, but may also include leaves, twigs, and smaller plants, the composition depending on the season and location. This results in highly heterogeneous waste material. So far, most of the green waste is composted or serves as raw material for energy production. However, the costs of collecting and transporting green waste exceed the value added by current reuse methods. Alternatively, green waste represents a promising biological feedstock (Langsdorf et al., 2021).

Recently, we explained efficient methods for the pretreatment of such materials (Varriale et al., 2022) and demonstrated the use of grass clippings as a substrate for the microbial production of the terpenoid α-humulene (Langsdorf et al., 2022). In continuation of this work, we hypothesized that green waste may also serve as a cost-effective source of enzymes. The extraction of technically relevant enzymes from waste materials is particularly interesting. The resource does not have to be cultivated but accumulates anyway. As a result, costs are reduced and acreage does not compete with food.

Peroxidases are enzymes in demand for a variety of industrial applications and are ubiquitously found in plants. They belong to the class of oxidoreductases (EC1.11.1.X) and are able to oxidize various substrates under the reduction of hydrogen peroxide (Pandey et al., 2017). Plant peroxidases are heme-dependent peroxidases and belong to the class III peroxidases of the peroxidase-catalase superfamily (Pandey et al., 2017). The numerous functions of plant peroxidases include germination, stress tolerance, cell wall metabolism, lignification, fruit ripening, and defense against pathogens (Pandey et al., 2017). Similarly diverse as the tasks of peroxidases in plants are the possible applications of (plant) peroxidases in industry, which have been summarized by several excellent reviews (Hamid and Khalil-ur-Rehman, 2009; Pandey et al., 2017; Sellami et al., 2022). Applications encompass biosensors, diagnostic and analytical kits, polymer synthesis, and synthetic dye

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degradation. Horseradish peroxidase (HRP) is the enzyme of choice in most of those applications. Nevertheless, alternative sources of peroxidases have been investigated. For example, peroxidases have been purified and characterized from turnip (Duarte-Vázquez et al., 2000), papaya fruit (Pandey et al., 2012), the fungus *Coprinus* (Ikehata et al., 2005), or the tree legume *Leucaena leucocephala* (Pandey and Dwivedi, 2011), amongst others. Peroxidases have also been isolated from different grasses such as Guinea grass (*Panicum maximum*) (Centeno et al., 2017), *Miscanthus x giganteus* (Dragana et al., 2017), or wheat grass (*Triticum aestivum*) (Lai et al., 2006).

Wastewater treatment applications of peroxidases do not necessitate highly purified enzymes (Feng et al., 2021), which is why crude enzyme extracts from commodity-scale biomass present a potential alternative. Target pollutants are mainly phenolic compounds, which must be removed from wastewater before it is released into the environment. Phenolic compounds are highly regulated, as many of them can be toxic or even carcinogenic and can accumulate in the food chain. Some major sources of industrial wastewater containing phenolic compounds are petroleum refining, coal refining, the textile and leather industry, and pulp and paper mills, amongst others (Kumaran and Paruchuri, 1997; Nicell et al., 1993). Conventional methods for removing phenolic contaminants from wastewater include distillation, adsorption, extraction, membrane processes, chemical as well as electrochemical oxidation, advanced oxidation processes, and biological treatment (Mohamad Said et al., 2021; Mohd, 2022; Villegas et al., 2016). Kilbanov et al. proposed enzymatic phenol removal as an alternative to bacterial degradation because it is less susceptible to factors such as substrate concentration, pH, temperature, and toxic contaminants (Klibanov et al., 1983). Peroxidases catalyze the formation of free radicals from various aromatic pollutants resulting in their polymerization and aggregation (Klibanov et al., 1983). In most cases, the oxidized products are less toxic and are more likely to be biodegradable compared to the phenolic starting compounds (Torres-Duarte and Vazquez-Duhalt, 2010). Due to the conversion of phenols into insoluble polymers, the products can be separated quite easily (Klibanov et al., 1983). While the advantages of enzymatic treatment are mainly the high transformation rate and high selectivity towards phenolic compounds (Mohamad Said et al., 2021), the enzymes are also highly dependent on the environmental conditions. In the case of oxidative polymerization of phenols, there is also enzyme inactivation by the end-product polymers, which adsorb to the enzymes and block access to the active site (Nakamoto and Machida, 1992).

The cost-effectiveness of enzymatic removal depends mainly on the properties and cost of the enzymes. To further optimize the process, different sources of plant peroxidases were investigated for wastewater treatment. Peroxidases from soybeans (Bódalo et al., 2006; Wright and Nicell, 1999; Steevensz et al., 2014) and turnips (Duarte-Vázquez et al., 2003) in particular have been suggested as promising alternatives to HRP for wastewater treatment. The application of peroxidases from alternative low-purity peroxidase sources was also already demonstrated as shown by Yadav et al. for the conversion of phenolic compounds with gourd (*Luffa aegyptiaca*) fruit juice (Yadav et al., 2017).

The aim of this study was to investigate grass clippings as the main component of green waste as a cheap and readily available source of peroxidases for phenol removal. Following the 12 principles of green chemistry according to Anastas and Warner, the use of enzymes from green waste for wastewater treatment can contribute especially in the form of using renewable raw materials and by using enzymes as "green" catalysts (Anastas and Warner, 2000). With regard to the UN Sustainable Development Goals (SDG), this will contribute in particular to SDG6 "Clean water and sanitation" as well as SDG12 "Responsible consumption and production". By removing pollutants from the environment, a contribution to the achievement of SDG14 "Life below water" and SDG15 "Life on land" can also be expected, while using waste materials for the production of enzymes contributes indirectly to SDG2 "Zero hunger".

2. Materials and methods

2.1. Grass cultivation

To investigate peroxidases from grass clippings, the two common grass species *Lolium perenne* (perennial ryegrass) and *Festuca arundinacea* (tall fescue) were used as a season-independent and reproducible model substrate for the otherwise highly heterogeneous material. Grass seeds were obtained from Feldsaaten Freudenberger GmbH & Co. KG (Krefeld, Germany). The two types of grass were planted separately in planter boxes with a volume of 6 l (40 (L) x 18 (W) x 13.7 (H) cm). COMPO SANA® lawn soil (N 50–300 mg/l, P_2O_5 100–370 mg/l, K_2O 200–500 mg/l,) with a pH value of 5.0–6.5 from COMPO GmbH (Münster, Germany) was used for cultivation. To eliminate potential fungus gnat larvae and other biological contaminants, the soil was treated at 90 °C for 30 min. Before use, the soil was cooled to room temperature. The planter boxes were filled with soil to 2 cm below the rim, the seeds were distributed homogeneously and covered with a 1 cm layer of soil, which was then lightly pressed down. The planter boxes were placed in a KBW 240 growth chamber from BINDER GmbH (Tuttlingen, Germany). An image of the planter boxes in the growth chamber is shown in Fig. S1. The grass was cultivated in a day-night cycle with 20 °C and daylight for 14 h and 15 °C without light for 10 h. The soil was kept moist with dH_2O until the grass was fully grown. After that, the grass was watered once a week with 300 ml dH_2O per planter box. After reaching about 10 cm in length, the grass was cut to about 4 cm each time. The grass samples were stored at −80 °C. In addition, lawn grass was harvested from a semi-shaded meadow in the city of Giessen, Germany (50°35′24" N, 8°40′55″ E) during autumn in 2021.

2.2. Homogenization of grass

The enzymes were released by mechanical cell disruption of the grass clippings. The grass was homogenized with an Ultra-Turrax TP18/2N (20,000 rpm, 75 W, 50 Hz) from Janke & Kunkel KG. (Staufen im Breisgau, Germany). 2 g of grass were homogenized in 20 ml of 50 mM potassium phosphate buffer at the fixed speed of 20,000 rpm for 1 min while being cooled on ice. The homogenized grass was passed through a cotton cloth and wrung out to separate most of the solids. The homogenate was then centrifuged at 3000 ×*g* and 4 °C for 30 min. The supernatant was collected and stored at −80 °C.

2.3. Ammonium sulfate precipitation

Fractionation by precipitation was performed in two steps. In the first step, the crude extract was brought to an ammonium sulfate saturation of 40 % by adding solid ammonium sulfate. The mixtures were incubated at 4 °C for 30 min and then centrifuged at 4 °C and 16,000 ×g for 30 min. The supernatant was collected and the pellet was discarded. In the second step, the supernatant was brought to 80 % saturation by adding solid ammonium sulfate. Again, the mixtures were incubated at 4 °C for 30 min and then centrifuged at 4 °C and 16,000 ×g for 30 min. The supernatant was discarded and the pellet containing the peroxidases was resuspended in 0.5 ml of 50 mM sodium phosphate buffer (pH 8.0) with 2 M $(NH_4)_2SO_4$.

2.4. Hydrophobic interaction chromatography

Chromatography experiments were performed using 50 mM sodium phosphate buffer (pH 8.0) with 2 M $(NH_4)_2SO_4$ as buffer A and 50 mM sodium phosphate buffer (pH 8.0) as buffer B at room temperature and a constant flow rate of 1 ml/min. 1 ml of the precipitation product was loaded onto a HiTrap™ Phenyl HP 1 ml column (GE Healthcare Bio-Sciences AB, Uppsala, Sweden). Before that, the column was equilibrated with 70 % buffer A and 30 % buffer B. After sample application, the column was washed with 8 CV of 30 % buffer B. The elution was

done with 100 % buffer B and fractions showing peroxidase activity were pooled. The peroxidase fractions were stored at −80 °C.

2.5. Desalting and concentration

After hydrophobic interaction chromatography, peroxidase fractions were desalted and concentrated with Amicon® Ultra-4 Ultracel® – 10 K (10 kDa MWCO) Centrifugal Filters (Merck Millipore Ltd., Ireland). The samples were concentrated 8-fold at 3000 ×g and 4 °C (4 ml of sample was concentrated to 0.5 ml) and then diluted 8-fold with 50 mM sodium phosphate buffer (pH 8.0). This was repeated three times to separate most of the salt. Finally, the sample was concentrated 20-fold from 4 ml to 0.2 ml. Desalted and concentrated peroxidase fractions were stored at −80 °C.

2.6. Enzyme activity assay

Peroxidase activity was measured via the oxidation of guaiacol to tetraguaiacol in the presence of H_2O_2. For this purpose, 20 mM guaiacol, 5 mM H_2O_2, and 5 µl enzyme sample were added to 50 mM potassium phosphate buffer (pH 7.0) to a final volume of 700 µl. The temperature was kept constant at 25 °C with a thermostat (Julabo F31-c, JULABO GmbH, Seelbach, Germany). The initial rate of tetraguaiacol production was recorded at 470 nm (Evolution 201 UV–Visible Spectrophotometer, Thermo Scientific, US-Waltham, Massachusetts, USA) as a triplicate. The concentration of tetraguaiacol was determined by the extinction coefficient of 26.6 mM^{-1} cm^{-1} at 470 nm (Maehly and Chance, 1954; Yadav et al., 2017). Enzyme activity is expressed as units, where 1 unit is defined as the formation of 1 µmol of tetraguaiacol per minute.

2.7. Determination of protein concentration

Protein concentration was determined via Bradford assay. A Bradford reagent was prepared from 109 ml 85 % phosphoric acid and 100 mg Coomassie® Brilliant Blue G-250 in 690 ml ddH_2O and stored under light exclusion at 4 °C. For the Bradford assay, 300 µl of Bradford reagent was added to 5 µl of the sample or standard solution. The mixture was incubated for 10 min at room temperature under light exclusion and measured at 595 nm in a plate reader (Infinite® M200 Pro NanoQuant, Tecan Group AG, Männerdorf, Switzerland). A standard curve with bovine serum albumin was used to calculate protein concentration from the measured absorption. All measurements for the determination of the protein concentration were performed in triplicates.

2.8. SDS-PAGE of the purified peroxidases

SDS-PAGE according to Laemmli was performed with the purified peroxidase fractions of both grass species to compare and evaluate the purification. For this, 5 µl of the peroxidase fractions (10 µl reduced sample) were applied to a 4–15 % Mini-PROTEAN TGX Gel (Bio-Rad Laboratories, USA). As molecular weight marker, the Precision Plus Protein™ All Blue Standards (Bio-Rad Laboratories, USA) was used. Protein bands were stained with Coomassie brilliant blue.

2.9. Characterization of the purified peroxidases

The temperature and pH optimum were determined for the peroxidase fractions of both types of grass. The determination was performed analogously to the enzyme activity assay. For the determination of optimal temperature, 20 mM guaiacol, 5 mM H_2O_2, and 5 µl enzyme sample were added to 50 mM potassium phosphate buffer (pH 7.0) to a final volume of 700 µl. The temperature was varied from 15 to 60 °C in increments of 5 °C. For the determination of optimal pH value, 20 mM guaiacol, 5 mM H_2O_2, and 5 µl enzyme sample were added to 50 mM sodium citrate buffer for the pH range from 3.0 to 5.5, 50 mM potassium phosphate buffer for the pH range from 6.0 to 7.5, or 50 mM TRIS buffer for the pH range from 8.0 to 9.0 to a final volume of 700 µl. The pH was varied in increments of 0.5 while keeping the temperature constant at 25 °C. The determination of the kinetics parameters was performed at the previously determined optimal temperatures and pH values. The initial reaction rate of the peroxidases was measured at different guaiacol concentrations. All other parameters were kept analogous to the enzyme activity assay. All measurements for the determination of the optimal reaction conditions and the determination of the kinetics parameters were performed in triplicates.

2.10. Conversion of phenolic compounds

To investigate the suitability of the grass peroxidases for wastewater treatment, the purified peroxidase fractions were used for the conversion of the phenolic compounds phenol, *m*-cresol, and 2,4-dichlorophenol. 20 mM stock solutions were prepared for the phenolic compounds in 50 mM potassium phosphate buffer (pH 6.0). 0.5 mM phenolic substrate, 2.5 mM H_2O_2, and 50 µl of the enzyme sample were added to 50 mM potassium phosphate buffer (pH 6.0) to a final volume of 1400 µl. The mixture was incubated on a shaker at 800 rpm and 30 °C and samples of 50 µl were taken every 5 min for 1 h. To stop the reaction, 10 µl of a 2860 U/ml catalase solution was added to the sample. The conversion of phenolic compounds was performed in triplicates in each case.

To optimize the conversion of the phenolic compounds, polyethylene glycol (PEG) was added to the reaction batch in some experiments. For this purpose, a stock solution of 1 g/l PEG with an average molecular weight of 20,000 g/mol was prepared in the reaction buffer. 100 mg/l PEG was added to the reaction batch.

The concentration of phenolic compounds was determined photometrically (Wu et al., 1997). For this purpose, two reagents, 83.4 mM $K_3Fe(CN)_6$ in 0.25 M $NaHCO_3$ (pH 10.0) and 20.8 mM 4-aminoantipyrine (AAP) in 0.25 M $NaHCO_3$ (pH 10.0), were prepared. The sample/catalase mixture (60 µl) was mixed with 100 µl of each of the two reagents and 740 µl of ddH_2O. The absorbance was measured at 510 nm against the reagent blank (without sample).

In addition to the purified peroxidase fractions, crude extracts of the grasses were prepared for phenol conversion. As described above in the chapter "2.2 Homogenization", crude extracts of *L. perenne* and *F. arundinacea* were prepared by homogenization, filtration, and centrifugation. In addition, a crude extract of grass from the "real" lawn grass was prepared in the same way. The crude extracts were stored at −80 °C until use.

3. Results and discussion

3.1. Purification of peroxidases from Lolium perenne and Festuca arundinacea

A purification scheme was developed for the peroxidases from the two grass species *Lolium perenne* and *Festuca arundinacea*, which could be applied equally to both types of grass. The use of individual grass species as a model substrate is especially important to obtain reproducible, season-independent results on the otherwise highly heterogeneous material. The two types of grass were incubated in a growth chamber in the laboratory and harvested regularly (see Fig. S1). After the homogenization of the grass samples and separation of the solids, the peroxidases were fractionated with ammonium sulfate in two steps. Subsequently, the peroxidases were further purified by hydrophobic interaction chromatography (HIC) and finally simultaneously desalted and concentrated. In addition to the final concentration step, the peroxidases were already concentrated by the second precipitation step, as the precipitate was dissolved in a smaller volume than before. In contrast, the samples were diluted by the HIC. A compromise of optimal conditions for both grass species was selected for the purification. Fortunately, the peroxidase fractions of the two model types of grass showed similar properties, enabling a common purification process. By optimizing the

process steps to the individual grasses, it is most likely that higher purification can be achieved. However, the purification process should be universally applicable to real grass clippings. To evaluate the quality of the purification, the specific activity was determined from the protein concentration and the enzyme activity. Table 1 shows the resulting total protein concentration, total activity, and specific activity after each purification step. The crude extract of *F. arundinacea* showed a three times higher specific activity compared to L. *perenne*. A similar ratio was also observed after the entire purification process. Based on the increase in specific activity, 3.8-fold purification was obtained for peroxidases from L. *perenne*. In comparison, a 2.8-fold purification could be achieved for *F. arundinacea*. An SDS gel showed three major bands at approximately 27, 37, and 55 kD in the peroxidase fractions of both species in addition to minor impurities (see Fig. S2). According to UniProt, *L. perenne* has a peroxidase with a mass of 36.5 kD (entry A2BCZ0). Thus, it can be assumed that the band around 37 kD is a peroxidase. For *F. arundinacea* no information exists in this regard. The lower protein concentration of the sample from *F. arundinacea* resulted in weaker bands when the same sample volume was applied. The similar band pattern further demonstrates the similarity of the enzyme fractions of both types of grass. A comparable crude extract from horseradish showed a specific activity of 2.65 U/mg (Lavery et al., 2010). With values of 3.7 and 12.3 U/mg the specific activity of the grasses after homogenization is in a comparable range. However, after extensive purification, a much higher specific activity of 772 U/mg was obtained for the highly purified HRP (Lavery et al., 2010).

3.2. Characterization of the purified peroxidases

The purified peroxidase fractions were compared with respect to their optimal reaction conditions, mainly temperature and pH. Fig. 1 shows the relative specific activity of the peroxidase fractions of *F. arundinacea* and L. *perenne* as a function of temperature and pH. The peroxidases from both types of grass exhibited an apparent temperature optimum around 30 °C with maximum specific activities of 17.7 U/mg for L. *perenne* and 36.0 U/mg for *F. arundinacea*. Regarding pH, the peroxidases from L. *perenne* displayed the maximal specific activity of approximately 17.0 U/mg in the range from pH 5.0 to pH 6.0, while the peroxidases from *F. arundinacea* showed a clear maximum of 40.1 U/mg at pH 6.0. Both grass species peroxidases share a pH optimum at pH 6.0. Pandey et al. summarized the properties of a variety of plant-derived peroxidases (Pandey et al., 2017). In most cases, the pH optimum is in the slightly acidic range around pH 5 to 6. Thus, the optima of grass peroxidases fit well within this range. The optimal temperature of most peroxidases, on the other hand, is somewhat higher than the grass peroxidases with temperatures around 40 °C. However, this is more of an advantage for the grass peroxidases, as they require a lower temperature and thereby lower energy input with an optimum of around 30 °C.

With the optimal reaction parameters of 30 °C and a pH of 6.0, experiments were conducted with different guaiacol concentrations to determine kinetics parameters according to Michaelis and Menten (see Fig. S3). For the peroxidase fraction of *F. arundinacea*, this results in a maximum reaction rate V_{max} of 41.4 μmol min^{-1} mg^{-1} and a Km value of 5.3 mM. The peroxidase fraction of L. *perenne* shows a lower maximum reaction rate V_{max} of 25.8 μmol min^{-1} mg^{-1} and a lower substrate affinity with a Km value of 7.5 mM. Most Km values of plant peroxidases for guaiacol range from about 0.3 mM to 9.5 mM (Pandey et al., 2017).

3.3. Application of grass peroxidases for the conversion of phenolic compounds

3.3.1. Conversion of phenolic compounds by purified peroxidase fractions

In the first step, the purified peroxidases from the two types of grass were investigated for the conversion of the common phenolic pollutants phenol, *m*-cresol, and 2,4-dichlorophenol. All reactions were carried out at the previously determined reaction conditions of 30 °C and pH of 6.0. In addition, it was investigated whether the conversion of the substrates could be improved by the addition of 100 mg/l PEG. It has been previously shown that 100 mg/l PEG is a sufficient dose to enhance the conversion of different phenolic compounds by protecting the peroxidases from inactivation (Quintanilla-Guerrero et al., 2008; Wu et al., 1997). In general, PEG with a higher molecular weight causes a stronger enhancement of phenol conversion (Kinsley and Nicell, 2000; Nakamoto and Machida, 1992), which is why a molecular weight of 20,000 was chosen for the PEG in our experiments.

Fig. 2 shows the removal of phenolic compounds by the peroxidase fractions of the two types of grass with and without the addition of 100 mg/l PEG. After one hour, over 70 % of phenol, over 80 % of *m*-cresol, and over 95 % of 2,4-dichlorophenol were converted by the purified peroxidases of *Lolium perenne*. After the same time, almost 70 % of phenol, almost 85 % of *m*-cresol, and over 95 % of 2,4-dichlorophenol were converted by the crude extract of *Festuca arundinacea*. Thus, the degree of conversion after one hour was similar with both extracts, although the fractions show clear differences in specific activity towards guaiacol. Accordingly, the enzyme activity applied in the experiment was 1.04 U/ml for L. *perenne* and 1.55 U/ml for *F. arundinacea*. However, this higher activity becomes apparent after the first 5 min of the reaction. The peroxidases from L. *perenne* converted about 35 % of the phenol and about 45 % of the *m*-cresol after 5 min, while the peroxidases from *F. arundinacea* had already converted more than 45 % of the phenol and about 55 % of the *m*-cresol during the period. In the case of 2,4-dichlorophenol, a conversion of over 95 % was already achieved after 5 min in both cases. The addition of 100 mg/l PEG increased both, the initial reaction rate and conversion of phenol and *m*-cresol after 1 h. Due to the already rapid conversion of 2,4-dichlorophenol, no improvement could be observed by the addition of PEG for the substrate. In the case of phenol and *m*-cresol, about 10 to 15 % were additionally converted after one hour by both grass extracts in the presence of PEG. In the first 5 min, the conversion could also be increased by approximately another 5 to 20 % depending on substrate and grass species. For a more accurate comparison of the conversion, a quotient was formed from converted phenolic compounds after 1 h and enzyme activity used. A higher value indicates a more efficient conversion of the phenolic compounds. For the substrates phenol, *m*-cresol, and 2,4-dichlorophenol, values of 0.24,

Table 1
Degree of peroxidase purification from *Lolium perenne* and *Festuca arundinacea*.

Purification step	Total volume [ml]		Protein concentration [mg ml^{-1}]		Total activity [U]		Specific activity [U $mg^{-1}_{protein}$]	
	Lolium perenne	*Festuca arundinacea*	*Lolium perenne*	*Festuca arundinacea*	*Lolium perenne*	*Festuca arundinacea*	*Lolium perenne*	*Festuca arundinacea*
Homogenization	310.0	298.0	0.400	0.187	466.1	685.3	3.756	12.312
Precipitation (40 %)	339.0	328.0	0.172	0.132	351.3	614.8	6.025	14.249
Precipitation (80 %)	8.5	8.2	3.922	2.329	284.9	424.5	8.570	22.230
HIC	118.7	114.8	0.174	0.119	207.6	295.6	10.050	21.668
Desalting and concentration	5.9	5.7	2.033	1.265	172.6	248.4	14.313	34.205

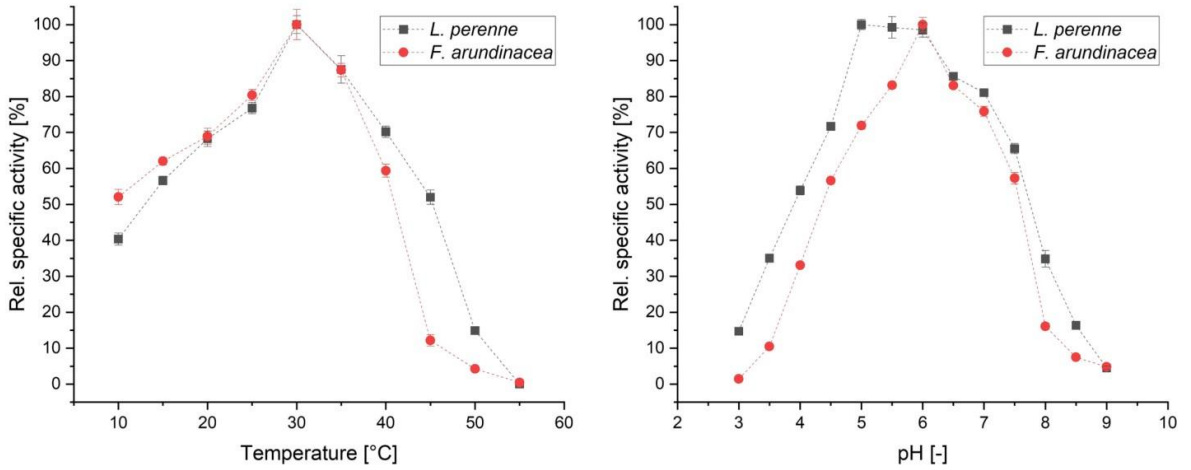

Fig. 1. Relative specific activity of the purified peroxidase fractions from *Lolium perenne* and *Festuca arundinacea* as a function of temperature (left) and pH (right). Maximum specific activity at optimal temperature is 17.7 U/mg for L. *perenne* and 36.0 U/mg for *F. arundinacea*, while at optimal pH it is 17.0 U/mg for *L. perenne* and 40.1 U/mg for *F. arundinacea*. All reactions were carried out with 20 mM guaiacol and 5 mM H_2O_2. For the determination of the optimum temperature, the pH was kept constant at 7.0, while the temperature was kept constant at 25 °C for the determination of the optimum pH ($n = 3$).

0.28, and 0.33 mM/U were obtained for L. *perenne* and 0.16, 0.19, and 0.22 mM/U for *F. arundinacea*, respectively. Thus, for the enzyme activity used, *L. perenne* was more effective. A possible explanation could be that the preparations contain different amounts of contaminants, which exhibit radical scavenging activity. By adding PEG, these values can be slightly increased to 0.28, 0.31, and 0.34 mM/U for *L. perenne* and 0.20, 0.22, and 0.23 mM/U for *F. arundinacea*, respectively.

Since the composition of the reaction mixture, especially enzyme and substrate concentration, as well as reaction conditions are different in each publication, an accurate comparison is difficult. For example, it was shown that 9.5 U/ml HRP can convert 5.2 mM 2,4-dichlorophenol in wastewater within 5 to 10 min under optimal conditions (Dec and Bollag, 1994). Wagner and Nicell demonstrated the removal of at least 95 % of 1 mM phenol and more than 96 % of 0.6 mM 2,4-dichlorophenol by 4.5 U/ml HRP after 3 h (Wagner and Nicell, 2002). The different efficiencies of removal of each substrate in our experiments may also be due to varying reaction optima. Caza et al. have shown that optimal pH values vary depending on the phenolic substrate (Caza et al., 1999). In the case of soybean peroxidase, the optimum pH for phenol is 6.0, while for both *m*-cresol and 2,4-dichlorophenol, it is 7.0. Interestingly, soybean peroxidase shows a similar affinity for the three substrates studied as in our case. Phenol requires the highest enzyme dose at 0.90 U/ml, followed by *m*-cresol at 0.75 U/ml and by a considerable margin 2,4-dichlorophenol at 0.08 U/ml the least (1 mM of each substrate) (Caza et al., 1999). Akhtar and Husain isolated peroxidase from bitter gourd (*Momordica charantia*) and applied the soluble and immobilized enzyme in the treatment of single phenolic compounds and model wastewaters (Akhtar and Husain, 2006). Contrary to the results with the grass peroxidases, the soluble and immobilized bitter gourd peroxidase showed the highest conversion towards phenol, followed by 2,4-dichlorophenol and *m*-cresol. However, a pH of 6 has also proven to be optimal with respect to all substrates, whereas the optimal temperature was again 40 °C as described previously.

3.3.2. Conversion of phenolic compounds by crude grass extracts

For the detailed characterization of the peroxidases, the enzymes had to be purified. However, this is probably too expensive for a real application. Dec and Bollag were the first to show that raw plant material can also be used for the conversion of phenols (Dec and Bollag, 1994). We wanted to test the conversion of phenolic compounds with a crude grass homogenate. The crude extracts of L. *perenne* and *F. arundinacea* exhibited a volumetric activity for guaiacol of 1315 U/l and 1375 U/l, respectively. This results in an activity of 9.9 U per gram of grass for L. *perenne* and 10.3 U per gram of grass for *F. arundinacea*. The conversion of the phenolic compounds with the crude extracts was carried out in the same way as in the experiments before. Since the positive effect of adding PEG was shown in the experiments with the purified peroxidases, PEG was also added in the experiments with the crude extracts. The reduction in reaction time as well as the increase in residual enzyme activity by the addition of PEG has already been confirmed for wastewater treatment with crude extracts from plant sources (Diao et al., 2011; Duarte-Vázquez et al., 2003). Fig. 3 shows the removal of phenolic compounds by the crude extracts of the two types of grass in the presence of 100 mg/l PEG. After one hour, approx. 10 % of phenol, 20 % of *m*-cresol, and almost 80 % of 2,4-dichlorophenol were converted by the crude extract of L. *perenne*. After the same time, approx. 10 % of phenol, over 15 % of *m*-cresol, and almost 75 % of 2,4-dichlorophenol were converted by the crude extract of *F. arundinacea*. Thus, in contrast to the purified peroxidases, the conversion of all substrates with both types of grass was poorer probably due to the lower volumetric activity of the crude extracts. Overall, the conversion by both crude extracts was very similar, as confirmed by the similar specific activity towards guaiacol. The results show that the peroxidases of the two types of grass do not differ significantly in their affinity towards the substrates. Instead, the rate and extent of conversion depended on the enzyme concentration indicated by the specific activity.

Again, a quotient of the converted phenolic compound and enzyme used was formed. The applied enzyme activities of 0.047 U/ml and 0.049 U/ml for L. *perenne* and *F. arundinacea*, respectively, were about 20- to 30-fold lower than that of the purified peroxidases. For the substrates phenol, *m*-cresol, and 2,4-dichlorophenol, values of 0.83, 1.71, and 6.01 mM/U were obtained for L. *perenne* and 0.61, 1.15, and 5.42 mM/U for *F. arundinacea*, respectively. As before with the purified peroxidases, the quotients of both grass species are similar due to similar enzyme activity used. The quotient should not be used as the only evaluation criterion, since higher quotients are generated here despite more incomplete conversion compared to the purified peroxidases.

Finally, we also wanted to test the conversion of phenolic compounds with "real" grass from a lawn in the next step. We prepared a crude extract exactly as described above. The volume activity of the homogenate from the lawn grass was 4080 U/l. Furthermore, the crude extract of the lawn grass showed an activity of 30.6 U per gram of grass. Again,

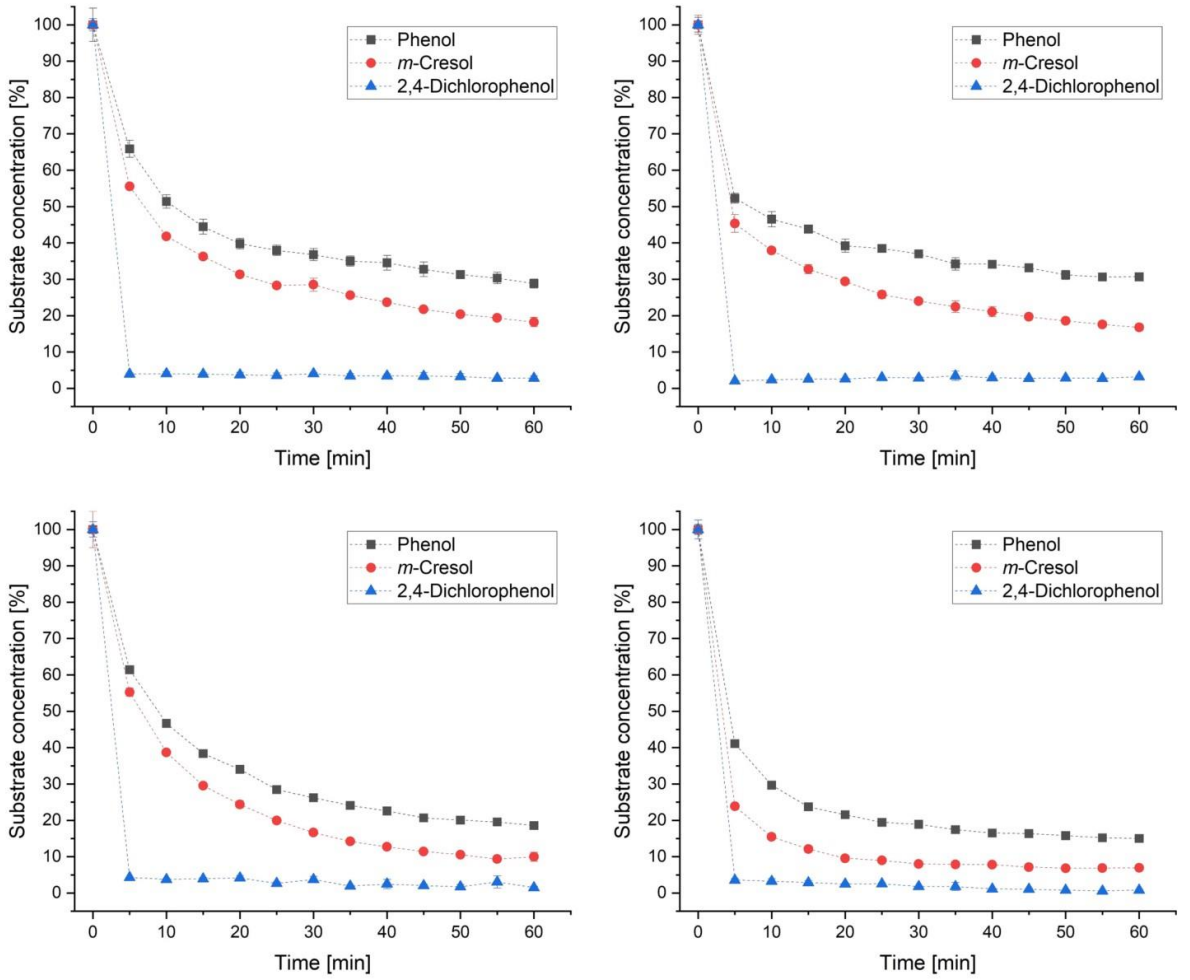

Fig. 2. Removal of the phenolic compounds phenol, *m*-cresol, and 2,4-dichlorophenol (each 0.5 mM) by purified peroxidase fractions of the cultivated grasses *Lolium perenne* (left) and *Festuca arundinacea* (right) without (top) and with the addition of 100 mg/l PEG (bottom) resulting in an applied enzyme activity of 1.039 U/ml for *Lolium perenne* and 1.546 U/ml for *Festuca arundinacea*. All reactions were carried out with 5 mM H_2O_2 at 30 °C and 800 rpm in a 50 mM potassium phosphate buffer with a pH of 6.0 (n = 3).

the conversion of the phenolic compounds with the crude extract was carried out in the same way as before. With 0.146 U/ml, the enzyme activity used was approx. three times higher in comparison to the laboratory grown grasses. Fig. 4 shows the removal of phenolic compounds by the crude extract of lawn grass with the addition of 100 mg/l PEG. After one hour, approx. 20 % of phenol, 40 % of *m*-cresol, and more than 95 % of 2,4-dichlorophenol were converted by the crude extract of lawn grass. Here, all three substrates were converted faster than with the crude extracts of the two types of grass grown in the laboratory. This may be attributed to the approx. three times higher volume activity of the lawn grass extract in comparison to the crude extracts of L. *perenne* and *F. arundinacea*. Again, *m*-cresol was converted somewhat faster than phenol, and 2,4-dichlorophenol was again clearly converted fastest. The quotients of phenol removal and enzyme activity used were 0.52, 1.03, and 2.38 mM/U for the substrates phenol, *m*-cresol, and 2,4-dichlorophenol, respectively. Similar values have been obtained for the other crude extracts. These results confirm that the conversion of phenolic compounds with peroxidases from grasses also works with real raw materials.

It was shown before that crude plant material shows similar results to isolated peroxidases in terms of phenol conversion (Dec and Bollag, 1994). Logically, high costs can be saved due to the low purification. Cooper and Nicell compared the removal of phenols from foundry wastewater using purified HRP and a crude HRP extract (Cooper and Nicell, 1996). In both cases, with the purified enzyme and with the crude extract, more than 95 % of the 3.5 mM phenol mixture was removed from wastewater. Duarte-Vázquez et al. produced a crude extract from turnip for the removal of phenolic compounds (Duarte-Vázquez et al., 2003). They were able to remove over 95 % of phenol, *m*-cresol, and 2,4-dichlorophenol with the addition of PEG within 10 min. Interestingly, they found decreased removal efficiency with increasing concentration of phenolic compounds for all compounds studied except 2,4-dichlorophenol. A similar effect can be seen here, in which the conversion of 2,4-dichlorophenol was hardly affected by the changed enzyme concentration in comparison to phenol and *m*-cresol. Yadav et al. demonstrated the use of gourd (*Luffa aegyptiaca*) fruit juice as a low-purity peroxidase source for the removal of guaiacol, phenol, and *m*-cresol amongst other substrates (Yadav et al., 2017). According to the Km

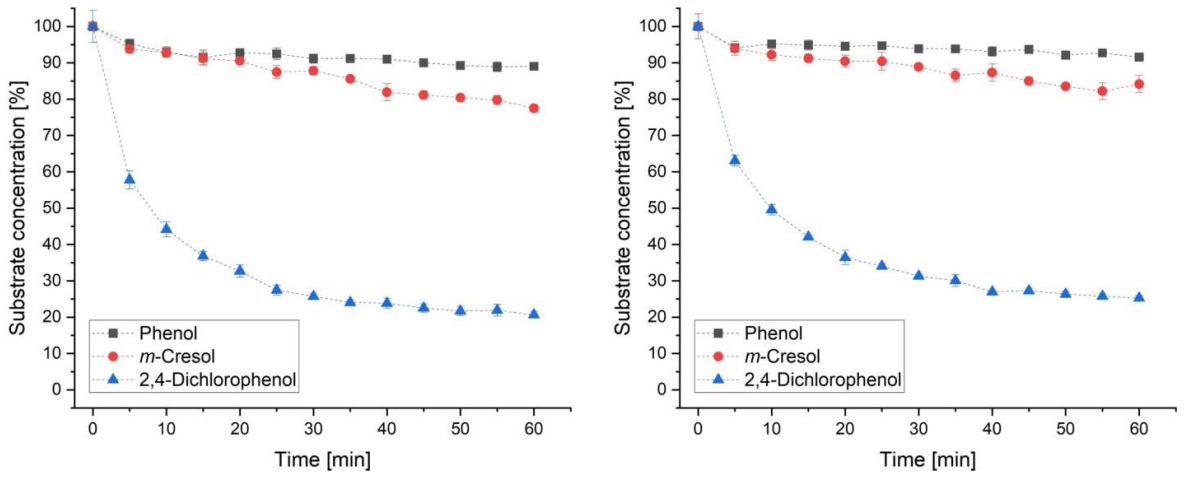

Fig. 3. Removal of the phenolic compounds phenol, *m*-cresol, and 2,4-dichlorophenol (each 0.5 mM) by crude extract of the cultivated grasses *Lolium perenne* (left) and *Festuca arundinacea* (right) with the addition of 100 mg/l PEG resulting in an applied enzyme activity of 0.047 U/ml and 0.049 U/ml, respectively. All reactions were carried out with 5 mM H_2O_2 at 30 °C and 800 rpm in a 50 mM potassium phosphate buffer with a pH of 6.0 (n = 3).

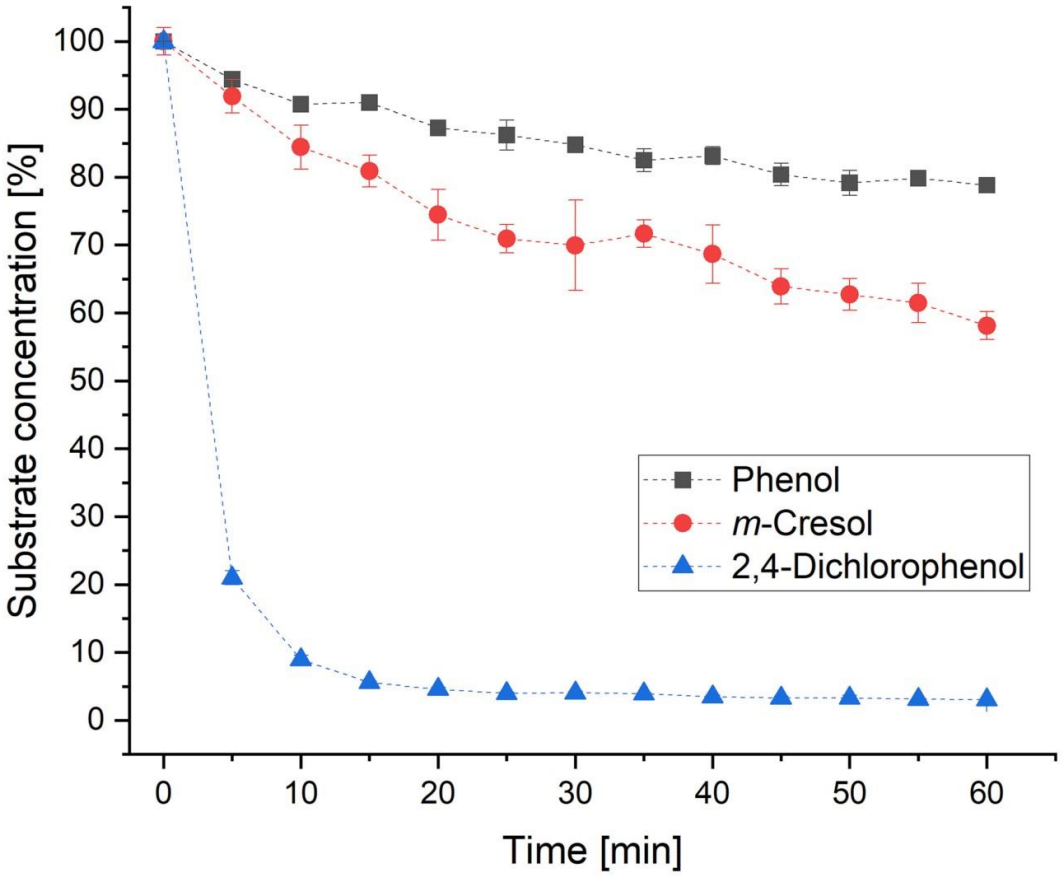

Fig. 4. Removal of the phenolic compounds phenol, *m*-cresol, and 2,4-dichlorophenol (each 0.5 mM) by crude extract of lawn grass with the addition of 100 mg/l PEG resulting in an applied enzyme activity of 0.146 U/ml. All reactions were carried out with 5 mM H_2O_2 at 30 °C and 800 rpm in a 50 mM potassium phosphate buffer with a pH of 6.0 (n = 3).

values, the fruit juice peroxidases have a higher affinity for phenol, rather than *m*-cresol in contrast to the grass peroxidases. Again, purity did not affect the effectiveness of enzymatic conversion. Kurnik et al. demonstrated the phenol removal with potato pulp peroxidases (Kurnik et al., 2015). They were able to achieve a phenol removal efficiency of over 95 % for up to 2 mM phenol after 2 h from synthetic wastewater with the potato pulp. With industrial effluent with much lower phenol concentrations of 0.02 to 0.1 mM, over 80 % of the phenol was removed by the addition of the potato pulp even without the addition of H_2O_2. This was explained by the adsorption of phenol to the pulp solids, as was also previously described for other crude plant materials (Duarte-Vázquez et al., 2003; Dec and Bollag, 1994). In the future, the efficiency of phenol removal with grass peroxidases should be compared to conventional peroxidases like HRP to help assess the performance of the crude extract from grass clippings.

In general, the use of a crude extract is the more economical option for the conversion of phenolic compounds. However, the removal of phenolic compounds can be further optimized by selecting the optimal parameters for the conversion of the respective substrates. Optimization of these parameters such as enzyme concentration, pH, H_2O_2 to substrate ratio, and PEG concentration was demonstrated by Caza et al. using soybean peroxidase (Caza et al., 1999). During our experiments, we observed that the removal of phenolic compounds is progressively worse at higher initial substrate concentrations, while the conversion was increased when a larger volume of enzyme extract was added to the reaction batch. Caza et al. showed that the degree of phenol conversion is strongly dependent on the enzyme concentration used (Caza et al., 1999). Quintanilla-Guerrero et al. have also confirmed that the removal efficiency decreases with increasing concentration of phenolic compounds (Quintanilla-Guerrero et al., 2008). Duarte-Vázquez et al. concentrated the crude extract from turnip roots 10-fold before use (Duarte-Vázquez et al., 2003). In the future, a concentration step should follow in the preparation of the crude extract to achieve higher enzyme concentrations. Additionally, the amount of enzyme required can be reduced by the addition of PEG (Caza et al., 1999; Cooper and Nicell, 1996; Wagner and Nicell, 2001). Furthermore, the optimal pH values and temperatures must be determined for the enzyme specific to the phenolic substrates. For example, the optimal conditions of the gourd fruit juice for the conversion of phenol were 28 °C and pH 7.0, while for the conversion of *m*-cresol, the optimum conditions were 35 °C and pH 6.0 (Yadav et al., 2017). It is promising that the two grass species show an equal pH and temperature optimum as far as the industrial use of the enzyme extract from grass mixtures is concerned. The substrate/H_2O_2 ratio can also be improved since it can have a great influence on the conversion as it has already been shown in numerous publications (Akhtar and Husain, 2006; Caza et al., 1999; Dahili et al., 2015; Wang et al., 2015; Wu et al., 1997). Wagner and Nicell were able to save 20 % of the enzyme by optimizing the H_2O_2 concentration (Wagner and Nicell, 2001). Hydrogen peroxide-mediated inactivation of peroxidases can be minimized by enzyme immobilization or *in situ* generation of H_2O_2 (Burek et al., 2019). Immobilization of enzymes is commonly used to improve their performance or properties (Quintanilla-Guerrero et al., 2008). Dahili et al. demonstrated the immobilization of peroxidase from a crude extract of horseradish (Dahili et al., 2015). However, enzymes in crude plant extracts may have a higher stability due to the fact that they are already immobilized in the plant material (Dec and Bollag, 1994).

Furthermore, there may be other phenolic substrates such as 2,4-dichlorophenol that are effectively converted by the peroxidases from grass clippings. It has been demonstrated that HRP can also be used for the removal of azo dyes (Mohan et al., 2005) or endocrine-disrupting compounds (Auriol et al., 2007). Using peroxidases from the perennial grass *Miscanthus x giganteus*, Dragana et al. have already demonstrated the degradation of dyes with a type of *Poaceae* (Dragana et al., 2017). In addition, the application with a model or real industrial wastewater has to be tested further on. Garg et al. summarized and compared different examples of phenol removal from industrial wastewater using various crude and purified plant peroxidases (Garg et al., 2020).

Apart from considering the enzymatic process, the acquisition and condition of the raw material should not be neglected. Several challenges still exist that make it difficult to effectively process green waste. When collecting green waste from public areas, contamination by waste is unavoidable and must be separated beforehand. Another major problem is the strong heterogeneity of green waste. The composition of green waste varies greatly depending on location and season. Even when harvesting the grasses planted in the laboratory, which consist of only one plant species, differences in peroxidase activity and protein concentration could be detected depending on when the grass samples were cut. This demonstrates the challenges of using heterogeneous plant feedstocks. For these reasons, the robustness of the process should also be studied depending on the characteristics of the raw material.

4. Conclusions

Peroxidases from grass clippings show potential for application in wastewater treatment. In our opinion, the enzymes need to be used as a crude extract because intensive purification would again entail high costs. These crude extracts can be used as a "green" supplementary part of peroxidases for the conversion of phenolic compounds since the plant material probably cannot meet the demand for peroxidases alone. Finally, the extraction of industrially relevant enzymes can contribute to a higher added value of green waste. However, a holistic ecological and economic utilization to close the material cycle as far as possible can only be realized by a cascade-like green waste-biorefinery.

CRediT authorship contribution statement

Alexander Langsdorf: Conceptualization, Methodology, Validation, Investigation, Writing – original draft, Visualization. **Marianne Volkmar:** Conceptualization, Methodology, Writing – review & editing. **Roland Ulber:** Conceptualization, Methodology, Writing – review & editing, Funding acquisition. **Frank Hollmann:** Conceptualization, Methodology, Writing – review & editing. **Dirk Holtmann:** Conceptualization, Methodology, Resources, Writing – review & editing, Supervision, Project administration, Funding acquisition.

Declaration of competing interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

Data availability

Data will be made available on request.

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Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.biteb.2023.101471.

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4.4 Electrodes from carbonized grass clippings for bioelectrochemical systems

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Electrodes from carbonized grass clippings for bioelectrochemical systems

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ABSTRACT

One major obstacle to the commercialization of electrobiotechnological systems is the cost of materials, including expensive electrodes. Smart recycling as well as the use of renewable resources can contribute to producing electrodes more ecologically and economically. Green waste is a biogenic residual material that occurs mainly in urban areas and is currently not recycled to a sufficient extent. Here we show the fabrication of electrodes from carbonized grass clippings and their application in microbial electrosynthesis as well as microbial fuel cells. While the electrodes cannot compete with established metal competitors for water electrolysis in microbial electrosynthesis, they perform comparably to commercial graphite electrodes in microbial fuel cells. With *Geobacter sulfurreducens*, a current response can be recorded for more than six weeks. To the best of our knowledge, this is the first time that carbonized green waste has been used as an electrode material for bioelectrochemical systems. This demonstrates the potential of carbonized biological materials as a raw material for electrodes and presents a recycling alternative for green waste.

1. Introduction

Electrobiotechnology is an emerging field that offers new opportunities in industry by combining electrochemical and biotechnological processes. By combining biotechnology with electrochemistry, bioprocesses can be intensified (Stöckl et al., 2023). Thanks to the new opportunities that are emerging, electrobiotechnology can substantially contribute to the realization of the UN Sustainable Development Goals (Gizewski et al., 2023). Bioelectrochemical systems use electroactive microorganisms capable of generating electric current or accepting electrons, for example, to convert CO_2 (Logan et al., 2019). Two of the most promising technologies are the microbial fuel cell and microbial electrosynthesis.

In the process of microbial electrosynthesis (MES), microorganisms can accept electrons from electrodes via different electron transfer mechanisms and use them as an energy source to produce basic chemicals. This allows the energy industry and the chemical or biotechnological industry to be linked. From various options for the MES, the use of secondary microbial electrochemical technology, meaning the integration of microbial conversions into electrosynthesis, is the most technically advanced, along with hybrid systems (Fruehauf et al., 2020). Indirect electron transport from electrodes to microorganisms can take place via hydrogen generated by water electrolysis. *Cupriavidus necator* is one such organism that is able to grow autotrophically by utilizing H_2 as electron donor, O_2 as electron acceptor, and CO_2 as carbon source. For example, we have shown that we could produce the sesquiterpene α-humulene via microbial electrosynthesis using a genetically modified *C. necator* strain (Krieg et al., 2018) or use Kolbe electrolysis waste gases for the synthesis of fuels and solvents (Teetz et al., 2022). By using exhaust gas CO_2 and renewable electrical energy, a green process can be created. However, for MES to be economically efficient, investment and operational costs must be reduced.

Microbial fuel cells (MFC) can be used in wastewater treatment plants to generate electricity using electroactive microorganisms to transfer electrons to electrodes by converting organic substances. In such real applications, mixed microbial cultures are usually present. In mixed cultures from different sources, *Geobacter sulfurreducens* is substantially responsible for the performance of the MFC (G. Sun et al.,

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2016). Therefore, the model organism *G. sulfurreducens* is often used in the MFC in laboratory experiments. It was shown that *G. sulfurreducens* is able to use graphite electrodes as the electron acceptor, allowing the production of electrical current through the oxidation of acetate (Bond and Lovley, 2003; Stöckl et al., 2019). The organism uses pili to form a biofilm on the electrode (Reguera et al., 2007). These also serve as electrical nanowires for direct electron transfer from the cell to the electrode surface (Reguera et al., 2005). As a result, the anode has a decisive influence on the performance of the MFC. The technical requirements for the individual components of a bioelectrochemical system including the electrodes are described in detail in the review article by Krieg et al. (Krieg et al., 2014). As described, there are different electron transfer mechanisms depending on the technology. Accordingly, the requirements for the electrodes vary depending on the bioelectrochemical system and the microorganisms used (*e.g.* biofilm formers or indirect electron transfer).

Carbon materials such as graphite have long been used as electrodes. The mining and production of graphite is an energy-intensive, time-consuming, and therefore expensive process (Jara et al., 2019). While graphite electrodes are traditionally made from fossil resources, biomass can serve as an alternative raw material for graphite electrode production. Biomass can be carbonized to obtain a biochar with a high graphite content (Zhang et al., 2021). The use of electrodes made from renewable raw materials can help to reduce investment and operating costs and can also have ecological advantages (Yang and Chen, 2020).

Many publications can be found on the hydrothermal carbonization of lignocellulosic waste biomass for different applications. However, the relatively low temperatures lead to a low degree of graphitization resulting in biochar with poor conductivity (Yang and Chen, 2020). Instead, additional pyrolysis of the biomass at higher temperatures is required. The production of biochar by pyrolysis has already been demonstrated from a variety of different lignocellulosic biomasses such as palm leaves (Ferreira et al., 2018), brewers' spent grain (Cancelliere et al., 2019), peanut shells (Zhan et al., 2021), *Cotinus coggygria* flowers (Li et al., 2020), maple wood (Zhang et al., 2014), vineyard residues (V. Hoffmann et al., 2019), or corncob (V. Hoffmann et al., 2019). In many cases, biochars have been investigated as an electrode material for supercapacitors e. g. from pyrolyzed peanut shells (Purkait et al., 2017), pyrolyzed fungi (Zhu et al., 2011), or pyrolyzed corn straw (Qiu et al., 2018). However, the application in bioelectrochemical systems requires additional prerequisites such as biocompatibility. Two excellent review articles on the topic of biochar electrodes for microbial fuel cells have been published by Yang and Chen (Yang and Chen, 2020) and by Chakraborty et al. (Chakraborty et al., 2020). In this case, the biochar may even have beneficial properties. The natural structure of plant materials can be exploited as demonstrated by Chen et al. using carbonized *Hibiscus cannabinus* as anode material for the MFC (Chen et al., 2012).

A lignocellulosic material that has not yet been adequately studied and utilized is green waste. Green waste is biogenic waste that is primarily generated in urban areas consisting mainly of grasses, leaves, and small branches. The material is usually composted or used in biogas plants, which is why the biomass is not sufficiently utilized. We have summarized the properties of green waste and possible material recycling methods in a review article (Langsdorf et al., 2021). Previously, grasses have been carbonized and investigated as electrode materials such as big bluestem for use in supercapacitors (Jin et al., 2014). Kabir et al. have demonstrated pyrolysis of real municipal green waste for the production of bio-oil, biochar, and syngas (Kabir et al., 2015). However, the production of biochar by pyrolysis from green waste as electrode material for bioelectrochemical systems has not yet been investigated. The use of carbonized green waste as electrode material in bioelectrochemical systems offers an opportunity to make the recycling of green waste more meaningful in the future. At the same time, the use of waste as electrode material can make the operation of bioelectrochemical systems more sustainable and cost-effective.

In this work, we aim to produce biochar from grass clippings as the main component of green waste for use as electrode material for bioelectrochemical systems. The production of biochar from green waste with the aim of using it as an electrode material has so far been insufficiently investigated. After carbonization, we demonstrate the production and characterization of electrodes before investigating their suitability for microbial electrosynthesis and microbial fuel cells. To the best of our knowledge, this is the first time that carbonized green waste has been used as an electrode material for bioelectrochemical systems.

2. Materials and methods

2.1. Carbonization of grass clippings

When selecting the raw material, care was taken to use a homogeneous and contamination-free starting material from one source in order to minimize any influences on the biochar characteristics. For this reason, grass clippings as the main component of green waste were used as a model substrate. The grass clippings used were sports and play turf, consisting of German pasture grass (*Lolium perenne*), regenerated with meadow grass (*Poa pratensis*). The grass clippings were harvested on the 4th of May 2020 from the VfB Zwenkau 02 e.V. sports field (Eythraer Weg 2, 04442 Zwenkau, Saxony, Germany; coordinates: 51°13′05.1″N 12°19′03.5″E) and then stored at -18 °C. To remove potential contaminants, the grass was first washed with water before being compacted for carbonization. In the first step, the grass was hydrothermally carbonized for 17 h at 250 °C and up to 55 bar pressure under a nitrogen atmosphere in an LA 500 Autoclave (VEB MLW Medizintechnik, Leipzig, Germany). The ratio of grass dry matter to process water was 1:8. After carbonization, the raw biochar was filtered from the process water, washed with water, dried to constant weight, and finally finely ground. Subsequently, the product was pyrolyzed at 850 °C for 3 to 6 min in an annealing tube furnace (ELTRA, Haan, Germany). The pyrolysis was carried out in a nitrogen countercurrent (250 L h^{-1}). After the final product had cooled down, it was finely ground again.

2.2. Manufacturing of electrodes from carbonized grass clippings

To produce the carbonized grass electrodes (CGE), a mixture of biochar and binder was applied to a metallic carrier. For this purpose, a binder solution containing 100 g L^{-1} PVDF in DMSO was prepared. Dimensionally stable anodes (DSA, titanium expanded metal electrodes coated with iridium mixed oxide from Metakem GmbH, Usingen, Germany) were used as the metallic carrier. The DSA carrier had dimensions of 25×55 mm and thus a total geometric surface area of 27.5 cm^2. 1 g of biochar was mixed with 1 mL of the 10 % PVDF solution and applied to 80 % of the DSA carrier surface area (when using the electrodes, only the coated part was immersed in the electrolyte). The electrodes were dried hanging for at least two days at room temperature.

2.3. Characterization of biochar and carbonized grass electrodes

The chemical analysis of the biochar produced was carried out by elemental analysis and determination of the ash content. Elemental analysis was used to determine the total content of the elements H, C, N, and S. The measurements were carried out in tin boats with a vario MACRO cube (Elementar Analysensysteme, Langenselbold, Germany). To determine the ash content, a defined quantity of the biochar was weighed into a ceramic crucible and the sample was incinerated at 815 °C in an oxygen atmosphere. The ash content was calculated by differential weighing of the used and remaining substance. The oxygen content was determined indirectly by summing the mass percentages of the elemental analysis and the ash content.

The conductivity of the pyrolyzed biochar was determined indirectly by an electrical resistance measurement. For this purpose, 0.66 g of biochar was placed in a non-conductive hollow cylinder with an internal

diameter of 8 mm, which was sealed on both sides with a brass cylinder. The carbon layer was pressed to a defined thickness of 9.9 mm. The electrical resistance of the carbon layer was measured using a multimeter.

The mass-specific surface area of the biochar was determined at a service unit at KIT using the BET method in a gas sorption meter (Autosorb-1, Quantachrome Instruments, Boynton Beach, FL, USA) with nitrogen as adsorbate. In preparation for the BET analysis, the samples were heated at 300 °C under vacuum for at least 18 h. The BET evaluation was done in the relative pressure range of $0.02 < p/p_0 < 0.12$. The BET analysis was carried out for three samples of pyrolyzed grass biochar in multiple determinations.

Scanning electron microscope (SEM, Carl Zeiss, Oberkochen, Germany) images were taken of the biochar and the CGE produced as well as commercial graphite electrodes to assess the surface structure.

Cyclic voltammetry was performed in 0.2 M potassium phosphate buffer (KH_2PO_4/K_2HPO_4) including 0.1 M KCl in an electrochemical reactor (shown in Figure S1) with 100 mM $K_3[Fe(CN)_6]$. For this purpose, a 3-electrode setup was employed with DSA or the CGE as the working electrode, DSA as the counter electrode, and an $Ag/AgCl/KCl_{sat}$ electrode (Xylem Analytics, Weilheim, Germany) as the reference electrode. The reference electrode was inserted via a Haber-Luggin capillary filled with saturated KCl. The working and counter electrodes each had a geometric surface area of 5 cm^2 (25×10 mm) immersed in the electrolyte. Prior to the experiments, the buffer was gassed with nitrogen for 30 min. The potential range from -0.2 V to 0.8 V *vs.* $Ag/AgCl/KCl_{sat}$ was scanned with a potentiostat (Interface 1010B, Gamry Instruments, Warminster, PA, USA) at scan rates of 2, 5, 10, and 20 mV s^{-1} starting at -0.2 V, with the third cycle being used for evaluation. The experiments were carried out as triplicates.

For linear sweep voltammetry (LSV), the same experimental setup was used as in cyclic voltammetry experiments. However, linear sweep voltammetry was carried out in the minimal medium for *Cupriavidus necator*, which is described below. In addition, the geometric surface area of the working and counter electrodes immersed in the electrolyte solution during the LSV was 15 cm^2 (25×30 mm) each. Linear sweep voltammetry was performed in the potential range from 0 to -2 V *vs.* $Ag/AgCl/KCl_{sat}$ at a scan rate of 20 mV s^{-1} with a potentiostat (Interface 1010B, Gamry Instruments, Warminster, PA, USA). The experiments were carried out as triplicates.

2.4. Application of carbonized grass electrodes in microbial electrosynthesis

First, the application of the CGE was investigated in microbial electrosynthesis (MES). The microorganism *Cupriavidus necator H16 PHB⁻4* (DSM-541, DSMZ, Braunschweig, Germany) was used for this purpose. By using the polyhydroxybutyrate (PHB)-deficient strain, the growth of the organism could be assessed via the optical density (OD_{600}) without being influenced by PHB production. As described above, the organism is able to grow autotrophically using H_2, O_2, and CO_2. H_2 and O_2 were produced *in situ* by water electrolysis, while CO_2 was fed into the reactor. The minimal medium suitable for bioelectrochemical systems for *C. necator* was described previously (Sydow et al., 2017). The medium consisted of 2.895 g L^{-1} Na_2HPO_4, 2.707 g L^{-1} $NaH_2PO_4 \times H_2O$, 0.17 g L^{-1} K_2SO_4, 0.097 g L^{-1} $CaSO_4 \times 2\ H_2O$, 0.8 g L^{-1} $MgSO_4 \times 7\ H_2O$, and 0.934 g L^{-1} $(NH_4)_2SO_4$ as well as trace elements: 750 µg L^{-1} $FeSO_4 \times 7\ H_2O$, 120 µg L^{-1} $MnSO_4 \times H_2O$, 120 µg L^{-1} $ZnSO_4 \times 7\ H_2O$, 24 µg L^{-1} $CuSO_4 \times 5\ H_2O$, 90 µg L^{-1} $Na_2MoO_4 \times 2\ H_2O$, 75 µg L^{-1} $NiSO_4 \times 6\ H_2O$, and 2 µg L^{-1} $CoSO_4 \times 7\ H_2O$.

The electrochemical reactor (shown in Figure S1) was filled with a total volume of 85 mL medium. An undivided two-electrode setup consisting of a working and counter electrode was chosen for the MES. Dimensionally stable anodes (DSA, titanium expanded metal electrodes coated with iridium mixed oxide from Metakem GmbH, Usingen, Germany) with a total geometric surface area of 27.5 cm^2 (25×55 mm) were used as counter electrodes. Either the CGE or, for comparison, a DSA was used as the working electrode. A PTFE mesh was used to separate the working and counter electrodes from each other but to keep them in parallel orientation with a distance of 2 mm. A photograph of the working and counter electrode assembly is shown in the supplementary materials in Figure S2. In the experiments, the electrodes had a geometric surface area of 20 cm^2 (electrode surface area immersed in the electrolyte; 25×40 mm) and were contacted with a titanium wire with a diameter of 0.5 mm. The reactors were stirred with a stirring bar at 200 rpm and tempered to 30 °C in an incubation hood (TH 30, Edmund Bühler GmbH, Bodelshausen, Germany). Reactors were gassed with N_2/CO_2 (80:20) at a gas flow rate of 20 mL min^{-1}. For water electrolysis, a current of -15 mA (-0.75 mA cm^{-2}) was applied via a potentiostat (Interface 1010B, Gamry Instruments, Warminster, PA, USA).

For inoculation of microbial electrosynthesis, a consecutive seed train of heterotrophic cultivation in LB medium, heterotrophic cultivation in minimal medium, and autotrophic cultivation in minimal medium is applied. The heterotrophic preculture in LB medium is inoculated from a cryo-culture of *C. necator H16 PHB⁻4*. The LB medium comprised 5 g L^{-1} NaCl, 5 g L^{-1} yeast extract, and 10 g L^{-1} trypticase peptone. 4 g L^{-1} fructose was added to the heterotrophic cultivation in the minimal medium as carbon source. A gas phase of $H_2/O_2/CO_2$ (64:16:20) was added to the autotrophic preculture in a septum flask at a pressure of 1.5 bar. The precultures were each inoculated from the previous preculture in late exponential to early stationary phase to an OD_{600} of 0.1 and were incubated at 30 °C and 180 rpm. With the autotrophic preculture in the late exponential to early stationary phase, the MES was inoculated to an OD_{600} of 0.2. Each day, 1 mL of sample is drawn to determine OD_{600} and pH value.

2.5. Application of carbonized grass electrodes in a microbial fuel cell

The microorganism *Geobacter sulfurreducens* (DSM-12,127, DSMZ, Braunschweig, Germany) was used to investigate the CGE in a microbial fuel cell. The medium for *G. sulfurreducens* consisted of 1.5 g L^{-1} NH_4Cl, 0.6 g L^{-1} Na_2HPO_4, 0.1 g L^{-1} KCl, 0.82 g L^{-1} Na-acetate, 2.5 g L^{-1} $NaHCO_3$, 1 % (v/v) mineral solution, and 1 % (v/v) vitamin solution. The mineral solution contained 1.5 g L^{-1} nitrilotriacetic acid, 3 g L^{-1} $MgSO_4 \times 7\ H_2O$, 0.5 g L^{-1} $MnSO_4 \times H_2O$, 1 g L^{-1} NaCl, 0.1 g L^{-1} $FeSO_4 \times 7\ H_2O$, 0.18 g L^{-1} $CoSO_4 \times 7\ H_2O$, 0.10 g L^{-1} $CaCl_2 \times 2\ H_2O$, 0.18 g L^{-1} $ZnSO_4 \times 7\ H_2O$, 0.01 g L^{-1} $CuSO_4 \times 5\ H_2O$, 0.02 g L^{-1} $KAl(SO_4)_2 \times 12\ H_2O$, 0.01 g L^{-1} H_3BO_3, 0.01 g L^{-1} $Na_2MoO_4 \times 2\ H_2O$, 0.03 g L^{-1} $NiCl_2 \times 6\ H_2O$, 0.3 mg L^{-1} $Na_2SeO_3 \times 5\ H_2O$, and 0.4 mg L^{-1} $Na_2WO_4 \times 2\ H_2O$. The pH value of the mineral solution was adjusted to 7.0 with KOH. The vitamin solution contained 2 mg L^{-1} biotin, 2 mg L^{-1} folic acid, 10 mg L^{-1} pyridoxine-HCl, 5 mg L^{-1} thiamine-HCl, 5 mg L^{-1} riboflavin, 5 mg L^{-1} nicotinic acid, 5 mg L^{-1} Ca-d-pantothenate, 0.1 mg L^{-1} vitamin B12, 5 mg L^{-1} p-aminobenzoic acid, and 5 mg L^{-1} (±)-α-lipoic acid. The whole medium had a pH value of 6.8.

The microbial fuel cell was conducted in an electrochemical H-cell (shown in Figure S3) consisting of two chambers separated by a proton exchange membrane (Nafion™ 117, QuinTech, Göppingen, Germany) with a surface area of 4.9 cm^2. The two chambers were each filled with 100 mL medium. However, mineral and vitamin solutions were only added in the anodic chamber. Either commercial cylindrical graphite rods (Graphite24, Bad Breisig, Germany) with a diameter of 15 mm or the CGE were used as both working and counter electrodes. The electrodes were contacted with a titanium wire with a diameter of 0.5 mm. The geometric electrode surface area at the start of the experiment was 15.8 and 22.5 cm^2 (25×45 mm) for the graphite electrodes and the CGE, respectively. An $Ag/AgCl/KCl_{sat}$ electrode (Xylem Analytics, Weilheim, Germany) was used as the reference electrode, which was introduced into the anode chamber by using a Haber-Luggin capillary filled with saturated KCl. The medium in the anode chamber was gassed with N_2/CO_2 (80:20) before starting the MFC. During the experiments, the medium in the anode chamber was also gassed with N_2/CO_2 (80:20) at a

gassing rate of 30 mL min^{-1}. A potential of 400 mV *vs.* $Ag/AgCl/KCl_{sat}$ was applied via a potentiostat (MultiEmStat3+, PalmSens BV, Houten, Netherlands). The anode and cathode chambers were stirred with a stirrer bar at 200 rpm and the entire setup was tempered to 30 °C in an incubation hood (CERTOMAT® HK, Sartorius, Göttingen, Germany).

For the preculture, 8 g L^{-1} Na_2-fumarate was added to the medium as electron acceptor. The preculture was cultivated from a cryo-culture of *G. sulfurreducens* in 50 mL medium with a gas phase of N_2/CO_2 (80:20) at a pressure of 1.4 bar in 250 mL septum flasks for four days at 30 °C and 180 rpm until it reached a final OD_{600} of approximately 0.5. The anode chamber was inoculated with 1 mL of the preculture. To determine the acetate concentration, 1 mL of sample was taken daily and stored at -20 °C until analysis. The acetate concentration was quantified by HPLC with an Aminex HPX-87H (Bio-Rad Laboratories, Hercules, CA, USA). The mobile phase was 5 mM H_2SO_4 with a flow rate of 0.6 mL min^{-1}. The column was heated to 60 °C and detection was performed at 210 nm.

3. Results and discussion

3.1. Abiotic characterization of the carbonized grass electrodes

The biochar produced from grass clippings using hydrothermal carbonization and pyrolysis was analyzed for its elemental composition. The biochar consists of 57.4 ± 5.9 % C, 4.0 ± 0.3 % N, 3.8 ± 1.5 % O, 1.4 ± 0.1 % H, 0.4 ± 0.2 % S, and 33.1 ± 6.0 % ash ($n = 8$). In another work, a carbon content of 87.5 % was achieved for mixed green waste biochar at 750 °C with a residence time of pyrolysis of 10 min (Ronsse et al., 2013). With higher pyrolysis temperature as well as duration, the carbon content of biochar can be increased (Ronsse et al., 2013). The electrical conductivity of biochar depends on the carbon content and the structure of the carbon (graphene/graphite) (Gabhi et al., 2017). Generally, the graphite content and thus the electrical conductivity also increase with higher pyrolysis temperature (Gabhi et al., 2020). However, with increasing pyrolysis time and temperature, the yield decreases, which is why it is necessary to find a compromise. Here, the biochar shows an electrical resistance of 0.71 ± 0.33 Ω cm^{-1} ($n = 8$). Similar values were obtained, for example, for carbonized coconut shells with 0.39 Ω cm^{-1} (Chen et al., 2016) or carbonized kenaf stalks with 0.67 Ω cm^{-1} (Chen et al., 2012).

The BET analysis of three grass biochar batches showed a specific surface area of 47.0 ± 4.5 m^2 g^{-1} ($n = 5$), 46.2 ± 1.6 m^2 g^{-1} ($n = 3$), and 51.0 ± 4.5 m^2 g^{-1} ($n = 4$). Previously, biochar from green waste (slow pyrolysis at 600 °C for 60 min) showed a specific surface area of 46 m^2 g^{-1} (Ronsse et al., 2013). Thus, the specific surface area of the carbonized grass clippings is in a very similar range to the biochar from mixed green waste (with different pyrolysis parameters). Chemical and thermal activation methods are described in the literature to increase the surface area of biochar (Jin et al., 2014; Dehkhoda et al., 2014; Ding et al., 2020; González-García et al., 2013). In our case, we did not use chemical activation with KOH, as it did not increase the final carbon content (data not shown). However, this could improve the properties of the biochar, depending on the field of application. Furthermore, the specific surface area of biochar also generally increases with higher pyrolysis temperatures (Ronsse et al., 2013).

We tested various methods for the production of electrodes from the carbonized grass clippings. We attempted to press electrodes from the biochar with and without binders and also without the use of a metallic carrier/electrode support. Compacting the biochar can further increase the electrical conductivity (Adinaveen et al., 2016). While the remaining lignin in biochar from hydrothermal carbonization serves as a binder when the biochar is pressed (Reza et al., 2012), pressing the biochar after pyrolysis without additional binder did not result in mechanically stable pellets in our case. Additionally, without a metallic carrier, electrically contacting the pressed electrodes was inconvenient. A proven method of fabricating electrodes from biochar is to apply the biochar to a metallic current collector using a binder such as PVDF or PTFE (Jin et al., 2014; Ding et al., 2020), which is why we chose that method. Fig. 1 shows one of the electrodes produced. The electrodes can be designed to be recyclable if a sustainable binder is used and by eliminating the metallic carrier. By additive manufacturing, electrodes with variable geometries can be created from the biochar (Idrees et al., 2018). To improve mass transfer in a bioelectrochemical system, the biochar could also be granulated (Ren et al., 2015) and used as a granular electrode in a fluidized bed reactor (Dong et al., 2018). More robust biochar electrode manufacturing processes suitable for mass production, such as screen printing, remain to be investigated.

The biochar and the CGE were examined in comparison to a commercial graphite electrode under the SEM regarding surface structure (Fig. 2). The graphite electrode shows a rough surface structure (a), while the biochar has clearer pores (b). The surface of the finished CGE is considerably coarser (c), which could promote biofilm formation. At a higher magnification, the polymer structure of the PVDF binder is clearly recognizable (d).

Fig. 3 shows the cyclic voltammograms of the redox probe $K_3Fe(CN)_6$ with a DSA as the working electrode (a) compared to a CGE as the working electrode (b). While the typical redox peaks can be measured in the cyclic voltammogram with the DSA, the peaks are less pronounced when using the CGE. The current at the lower and upper potentials might be attributed to unspecified side reactions or the pattern of an ohmic resistance. Nevertheless, it can be stated that both oxidation of Fe^{2+} and reduction of Fe^{3+} are detectable at the fabricated electrodes. In a linear sweep experiment in the medium for the MES (Figure S4), an earlier and greater current response with increasing potential can be observed using the CGE compared to the DSA, which might be mainly attributed to a higher electrode resistance and an ohmic behavior. With both electrodes, visible bubble formation on the working electrode indicating hydrogen evolution can only be seen from around -700 to -800 mV in all experiments.

3.2. Application of carbonized grass electrodes in microbial electrosynthesis

First, the carbonized grass electrodes were investigated for use in microbial electrosynthesis. For this purpose, *Cupriavidus necator PHB⁻4* was cultivated electroautotrophically at a constant current density of -0.75 mA cm^{-2} over seven days. The CGE was compared with a DSA as

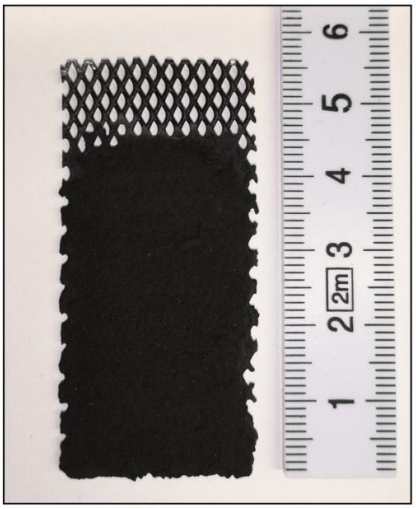

Fig. 1. Manufactured carbonized grass electrode: 1 g of biochar mixed with 1 mL of the 10 % PVDF solution in DMSO applied to 80 % of the DSA metal carrier with dimensions of 25×55 mm.

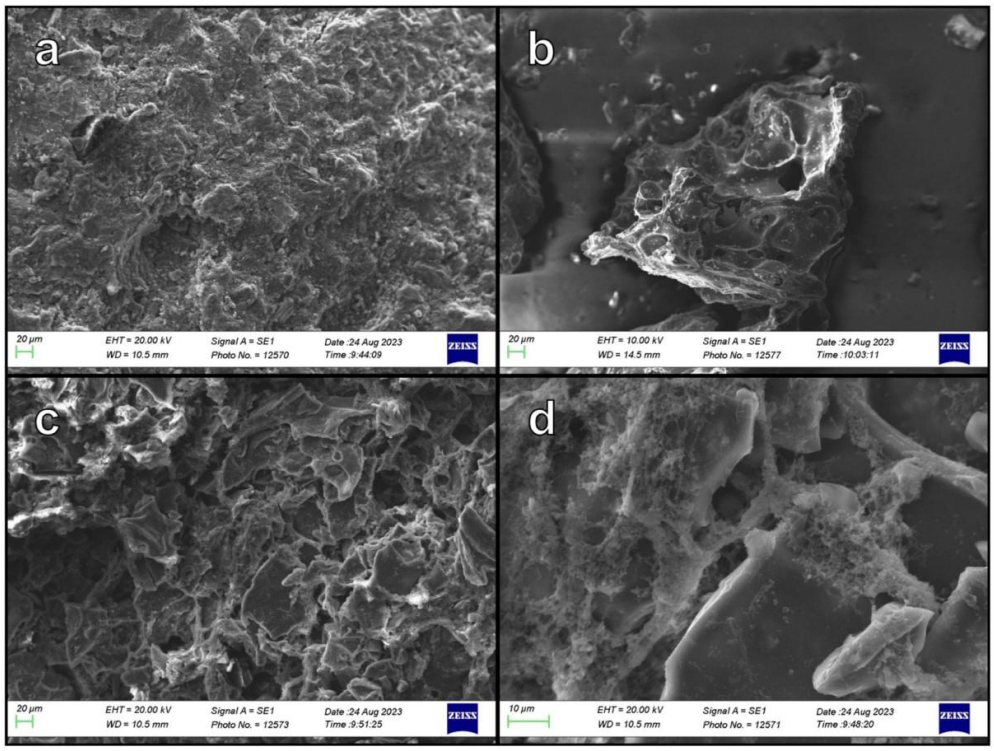

Fig. 2. SEM images of a commercial graphite rod (a), a grass biochar particle (b), and a carbonized grass electrode at lower (c) and higher magnification (d).

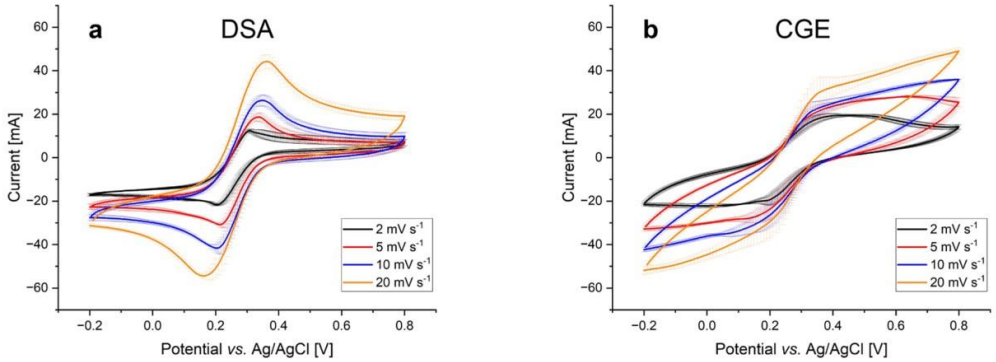

Fig. 3. Cyclic voltammetry of 100 mM $K_3Fe(CN)_6$ with DSA (a) or carbonized grass electrode (CGE) (b) as working electrode at scan rates of 2, 5, 10, and 20 mV s^{-1} (n = 3). DSA as counter electrode each, geometric surface area of both working and counter electrode of 5 cm^2, Ag/AgCl/KCl_{sat} reference electrode, and 0.2 M potassium phosphate buffer with 0.1 M KCl as electrolyte.

the working electrode for the hydrogen evolution reaction. Fig. 4 shows the course of OD_{600} (a) as well as pH value and potential *vs.* Ag/AgCl/KCl_{sat} (b) of the two experiments. Faster microbial growth can be observed when the DSA is used as a working electrode. While an average OD_{600} of 1.7 ± 0.4 is achieved with the DSA after seven days, the use of the CGE only results in an OD_{600} of 1.1 ± 0.3. Differences can also be seen in the pH value and potential curves. The pH value of the DSA experiment drops from 6.6 ± 0.0 to 6.2 ± 0.1 during the experiment. When using the CGE, the pH value drops from 6.6 ± 0.1 to 5.5 ± 0.2. Since the organism in the experiment with the CGE shows lower growth, it is unlikely that the pH value drop has a biological origin but rather an electrochemical one. However, the pH value is still in a range in which it should not have a notable influence on the growth of the microorganism. The potential curve is relatively linear during both experiments, although it is about 0.35 V more negative for the CGE. The higher potential indicates either a higher electrode resistance or a higher overpotential for the desired hydrogen evolution reaction at the CGE. During the linear sweep experiment (Figure S4), a higher current response with increasing potential and a current response before the actual water electrolysis can be seen with the CGE compared to the DSA. The results are reflected in the electrical potential during the MES. It was not to be expected that the CGE would outperform the DSA, as the DSA is

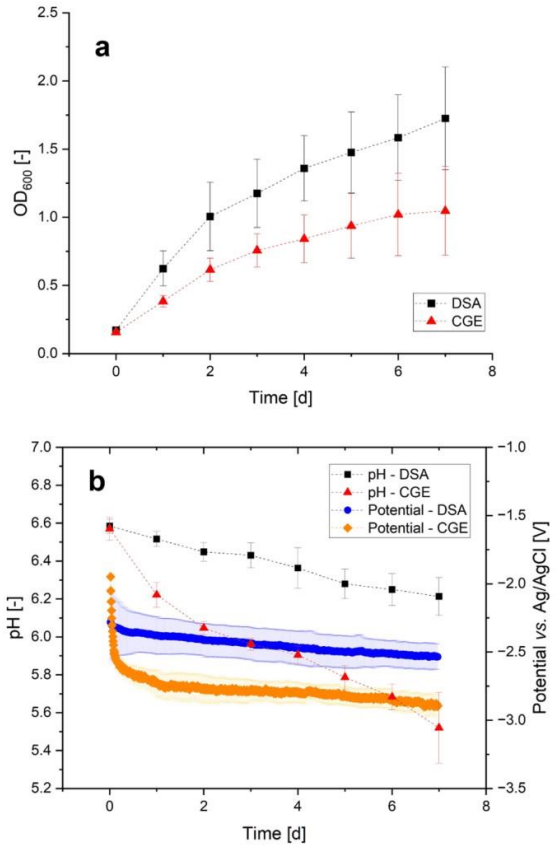

Fig. 4. Course of OD_{600} (a) as well as pH value and potential *vs.* $Ag/AgCl/KCl_{sat}$ (b) during electroautotrophic cultivation of *Cupriavidus necator PHB*$^{-}$*4* with a DSA and a carbonized grass electrode (CGE) as working electrode (n = 3). Temperature of 30 °C, mixing at 200 rpm, minimal medium with an initial volume of 85 mL, gassing rate of 20 mL min^{-1} with N_2/CO_2 (80:20), constant current density of -0.75 mA cm^{-2} for water electrolysis, DSA as counter electrode each, geometric surface area of both working and counter electrode of 20 cm^2, and $Ag/AgCl/KCl_{sat}$ reference electrode. Dashed lines serve as a guide to the eye.

optimized for the oxygen evolution reaction in water electrolysis and has a lower resistance. The lignocellulosic biochar also cannot compete with specialized carbon-based electrodes, such as customized carbon nanotubes or nanorods designed specifically for the hydrogen/oxygen evolution reaction (Liu et al., 2024; You et al., 2023; Deng et al., 2023) or battery solutions (Zhong et al., 2023; Zhang et al., 2022). However, the electrochemical performance of the CGE could be optimized by adapting the carbonization process, by chemical pretreatment prior to carbonization or by incorporating conductive material into the CGE during manufacture.

3.3. Application of carbonized grass electrodes in a microbial fuel cell

After the application in microbial electrosynthesis, the carbonized grass electrodes were investigated for use in microbial fuel cells. For this purpose, a microbial fuel cell with *Geobacter sulfurreducens* was conducted either with graphite electrodes (GE) or the CGE. Fig. 5a shows the current density and the acetate concentration throughout the two experiments. Compared to the graphite electrodes, an earlier current response can be seen in the experiment with the CGE. As a result, acetate can no longer be detected one day earlier. One reason for this could be faster biofilm formation due to more advantageous surface properties. The coarser surface structure was confirmed with the SEM (see above). Since different precultures were used for the triplicates, the influence of the preculture on the current response can be excluded. The absolute current response is higher with the graphite electrodes. The integral of the current curve results in an electric charge of 39.4 ± 1.9 and 25.1 ± 4.1 As cm^{-2} for the graphite electrodes and CGE, respectively. This could be due to the higher conductivity of the graphite electrodes, benefiting electron transfer. Nevertheless, we were able to show that the electrodes from carbonized grass clippings are suitable for microbial fuel cells and may have advantageous properties.

In a subsequent experiment, we investigated whether the electrodes are also suitable over a longer period of time. For this purpose, three microbial fuel cells were set up with the CGE as before. The current response was recorded over several weeks. 20 mM acetate was added when the current dropped. Fig. 5b shows the current density curve of the three MFCs. Cells A and C ran successfully for 44 and 45 days, respectively. Due to technical difficulties with cell B, which were unrelated to the CGE, the experiment had to be aborted after 25 days. No restrictions due to the electrodes were observed throughout the experiment. The experiment could likely have run for much longer, indicating a stable electrode performance. Commault et al. demonstrated an MFC with (modified) graphite rods as electrodes and a *Geobacter*-dominated biofilm using acetate/ethanol medium and synthetic wastewater that was active for 75 days (Commault et al., 2015). Throughout the experiment shown, varying current responses were observed, particularly in cell C, which could be due to varying substrate concentrations. In addition, adaptation effects of the microorganism could already occur over the duration of the experiment. Differences in the biofilm may also be a reason, as only the living cells are responsible for the electrochemical activity (D. Sun et al., 2016).

The results of the MFC have shown that electrodes made from carbonized grass clippings can certainly be an alternative to fossil graphite. However, different challenges could arise when using residual materials. One challenge could be possible contamination by *e.g.*, plastic in the raw material. Furthermore, it remains to be investigated what influence the heterogeneity of the starting material due to seasonality has on the result of the carbonization. Another major obstacle to the use of pyrolyzed waste materials as electrode material is the high energy input required for carbonization. Electrode fabrication seems to be a promising method in colder seasons because there is less liquid in the green waste during that time. In addition, much of the green waste in winter is foliage, which contains little lignin and thus allows for easier carbonization. To further increase the efficiency of pyrolysis, the solid part could be separated from the liquid part of the green waste beforehand, for example by pressing. Then only the solid part could be pyrolyzed, while the liquid part is recycled elsewhere. Previously, we have shown that the juice from grass clippings can serve as a growth medium for *C. necator* (Langsdorf et al., 2022). The combination of biorefineries for the use of biogenic feedstocks and bioelectrochemical transformations for coupling electrical energy with the chemical industry offers huge potential for a future bio-based economy (Harnisch and Urban, 2018).

4. Conclusions

For the first time, we demonstrated the manufacturing of electrodes from carbonized grass clippings and their application in bioelectrochemical systems. The greatest potential of biochar electrodes lies in technologies that use graphite or similar materials as electrodes. We were able to show that electrodes from carbonized grass clippings perform similarly to commercial graphite electrodes in the microbial fuel cell and that they can be used for more than six weeks, probably even much longer. This work once again shows the potential of renewable raw materials as a starting material for electrodes as well as another

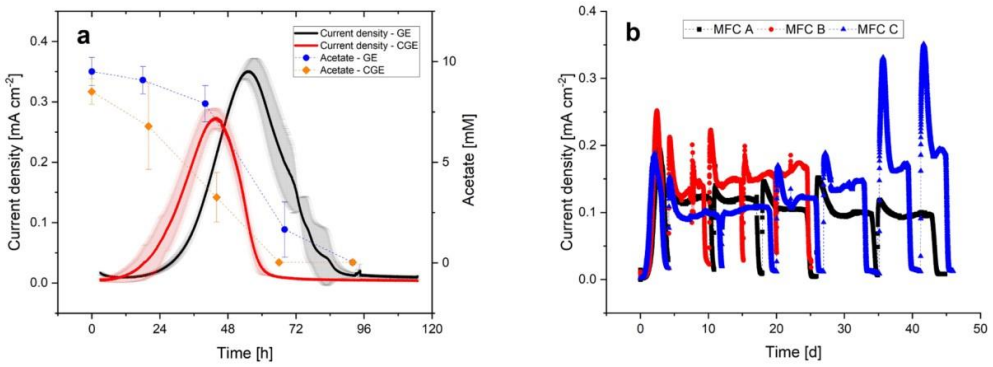

Fig. 5. Current density and acetate concentration of the MFC with *G. sulfurreducens* with graphite electrodes (GE) and carbonized grass electrodes (CGE) each as working and counter electrode (a, $n = 3$) and current density of three MFC experiments titled A, B, and C with carbonized grass electrodes (b, $n = 1$). Temperature of 30 °C, mixing at 200 rpm, initial medium volume of 100 mL in anode and cathode chamber, separation of the anode and cathode chambers by a proton exchange membrane (Nafion™ 117) with a surface area of 4.9 cm^2, gassing rate of 30 mL min^{-1} with N_2/CO_2 (80:20), constant potential of 400 mV *vs.* $Ag/AgCl/KCl_{sat}$, geometric surface area of GE and CGE of 15.8 and 22.5 cm^2 respectively, and $Ag/AgCl/KCl_{sat}$ reference electrode. Dashed lines serve as a guide to the eye.

method of recycling green waste materially.

CRediT authorship contribution statement

Alexander Langsdorf: Conceptualization, Methodology, Investigation, Writing – original draft, Visualization. **Michael Halim:** Methodology, Investigation, Writing – review & editing. **Marianne Volkmar:** Conceptualization, Writing – review & editing. **Markus Stöckl:** Conceptualization, Writing – review & editing. **Ralf Harnisch:** Investigation, Writing – review & editing. **Peter Hahn:** Conceptualization, Methodology, Writing – review & editing, Supervision. **Roland Ulber:** Conceptualization, Writing – review & editing, Funding acquisition. **Dirk Holtmann:** Conceptualization, Methodology, Writing – review & editing, Project administration, Funding acquisition.

Declaration of competing interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

Data availability

Data will be made available on request.

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Supplementary materials

Supplementary material associated with this article can be found, in the online version, at doi:10.1016/j.clce.2024.100118.

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4.5 Production of polyhydroxybutyrate from industrial flue gas by microbial electrosynthesis

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Production of polyhydroxybutyrate from industrial flue gas by microbial electrosynthesis

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ABSTRACT

The aim of this work was to produce the biopolymer polyhydroxybutyrate (PHB) from industrial flue gas as CO_2 source and electrolysis originating hydrogen via microbial electrosynthesis. Besides the laboratory experiments, the experiments were carried out under industrial conditions directly on-site in a cogeneration plant. We were able to demonstrate that the use of flue gas as a CO_2 source has no detectable negative effect on bacterial growth and PHB production in comparison to a pure gas mixture. In an electrochemical H-cell 333 ± 44 mg L^{-1} PHB were obtained, which corresponds to a PHB content of 43 ± 3 % of the cell dry weight. By using flue gas for the production of the biopolymer PHB, not only CO_2 emissions are reduced, but also possible environmental pollution from non-biodegradable plastics. To the best of our knowledge, this is the first time that the production of PHB from flue gas has been demonstrated using *Cupriavidus necator*.

1. Introduction

Reducing anthropogenic CO_2 emissions to combat climate change is one of the greatest challenges of our time. By recycling CO_2 from exhaust gases as a resource for the industry, these emissions can be lowered. At the same time, this can reduce the use of fossil resources. For example, CO_2 can be a resource for the microbial production of the biodegradable polymer polyhydroxybutyrate (PHB). This not only allows us to move away from petroleum-based polymers but also to make the disposal of polymers more sustainable. Biopolymers are becoming increasingly important as a biodegradable alternative due to the growing environmental impact of plastics [1]. Therefore, the electro-biotechnological conversion of CO_2 could help to achieve the UN Sustainable Development Goals, in particular SDG 13 "Climate Action" [2]. The production of PHB as a biodegradable plastic alternative also contributes to SDGs 12 "Responsible Consumption and Production", 14 "Life below Water", and 15 "Life on Land".

PHB belongs to the short-chain length polyhydroxyalkanoates [3]. Due to their biodegradability and biocompatibility, polyhydroxyalkanoates are a promising alternative to conventional polymers [3]. Unfortunately, PHB is not yet cost-competitive with conventional polymers. The use of waste materials can help to make PHB production less expensive and more sustainable. Various waste materials have already been used for the microbial production of PHB [4]. Recently, a review article has been published outlining the state of the art in microbial PHB production from CO_2 [5]. Polyhydroxyalkanoates can be produced by a range of bacteria including *Cupriavidus*, *Alcaligenes*, *Pseudomonas*, recombinant *E. coli,* and methylotrophs [6]. *Cupriavidus necator* is considered a promising aerobic chemoautotrophic organism for third-generation biorefineries due to its ability to store CO_2 in the form of polyhydroxybutyrate [7]. Under lack of essential macroelements such as nitrogen as well as an excess of a carbon source, *C. necator* produces PHB as an energy reserve [8]. The organism is known for its broad substrate spectrum. Amongst others, PHB production has already been demonstrated on various carbon sources such as acetic acid [9], lactic acid [10], oleic acid [11], glycerol [12], or formate [13,14]. In terms of circular economy, *C. necator* is also capable of producing PHB from different carbon-containing waste

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streams [15]. Sequencing of the genome of *C. necator* H16 enables genetic engineering to further exploit the potential of the organism [16] and as a result the production of other higher-value products such as terpenes [17–19] or fuels and solvents [20] from CO_2 in perspective. *C. necator* is able to grow autotrophically with CO_2 as carbon source, hydrogen as electron donor, and oxygen as terminal electron acceptor. Hydrogen and oxygen can be produced *in situ* using water electrolysis. For microbial CO_2 fixation, H_2 is a promising electron donor because it can be produced electrochemically efficiently and has a low reduction potential [21]. The electrochemical process of water electrolysis and microbial production can be combined in microbial electrosynthesis (MES). When CO_2 is used from an exhaust stream and hydrogen and oxygen are produced by water electrolysis using renewable energy (e.g. photovoltaics), an overall "green" process can be created. While PHB production with *C. necator* under autotrophic conditions has been extensively studied, research on electroautotrophic cultivation, especially with a real exhaust CO_2 source, is still lacking. Harmful substances such as nitrogen oxides or sulfur oxides are often present in these exhaust gas streams and might affect the process. Garcia-Gonzalez and De Wever investigated the impact of real off-gases on autotrophic PHB production with *C. necator* [22]. They observed that impurities of real off-gases did not affect the bacterial performance or the properties of the produced PHB in comparison to pure CO_2. Wu et al. demonstrated the microbial electrosynthesis of lycopene from flue gas using a genetically modified *C. necator* strain [23]. Previously, we have shown microbial PHB production based on CO_2 by *C. necator* using drop-in electrolysis [13,14]. In this process, CO_2 was electrochemically reduced to formate, which served in a second step as the sole substrate for *C. necator*. However, for the direct electrochemical conversion of flue gasses, a gas purification is usually required since components other than CO_2 (such as O_2) lead to significant decrease of CO_2 electrolysis. In contrast, the direct microbial utilization of CO_2 in flue gas is a rather robust approach, as microbial growth and synthesis are generally less sensitive towards impurities or toxic substances in the flue gas. Especially for the application of aerobic microorganisms, the oxygen in the flue gas stream can even be beneficial.

The objective of this study was to produce PHB using a bio-electrochemical process with *C. necator* based on CO_2 from a coal-fired cogeneration plant. Data from the industrial plant experiments are compared with analogous investigations from laboratory experiments.

2. Materials and methods

2.1. Precultures for microbial electrosynthesis

A seed train of three precultures was used to inoculate the microbial electrosynthesis. From a cryo-culture of *Cupriavidus necator* H16 (DSM-428, DSMZ, Braunschweig, Germany), a preculture was inoculated in LB medium. The LB medium consisted of 5 g L^{-1} NaCl, 5 g L^{-1} yeast extract, and 10 g L^{-1} trypticase peptone and had a pH value of 7.1. This was followed by heterotrophic and autotrophic preculture in the minimal medium used in the bioelectrochemical system. The minimal medium for the cultivation of *C. necator* in bioelectrochemical systems was developed in a previous work [24]. It contains 2.895 g L^{-1} Na_2HPO_4, 2.707 g L^{-1} NaH_2PO_4*H_2O, 0.17 g L^{-1} K_2SO_4, 0.097 g L^{-1} $CaSO_4$*$2H_2O$, 0.8 g L^{-1} $MgSO_4$*$7H_2O$, and 0.934 g L^{-1} $(NH_4)_2SO_4$ as well as trace elements: 750 µg L^{-1} $FeSO_4$*$7H_2O$, 120 µg L^{-1} $MnSO_4$*H_2O, 120 µg L^{-1} $ZnSO_4$*$7H_2O$, 24 µg L^{-1} $CuSO_4$*$5H_2O$, 90 µg L^{-1} Na_2MoO_4*$2H_2O$, 75 µg L^{-1} $NiSO_4$*$6H_2O$, and 2 µg L^{-1} $CoSO_4$*$7H_2O$. The pH value of the minimal medium was 6.6. For heterotrophic preculture, 4 g L^{-1} fructose was added to the medium. For autotrophic preculture, a gas atmosphere of $H_2/CO_2/O_2$ (64:16:20) at 1.5 bar was applied. Heterotrophic and autotrophic preculture in the minimal medium were inoculated from the previous culture in late exponential or early stationary phase to an optical density (OD_{600}) of 0.1. All precultures were incubated at 30 °C and 180 rpm.

2.2. Microbial electrosynthesis with C. necator

Microbial electrosynthesis was performed in an undivided electrochemical reactor with a 3-electrode setup. Platinized titanium expanded metal electrodes (50 g Pt per m^2, Metakem GmbH, Usingen, Germany) with a geometric area of 22.5 cm^2 (45 mm×25 mm) each were used as working and counter electrodes. The working electrode was contacted with a platinum wire with a diameter of 0.5 mm and the counter electrode with a stainless steel wire with a diameter of 0.5 mm. The working and counter electrodes were held at a distance of 2 mm and were separated from each other using a PTFE mesh. A photograph of the electrodes is provided in Figure S1. An Ag/AgCl electrode (Xylem Analytics, Weilheim, Germany) was used as the reference electrode, which was introduced into the cells through a Haber-Luggin capillary filled with saturated KCl solution. The reactors were autoclaved and filled to a total volume of 110 mL with the minimal medium described in the previous chapter. A constant current of −15 mA (-0.67 mA cm^{-2}) was applied for water electrolysis via a potentiostat (Interface 1010B, Gamry Instruments, Warminster, PA, USA). The MES took place in an incubation hood (CERTOMAT® HK, Sartorius, Göttingen, Germany) at 30 °C. A stir bar was used for mixing at 300 rpm. In the laboratory experiments, a pure gas mixture consisting of 85 % N_2, 10 % CO_2, and 5 % O_2 (Nippon Gases Deutschland, Düsseldorf, Germany) was continuously supplied via a cannula at a gas flow rate of 25 mL min^{-1}. In addition, a laboratory experiment was carried out with 100 % CO_2 and a gas flow rate of 2.5 mL min^{-1}. In the experiments with flue gas from the cogeneration plant, the gas flow rate was also 25 mL min^{-1} to keep the supplied volume of CO_2 approximately identical. The gas supply was passed through a sterile filter. The flue gas was continuously withdrawn after flue gas desulfurization from a sampling line intended for exhaust gas analysis in the cogeneration plant using an internal pump and fed directly into the reactors. Besides nitrogen, the flue gas consisted mainly of oxygen (7 – 11 %) and carbon dioxide (approx. 10 %). It also contained traces of NO (39 – 302 mg m^{-3}), SO_2 (3 – 279 mg m^{-3}), and CO (2 – 32 mg m^{-3}) The gas concentration data are given under standard conditions and were provided by Mainova AG. Figure S2 shows photos of the experimental setup in the cogeneration plant of Mainova AG. The cogeneration plant is located in Frankfurt am Main, Germany. It is operated with hard coal or natural gas, whereby the experiments have only taken place on the units for hard coal. Annually, about 350,000 tons of hard coal are consumed in the cogeneration plant, which together with the combustion of natural gas leads to CO_2 emissions of the cogeneration plant of about 800,000 tons (for the year 2022) [25].

MES was inoculated from the autotrophic preculture in late exponential or early stationary phase to an OD_{600} of 0.2. The process is divided into a microbial growth phase (first phase) and a PHB formation phase (second phase). The growth phase each lasted 4 days in the laboratory experiments and in the cogeneration plant experiment, whereby an additional experiment with a growth phase of 5 days was carried out in the cogeneration plant. In the second phase, the production of PHB is initiated by switching to an ammonium-free medium. For this purpose, the entire reaction volume was centrifuged at 3000xg for 30 min and the pellet was resuspended in 110 mL of ammonium-free minimal medium. For the determination of OD_{600} and pH value, 1 mL of sample was taken daily. The terminal voltage was also measured daily with a digital multimeter (VC165 VOLTCRAFT®, Conrad Electronic SE, Hirschau, Germany). All experiments were performed in triplicates.

2.3. Determination of cell dry weight and polyhydroxybutyrate concentration

For the determination of the cell dry weight and the PHB concentration, 5 mL of sample were taken daily and stored at −20 °C until analysis. The 5 mL sample was first centrifuged at 3000xg for 15 min. The pellet was then resuspended in 1 mL ddH_2O and centrifuged again in a smaller reaction tube at 16000xg for 5 min. The supernatant was

discarded, and the cell pellet was dried at 100 °C for 24 h. The difference in weight of the reaction vessels with and without samples was determined. After determination of the cell dry weight, 1 mL of 96 % sulfuric acid was added to the dried cell pellet. The mixture was incubated at 99 °C and 500 rpm for one hour to convert the PHB to crotonic acid. After conversion, samples were diluted 1:50 with ddH_2O and filtered (0.22 µm). The crotonic acid was quantified by HPLC with an Aminex HPX-87 H (Bio-Rad Laboratories, Hercules, CA, USA). The mobile phase was 5 mM H_2SO_4 with a flow rate of 0.6 mL min^{-1}. The column was heated to 60 °C and detection was performed at 210 nm. For calibration, standards from commercial PHB (Sigma-Aldrich, St. Louis, MO, USA) were prepared and quantified in the same way. This resulted in the following calibration curve: PHB [mg] = $4.0707*10^{-6}$ * $Area_{crotonic\ acid}$ [mAU].

3. Results and discussion

To evaluate the performance of microbial electrosynthesis in an industrial environment with flue gas as the CO_2 source, the identical experiment was first performed in the laboratory under controlled and optimized conditions (e.g. by using an artificial gas mixture with a constant composition of 85 % N_2, 10 % CO_2, and 5 % O_2). As described above, MES was divided into a microbial growth phase and a PHB formation phase. The course of the OD_{600} as well as the PHB concentration in the laboratory experiment is shown in Fig. 1a. The OD_{600} increases continuously from 0.19 ± 0.00–1.6 ± 0.1 by the end of the growth phase on day four. After the medium change to ammonium-free medium, the OD_{600} decreases to 1.1 ± 0.1 due to the dilution of the cell pellet with the initial medium volume (volume decrease during the experiment by sampling) and increases again up to an average maximum OD_{600} of 2.7 ± 0.5 after 240 h. During the growth phase, only small amounts of PHB of <10 mg L^{-1} are detectable. In the second phase, the PHB concentration follows the OD_{600}, suggesting that the increase in OD_{600} is mainly due to PHB production and not cell multiplication. It was previously shown that the actual cell mass without PHB, expressed as residual cell concentration, remains more or less constant in the PHB production phase [26]. PHB concentration increases from 2.7 ± 0.2 mg L^{-1} to a value as high as 347 ± 65 mg L^{-1} on average after six days following medium exchange. This corresponds to an PHB content of 59 ± 1 % of cell dry weight. The PHB concentration then decreases in proportion to the OD_{600}. In addition, we carried out a laboratory experiment with 100 % CO_2 at a gassing rate of 2.5 mL min^{-1} (Figure S3). The experiment showed that without additional gassing with O_2, PHB production already sets in during the growth phase due to oxygen limitation. As a result, a lower maximum PHB concentration of 275 ± 81 mg L^{-1} is achieved on average at a maximum OD_{600} of 2.2 ± 0.4.

In the literature, PHB concentrations of 23.9 g L^{-1} [27] and 61.9 g L^{-1} [28] could be achieved in autotrophic processes. In heterotrophic fed-batch processes, up to 121 g L^{-1} [29] and 125 g L^{-1} [30] of PHB could be obtained. The highest content of PHB in cell mass was reported by Tanaka and Ishizaki with a PHB content of 82.1 % [27], although it is usually lower. The PHB concentrations achieved with the electroautotrophic setup are far below the presented values, as it only serves as a proof of concept and still has plenty of room for optimization (e.g., electrode-to-volume ratio, residence time of the gasses in the reactor, gas pressure, and process mode).

After the successful microbial electrosynthesis in the laboratory, experiments with the same setup were performed in a cogeneration plant with flue gas as CO_2 source. Fig. 1b shows the course of OD_{600} and PHB concentration in the cogeneration plant experiment. The additional experiment with a growth phase of 5 days can be found in the supplementary materials (Figure S4), which will also be discussed below. In the presented results, the OD_{600} increases from 0.19 ± 0.02–2.0 ± 0.2 during the growth phase in the first 4 days. Thus, we achieved an even faster growth than in the laboratory experiment with the final OD_{600} being 0.4 higher. It should be noted that there is no evidence of growth inhibition due to contaminants in the flue gas. In the experiment with a growth phase of 5 days, an OD_{600} value of 1.7 ± 0.0 was achieved at the end of the growth phase (Figure S4a). Therefore, the extension of the growth phase from 4 to 5 days did not lead to an increase in microbial growth. As in the laboratory experiments, dilution occurs due to the medium exchange. As a result, the OD_{600} in the shown experiment drops to 1.2 ± 0.1 before rising to 3.3 ± 0.4 in the PHB formation phase after another 7 days and showing evidence of reaching a plateau. No PHB formation is detectable during the growth phase of both experiments at the cogeneration plant. After switching to ammonium-free medium, PHB is produced continuously. Over the 7 days, a maximum of 333 ± 44 mg L^{-1} PHB was formed on average in the shown experiment, which corresponds to a PHB content of 43 ± 3 % of the cell dry weight. Therefore, the PHB concentration is in a comparable range to the laboratory experiment employing the artificial gas mixture since the standard deviation of both experiments is considerably higher than the difference in the PHB concentration. In the experiment with a growth phase of 5 days, only a maximum PHB concentration of 265 ± 46 mg L^{-1} was formed with a PHB content of 51 ± 4 % of the cell dry

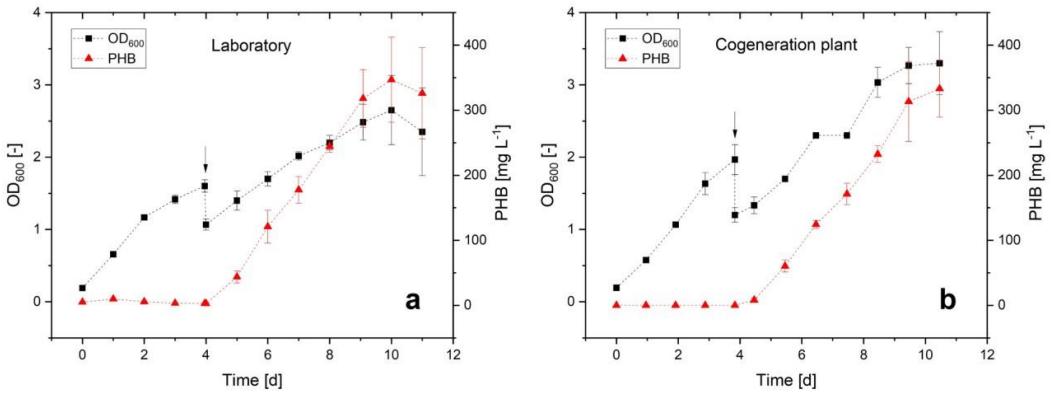

Fig. 1. Optical density and PHB concentration of the MES in the laboratory with a pure gas mixture consisting of 85 % N_2, 10 % CO_2, and 5 % O_2 (a) and the experiment in the cogeneration plant with flue gas (b) (n=3). Reaction parameters: 30 °C, mixing at 300 rpm, minimal medium with an initial volume of 110 mL, gassing rate of 25 mL min^{-1}, constant current density of 0.67 mA cm^{-2} for electrolysis, platinized titanium expanded metal electrodes as working and counter electrodes, Ag/AgCl reference electrode. The arrow indicates the change to ammonium-free medium after 4 days and the start of the PHB production phase. Dashed lines serve as a guide to the eye.

weight

In contrast to the setup of Wu et al. who fed gas into a reactor every 24 h for 30 min [23], we continuously supplied both the pure gas mixture and the flue gas into the bioelectrochemical reactor. Nevertheless, Wu et al. also found no influence of the use of flue gas as a CO_2 source on the growth of *C. necator*. The composition of the flue gas was comparable to the flue gas used in this work and thus, it can be concluded that even continuous exposure to the flue gas does not result in growth inhibition. A similar example with different microorganisms was also shown by Rovira-Alsina et al., in which they demonstrated that the use of real exhaust gas had no relevant effect on the bioelectrochemical conversion of CO_2 to acetate with a microbial community from an anaerobic digester [31].

Besides the OD_{600} and the PHB concentration, the course of pH value as well as the resulting electrode potential *vs.* Ag/AgCl of the laboratory experiment with the pure gas mixture and the experiment in the cogeneration plant with flue gas were monitored. The respective data are presented in Fig. 2. In the laboratory experiment, the pH value slightly decreases from 6.61 ± 0.02–6.33 ± 0.03 during the growth phase and remains relatively constant during the PHB formation phase. The laboratory experiment with 100 % CO_2 (Figure S3b) shows a similar course of the pH value. Accordingly, the pH value cannot be a limiting factor in the laboratory experiments. The pH value of the experiment with flue gas decreases continuously until the end of the growth phase from 6.70 ± 0.01 to about 5.90 ± 0.09. After the medium change, the pH value starts at about 6.77 ± 0.05 and decreases again continuously. After approximately 179 h the pH value drops considerably faster to a pH value of 3.39 ± 0.28 after 251 h. Apparently, a component of the flue gas is responsible for the drop in pH value either directly, through electrochemical conversion, or the metabolism of the microorganism. A similar course of the pH value can also be observed in the other experiment with flue gas (Figure S4b). Thus, regulating the pH value appears to be an important aspect in the further optimization of the process when using flue gas. In contrast to the pH value, the resulting courses of the electrode potentials of the laboratory and the industrial experiments are described by rather similar patterns. On average, the potential in the laboratory experiment is around −1.0 to −1.1 V *vs.* Ag/AgCl, which is slightly lower than the potential in the cogeneration plant at around −0.8 to −1.0 V *vs.* Ag/AgCl, respectively. However, the potential of the laboratory experiment with 100 % CO_2 (Figure S3b) lies in a similar range as the experiments with flue gas.

Based on the average terminal voltage (Figure S5) and the applied current of −15 mA, the consumed electrical energy can be calculated for the experiments (Equation S1). The period up to the maximum PHB concentration is taken into account, thus up to 240 h in the laboratory and up to 251 h in the cogeneration plant. This amounts to an electrical energy input of 9.67 ± 0.25 Wh for the laboratory experiment and 9.35 ± 0.24 Wh for the experiment in the cogeneration plant. Based on these results, the electrical energy required to produce 1 kg of PHB in the laboratory and the cogeneration plant is 410 ± 97 and 444 ± 67 kWh, respectively (Equation S2). Assuming an electricity price of 0.182 € per kilowatt hour in industry [32], the electricity costs amount to around 75 ± 18 or 81 ± 12 € per kg of PHB (Equation S3). Although the values between the laboratory and the cogeneration plant hardly differ, at this stage the energy costs for PHB production alone are far too high for possible commercialization.

The use of CO_2 as a carbon source in combination with electrochemically produced H_2 is considered to have a high technology readiness level since electrochemical hydrogen production is well advanced [33]. The bottleneck now lies in microbial conversion, which can probably be significantly improved above all by genetic engineering of suitable autotrophic microorganisms. Kim et al. were able to increase biomass accumulation by 11 % and PHB production by 28 % through genetic modifications [34]. In addition to the modification of the biocatalyst, the parameters on the process side are also crucial. The low gas solubility and the resulting limitation of gas transfer restrict microbial growth and PHB production in autotrophic cultivation. It has been reported that oxygen transfer can limit PHB accumulation at high cell mass concentrations [26]. Gas mass transfer can be enhanced through higher operation pressure, which can increase productivity [35]. In our case, the experiments were performed at ambient pressure. Designing a suitable bioreactor, for example, an airlift reactor or a bubble column can improve limitations such as gas transfer [36]. Furthermore, our results have also shown that the continuous use of flue gas can lead to a drop in the pH value. This phenomenon can be avoided by using an appropriate bioreactor with pH control. Concerning the gaseous substrates, both the ratio of the individual gases and the gas flow can be optimized to ensure optimal utilization of gases. However, one challenge in aerobic cultivation using hydrogen as an electron donor is the existing risk of explosion. Lambauer and Kratzer have shown how to design a laboratory-scale explosion-proof reactor for PHB production from CO_2 using *C. necator* [37]. It has also been suggested that the growth phase

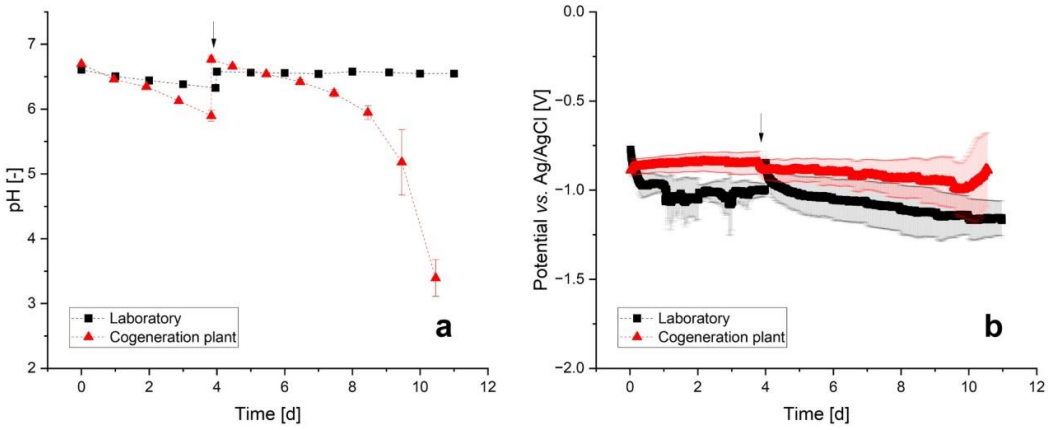

Fig. 2. Course of pH value (a) and potential *vs.* Ag/AgCl (b) of the MES in the laboratory with a pure gas mixture consisting of 85 % N_2, 10 % CO_2, and 5 % O_2 and the experiment in the cogeneration plant with flue gas (n=3). Reaction parameters: 30 °C, mixing at 300 rpm, minimal medium with an initial volume of 110 mL, gassing rate of 25 mL min^{-1}, constant current density of 0.67 mA cm^{-2} for electrolysis, platinized titanium expanded metal electrodes as working and counter electrodes, Ag/AgCl reference electrode. The arrow indicates the change to ammonium-free medium after 4 days and the start of the PHB production phase. Dashed lines serve as a guide to the eye.

should first be carried out heterotrophically with, for example, fructose as substrate and for PHB production to switch to autotrophic cultivation [27,38]. To make such a process more sustainable, substrates from waste materials can be used in the growth phase as we have shown with a growth medium from grass clippings for the cultivation of *C. necator* [39]. A heterotrophic growth phase may result in higher cell mass in less time and thus presumably generate more PHB from CO_2. However, it has also been reported that high cell dry weight at the beginning of the second phase can negatively affect PHB productivity [22]. The efficiency of water electrolysis can be further increased by reducing the internal resistance, for example through an optimized electrode setup or increased conductivity of the medium. A key parameter is to match the rate of hydrogen generation and hydrogen consumption by the microorganisms. In the future, an enhanced gas analysis of the gas outlet could be used to determine how much hydrogen is left to optimize the efficiency of water electrolysis. This could also provide information on CO_2 consumption. In addition, it is likely to be advantageous to separate water electrolysis and fermentation spatially in order to make both processes individually more efficient and thus achieve higher productivity. The combination of both processes requires a compromise between electrolyte and growth medium. A similar approach is the electrochemical reduction of CO_2 to one-carbon compounds such as carbon monoxide, formic acid, or methanol, which can be used subsequently as a simultaneous carbon source and electron donor for microorganisms [33]. However, direct utilization of CO_2 by microorganisms and *in-situ* water electrolysis can bypass this additional step.

4. Conclusions

We successfully realized the microbial electrosynthesis of PHB with *Cupriavidus necator* by using real flue gas directly on-site in a cogeneration plant. In addition, no detectable inhibitory effects on bacterial growth or PHB production were observed compared to the same experiments carried out in the laboratory with a pure gas mixture. However, the results presented here are only a proof of concept that still leaves room for optimization. Limitations lie primarily in microbial conversion, which can be addressed by genetically modifying the biocatalyst and optimizing the process regarding gas transfer. Further consideration should also be given to practical implementation on a larger scale in the industry. To realize such a concept in the future, a corresponding bioreactor with all necessary infrastructure would have to be implemented in power plants. A detailed analysis of the CO2 reduction effects and the associated costs must also be carried out. Nevertheless, the results show a promising possibility of using exhaust gas CO_2 as an alternative resource and reducing emissions at the same time.

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CRediT authorship contribution statement

Julian Philipp Schütz: Writing – review & editing, Validation, Methodology, Investigation. **Alexander Langsdorf:** Writing – original draft, Visualization, Validation, Methodology, Investigation, Conceptualization. **Dirk Holtmann:** Writing – review & editing, Supervision, Resources, Project administration, Methodology, Funding acquisition, Conceptualization. **Markus Stöckl:** Writing – review & editing, Supervision, Resources, Methodology, Conceptualization. **Roland Ulber:** Writing – review & editing, Conceptualization.

Declaration of Competing Interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper

Data availability

Data will be made available on request.

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Appendix A. Supporting information

Supplementary data associated with this article can be found in the online version at doi:10.1016/j.jcou.2024.102800.

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5 Further results and general discussion

5.1 Green waste as a feedstock for fermentation

In this work, the successful cultivation of *C. necator* pKR-hum on juice from grass clippings as complete growth medium and the production of α-humulene were demonstrated. In the future, more consideration should be given to the production of the juice from grass clippings or green waste. The method can certainly influence the ingredients and their concentration in the grass juice. It is also important to consider that the chosen method is quite simple to perform on a larger scale. Different production methods for grass juice could be investigated, although an impression of the most promising options has already been gained. A screw press has proven to be the most suitable method for separating the liquid and solid phases of green waste (Volkmar et al., 2023). Accordingly, mass yields of 50 to 80% juice are possible with suitable mechanical homogenizers such as a screw press. A screw press has also been chosen in other publications dealing with this topic (Cerrone et al., 2015; Schwarz et al., 2018), suggesting that a screw press might be the most suitable choice. In the conducted experiments as well as in the literature it has been shown that *C. necator* can be cultivated with non-sterilized (Schwarz et al., 2018), sterile-filtered (Koller et al., 2005), and autoclaved (Cerrone et al., 2015) juice from (ensiled) grass. On a larger scale, filtration is the least suitable method of sterilization. In addition, the major proportion of the solid matter would have to be removed beforehand anyway, as the filters would otherwise become blocked. The advantage of filtration is that the ingredients are hardly affected compared to sterilization by temperature. Autoclaving mainly affects the sugars contained in the product through the Maillard reaction. Other ingredients such as vitamins or trace elements can also be sensitive to temperature and thus be affected. In contrast to the common acidification of the fermentation broth in the course of an unregulated bioprocess, the pH value became alkaline when grass juice was used as the sole growth medium in this work. This may be due to the fact that amino acids are utilized as a carbon source whenever there is a lack of carbon but sufficient nitrogen. By using amino acids as a carbon source, ammonia is split off, which forms an ammonium ion and causes alkalization of the medium. This may indicate that most of the available sugars were destroyed by autoclaving. Gamma radiation sterilization of the medium could be tested on an industrial scale to avoid affecting the ingredients. Apart from the sterilization method, no typical pretreatment method for green waste has been investigated in this work. Pretreatment can make sugars from cellulose and hemicellulose accessible for the microorganisms but can also produce toxic compounds. The influence of different pretreatment methods and the fermentative use of the resulting sugars was already investigated for green waste (Varriale et al., 2022; Volkmar et al., 2023). Additional sugars for fermentation on the press juice growth medium could be obtained from enzymatic hydrolysis of the residual press cake. This would enable comprehensive use of the biomass. After choosing the most suitable production method of the growth medium, the components of the press juice should be analyzed as comprehensively as possible and the substances that can be used by the microorganism should be identified. The limiting substances should be determined based on these results. A medium optimization based on statistical design of experiments (DoE) can be carried out for this purpose. The limiting substances can be supplemented to improve fermentation efficiency. Based on the present results, in the case of the ultimately used autoclaved growth medium, a carbon source such as fructose must probably be added first. In addition to the utilizable carbon and nitrogen sources, a large proportion of the elements potassium, magnesium, and calcium would be particularly important. Trace elements could also be added to the medium rather

inexpensively. Furthermore, it remains to be investigated whether a concentration of the medium would be useful or necessary for cultivation. With regard to feeding with the press juice, a higher concentration could be advantageous so that less volume is introduced into the bioreactor. Since the results showed that the autoclaved grass juice can also be used by *C. necator*, a possible concentration, could be carried out by evaporation through increased temperatures. This would avoid concentration on a larger scale employing filtration. The concentration of grass juice by evaporation was previously shown by Cerrone et al. (Cerrone et al., 2015). Concentration would also be advantageous in terms of any necessary transportation of the growth medium and the necessary storage space. In this regard, processing of the green waste and fermentation ideally takes place at the same location. The issue of the robustness of fermentation must be considered due to the strong heterogeneity of green waste. A simple approach for an initial assessment would be a broad DoE with green waste from different locations and seasons. Certain plants could also contain substances that might have antibacterial effects. Experiments with green waste from various sources must also be carried out in order to investigate the influence of contamination and the need for purification processes. For conventional recycling methods, green waste is already being processed and shredded. Such existing procedures should be used when implementing new procedures. Accordingly, purification processes may already be sufficient for the novel processes.

As part of further experiments, apple pomace residues, which had previously been subjected to hot water extraction, were also examined as a substrate for fermentation. A growth medium was produced from the material in the same way as from the grass clippings and was tested for the cultivation of *Cupriavidus necator* H16. In this case, however, the microorganism could not grow on just the medium from apple pomace. In combination with the minimal medium for *C. necator* (without a carbon source), the apple pomace juice could at least be used as a carbon source for fermentation. This shows the potential of green waste compared to other residual materials, as green waste contains a large number of nutrients. Otherwise, many biological residues are used either as a source of carbon and/or nitrogen. In a recently published joint work the use of grass press juice and an enzymatic hydrolysate of pretreated lignocellulosic biomass as growth medium or medium supplement for the cultivation of the microorganisms *S. cerevisiae*, *Lactobacillus delbrueckii subsp. lactis*, *Ustilago maydis*, and *Clostridium acetobutylicum* was investigated (Volkmar et al., 2023). Similar or higher yields of various basic chemicals were achieved compared to reference processes with established growth media. There are no other comparable results in the literature in which fresh grass juice was also used as a complete growth medium. The most similar results are from Schwarz et al. who used the juice of grass silage as a growth medium for *C. necator* (Schwarz et al., 2018). However, the comparison is difficult, as the composition of fresh grass juice differs greatly from ensilaged grass juice (Koller et al., 2005). In the ensiled grass juice, the generated acids such as acetic acid or lactic acid are the main sources of carbon (Schwarz et al., 2018). Processing the green waste immediately probably makes more sense, as otherwise vast amounts of storage capacity in the form of silos would be required. Furthermore, growth with the silage grass juice is significantly slower than with fresh grass juice, as shown by the maximum growth rate of 0.06 h^{-1} with grass silage compared to 0.43 h^{-1} with fresh grass in the shown experiments. In other cases, (ensiled) grass juice is only added as a source of carbon or nitrogen.

Regarding product formation, the concentration of α-humulene was very low at 2 mg L^{-1} compared to previously reported 2 g L^{-1} in a fed-batch process with fructose as the carbon source (Milker et al., 2021). However, the concentration of 10 mg L^{-1} α-humulene in the

experiment with LB medium was in a similar range to the experiment with grass juice and, in addition, the experiments were designed only to serve as proof of concept. Fed-batch experiments should therefore be carried out in a controlled bioreactor, which would probably allow similar concentrations as 2 g L^{-1} of α-humulene to be achieved. A fed-batch process with the feeding of ensiled grass juice as a carbon source has previously been demonstrated for the cultivation of *Pseudomonas chlororaphis* and *Burkholderia sacchari* (Cerrone et al., 2015). In further experiments, the choice of extraction agent for *in situ* product removal should also be investigated. The use of *n*-dodecane in combination with grass juice is unsuitable as an emulsion is formed in the aqueous phase with the extraction agent due to an unknown ingredient in the grass juice. This phenomenon can be investigated further and based on the results another suitable extraction agent should be selected. The choice of an alternative solvent to *n*-dodecane should also be selected with regard to health, safety, and environmental impact to ensure that it can be used on an industrial scale without concern (Prat et al., 2016). As an alternative to the presented complex process design with *in situ* product removal for the production of α-humulene, a different product could be chosen. This should simplify the process without reducing the added value. The sequencing of the genome of *C. necator* (Pohlmann et al., 2006) opens up further possibilities for optimizing the organism with regard to the utilization of waste material or the production of high-quality products. The necessary infrastructure for production, potential purification, storage, and transportation of the product should also be taken into account.

5.2 Peroxidases from green waste for wastewater treatment

Another material utilization route that was demonstrated was the use of peroxidases from grass clippings for the conversion of phenolic substances for wastewater treatment. Prior to their use in wastewater treatment, the peroxidases were purified and characterized. The specific activity of the peroxidase fraction was only moderately increased by purification. The aim of the purification was to isolate the peroxidases for a conclusive characterization. However, the entire purification process should focus more strongly on minimal yield loss. A compromise was chosen for the purification process for both grasses and additionally the purification process was not designed for maximum specific activity. Accordingly, a higher specific activity could be achieved by an adapted purification scheme. Further chromatography resins should be tested for purification, as this aspect was not given much attention in the work. Hydrophobic interaction chromatography can be followed by another chromatography step with an ion exchange resin, probably anion exchange chromatography since most proteins have a basic isoelectric point, to improve purification. The optimum chromatography conditions such as pH value or salt concentration must then be determined. Within the scope of the characterization, optimal reaction conditions and kinetic parameters for the conversion of guaiacol were determined. The optimum pH value of the grass peroxidases is within the previously reported range of 5 to 6, while the optimum temperature of around 30 °C is slightly lower than the reported 40 °C (Langsdorf et al., 2023; Pandey et al., 2017). However, the lower optimum temperature can be an advantage for the grass peroxidases, as the wastewater treatment could be efficient at ambient temperature. The peroxidases from *Festuca arundinacea* and *Lolium perenne* showed K_m values of 5.3 and 7.5 mM, respectively (Langsdorf et al., 2023), which are not particularly low, but are at least in the middle of the reported range of 0.3 to 9.5 mM for plant peroxidases for the substrate guaiacol (Pandey et al., 2017). The characterization of grass peroxidases should be expanded. It is still unknown whether a single

peroxidase or several peroxidases are responsible for the corresponding reactions in the individual grasses. The proteins present can be separated by molecular weight and isoelectric point using 2D gel electrophoresis. Mass spectrometry or native gel electrophoresis could be used to determine the molecular weight of the peroxidases. Additionally, native gel electrophoresis could be used to examine the individual bands for peroxidase activity and clarify the question of whether one or more peroxidases are present.

The experiments clearly showed that the substrate 2,4-dichlorophenol is converted much more effectively with the grass peroxidases than the other two substrates phenol and *m*-cresol. Similar behavior was previously observed for soybean peroxidase against the three substrates that were also investigated in this work. Using the same substrate concentration, 2,4-dichlorophenol required by far the lowest amount of enzyme for conversion, while *m*-cresol and phenol required about the tenfold enzyme concentration (Caza et al., 1999). However, the conversion of the three substances was also shown with peroxidase isolated from bitter gourd (*Momordica charantia*), where phenol was converted fastest, followed by 2,4-dichlorophenol and *m*-cresol (Akhtar and Husain, 2006). This shows that peroxidases from other sources behave differently. Generally, a quantitative comparison with other publications is difficult because, apart from the reaction conditions and time, especially the amount of enzyme used and the substrate concentration differ greatly. In one example, 4.5 U mL^{-1} of HRP was used to convert over 95% of initial 1 mM phenol and over 96% of initial 0.6 mM 2,4-dichlorophenol within 3 hours (Wagner and Nicell, 2002). Dec and Bollag demonstrated the conversion of 5.2 mM 2,4-dichlorophenol within 5 - 10 minutes with 9.5 U mL^{-1} of HRP (Dec and Bollag, 1994). The effectiveness of the conversion of phenolic substances is first of all dependent on the amount of enzyme used (Caza et al., 1999). Correspondingly, it was shown that the efficiency of the conversion decreases with increasing substrate concentration (Quintanilla-Guerrero et al., 2008). It has also been observed that high substrate concentrations combined with comparatively low enzyme concentrations can lead to substrate inhibition of the grass peroxidases. Due to the lower enzyme concentration in the crude extract, this effect was more pronounced in that case. When using a crude extract from turnip for the conversion of phenolic substances, a reduced conversion with increasing substrate concentration was also noted (Duarte-Vázquez et al., 2003). One exception was the substrate 2,4-dichlorophenol, for which no decreasing conversion efficiency was detected in the investigated range. Similar behavior was observed in this work for the substrate 2,4-dichlorophenol in comparison to phenol and *m*-cresol (Langsdorf et al., 2023).

In biotechnological processes, purification procedures are usually the bottleneck of a production process and are responsible for a large part of the overall process costs. With regard to the isolation of peroxidases from plant material, it is therefore also advantageous to limit purification to the bare minimum. In the case of analytical or diagnostic applications, highly pure enzymes are particularly necessary, as these could otherwise influence the result. The same applies to biochemical syntheses. It was previously shown that high-purity enzymes are not required for wastewater treatment (Feng et al., 2021). The application of a crude extract for wastewater treatment is in any case the more economical and therefore preferred variant for the use of peroxidases. As already shown in other studies, an effective conversion of the phenolic substances with an unpurified crude extract of grasses could be achieved. Using fruit juice from gourd (*Luffa aegyptiaca*) as a crude peroxidase extract, the conversion of phenol and *m*-cresol, among others, was demonstrated (Yadav et al., 2017). The purity of the peroxidases did not influence the conversion. Within this work, a specific activity for guaiacol of 3.7 and 12.3 U mg^{-1} for the crude extracts of the two grass species was obtained (Langsdorf

et al., 2023). In another study, a specific activity of 2.65 U mg^{-1} was achieved with a crude extract of HRP (Lavery et al., 2010). Accordingly, the specific activity of the crude grass extracts is in a similar range to that of the crude extract of the conventionally used HRP. Ultimately, however, a HRP solution with 772 U mg^{-1} was obtained after a complete purification scheme in the same work (Lavery et al., 2010). Since an infinitely high volume of enzyme extract cannot be added to wastewater to be treated, concentration may be necessary. Concentrating a crude extract can be more problematic than with a previously purified enzyme extract due to the large number of ingredients and possible solids present. Such a concentration had previously been demonstrated using a crude extract from turnip, which was concentrated tenfold (Duarte-Vázquez et al., 2003).

Regardless of whether purified peroxidases or a crude extract is used, the enzyme reaction for wastewater treatment can be optimized in many ways. For the profitability of the process, the quantity of enzyme used must be reduced primarily, as this is the main cost factor. This can be done by selecting the optimum reaction parameters. The optimization of the parameters enzyme concentration, pH value, PEG concentration, and H_2O_2 to substrate ratio was demonstrated in the case of the conversion of phenolic substances with soybean peroxidase (Caza et al., 1999). In the experiments, the optimum temperature and pH value, which were determined in the assay with guaiacol, were applied. However, the reaction optima can vary for the individual substances and must still be determined for the respective phenolic substance in order to optimize the conversion. While a pH value of 6.0 was optimal for phenol in the case of soybean peroxidase, the pH optimum for *m*-cresol and 2,4-dichlorophenol was 7.0 (Caza et al., 1999). In another work, the optimal pH value for all three substances was 6.0 while using a peroxidase from bitter gourd (Akhtar and Husain, 2006). In the experiments using a gourd fruit juice as a source of peroxidases by Yadav et al., the peroxidases showed optima towards phenol at 28 °C and pH 7.0 and towards *m*-cresol at 35 °C and pH 6.0 (Yadav et al., 2017). The addition of an additive allows the necessary enzyme concentration to be further reduced by increasing conversion efficiency. The addition of PEG resulted in more efficient conversion in this work as well as other work previously (Caza et al., 1999; Cooper and Nicell, 1996; Wagner and Nicell, 2001). As described previously (Diao et al., 2011; Duarte-Vázquez et al., 2003), the positive influence through the addition of PEG in terms of a reduced reaction time when using a crude extract compared to a pure enzyme could be confirmed. The influence of different molecular weights of PEG could first be examined in order to then determine the optimal concentration of PEG in the reaction mixture. Another important parameter is the H_2O_2 concentration used. In this work, an excess of H_2O_2 was used so that there were no limitations. However, the ratio of substrate to H_2O_2 can also have an influence on the conversion, which is why this ratio also needs to be optimized following the previous parameters. The influence of the substrate to H_2O_2 ratio has been investigated in a number of publications (Akhtar and Husain, 2006; Caza et al., 1999; Dahili et al., 2015; Wang et al., 2015; Wu et al., 1997). By optimizing the H_2O_2 concentration, 20% of the enzyme could be saved (Wagner and Nicell, 2001). In order to have a permanent optimal concentration of H_2O_2 and thus promote a continuous process, H_2O_2 can be produced electrochemically *in situ* (Burek et al., 2019).

The use of waste materials as a source of enzymes creates additional challenges. Process robustness is an important aspect in this context. Specific lawn grass species were planted and used for the experiments in order to obtain reproducible results and not be influenced by varying raw materials. In the case of grass from lawns, it is also not possible to say with certainty which grass species is present. The influence of varying raw materials on enzyme extraction and enzyme activity is to be investigated. Contamination can occur in public waste,

which must be separated before enzyme extraction. There may be plants with ingredients that affect the enzymes by inhibiting peroxidase activity. Fluctuating peroxidase activities depending on the time at which the grass was harvested have already been detected in the laboratory. Seasonal variations must also be taken into account. In winter, green waste consists mainly of woody materials and foliage. In preliminary tests, no peroxidase activity could be detected in foliage. Another important aspect for further work is to test the grass peroxidases for the conversion of phenolic substances in real wastewater. In this work, solutions of individual phenolic substances were used. In real wastewater, the actual concentrations of phenolic substances can vary and mixtures of different substances are usually present. Furthermore, the enzymes must be stable in the wastewater. Various parameters can be optimized regarding the treatment of real wastewater such as choosing suitable enzymes for the substances present and the existing reaction conditions. For example, the grass peroxidases have shown high activity towards 2,4-dichlorophenol compared to the other substrates phenol and *m*-cresol. In real applications, when several phenolic substances are likely to be present, a compromise must be found regarding temperature and pH value. Advantageously, the peroxidases of the two grass species examined showed similar temperature and pH optima in the enzyme assay with guaiacol. This promises that in real applications a compromise can be found with regard to the reaction conditions when mixtures of different grasses or plants in general are present. Plants that grow in similar environmental conditions are likely to have similar optimal reaction conditions for peroxidases, at least in terms of temperature. It must be investigated how peroxidase mixtures behave when shrubs, bushes, or leaves are added besides grass clippings.

In the future, more substrates should be investigated in order to possibly find further phenolic substances that can be converted by the grass peroxidases as effectively or even faster than 2,4-dichlorophenol. Peroxidases might also be used in the special case of the conversion of endocrine-disrupting compounds (Auriol et al., 2007) or the conversion of azo dyes (Mohan et al., 2005). The conversion of dyes was previously demonstrated with *Miscanthus x giganteus* using a *Poaceae* (Dragana et al., 2017). The performance of the grass peroxidases should be compared in the following with the established HRP. It would be reasonable to further use a crude extract of horseradish as a comparison. Ultimately, a cost calculation and life cycle assessment must be carried out for a suitable real-life application in order to determine the profitability in comparison to commercial HRP.

5.3 Electrodes from carbonized green waste for bioelectrochemical systems

The carbonization of grass clippings and the production of electrodes from the resulting biochar, which were examined for use in microbial electrosynthesis and microbial fuel cells, were investigated. First of all, the carbonization process holds potential for optimization. The parameters of hydrothermal carbonization and pyrolysis have a decisive influence on the properties of the resulting biochar. The carbon content of the biochar generally increases with the duration of pyrolysis and higher pyrolysis temperature (Ronsse et al., 2013). Similarly, the graphite content also increases with higher pyrolysis temperature (Gabhi et al., 2020). The carbon structure (graphite/graphene) primarily determines the electrical conductivity of the biochar (Gabhi et al., 2017). Raman spectroscopy could be used to determine the degree of order of the carbon to optimize the carbonization process in this regard. In a Raman spectrum, a D peak (“disordered”) can be found, whose intensity indicates disordered carbon, while the

intensity of the G peak (“graphene/graphite”) reflects the ordered carbon structure. The D peak lies at about 1348 cm^{-1} and the G peak at about 1582 cm^{-1} (Zhan et al., 2021). Correspondingly, with increasing pyrolysis temperature, the extent of the G peak also increases (Gehring et al., 2019). A higher pyrolysis temperature can also increase the specific surface area of the biochar (Ronsse et al., 2013). The pyrolyzed biochar from grass clippings did show a specific surface area very similar to previously reported values of pyrolyzed mixed green waste although pyrolysis temperature and duration were considerably different (Ronsse et al., 2013). Extended pyrolysis time and a higher temperature, however, also lead to a lower yield and higher energy consumption and therefore higher operating costs. Consequently, a compromise must be found with regard to the choice of pyrolysis duration/temperature and the desired properties of the biochar. In addition to varying the carbonization parameters, chemical activation of the biochar can be tested to generate favorable properties. Preliminary tests have shown that the carbon concentration hardly changed with the additional step of chemical activation with KOH. As it was primarily important to produce highly conductive carbons, this additional step was therefore omitted in the experiments. However, chemical activation can be used to increase the specific surface area of the biochar (Ding et al., 2020; González-García et al., 2013; Jin et al., 2014). Furthermore, the carbonization experiments were carried out with quite homogeneous grass clippings, in order to achieve the most reliable results possible. In the following experiments, the influence of different compositions of green waste with regard to the biochar properties must be investigated to assess the robustness of the carbonization process. Also, when using publicly collected waste material, the risk of non-biological contaminants such as plastic waste must be considered. These contaminants must be sorted out before the material is carbonized. The existing infrastructure for green waste disposal and recycling can be used to collect and process the material.

Various methods were tested for the production of usable electrodes from biochar for bioelectrochemical systems. The two most successful methods were pressing of the biochar and the application of the biochar onto a metallic carrier/current collector. A binder (PVDF) was necessary for both methods in order to obtain a stable electrode. In experiments with biochar after hydrothermal carbonization, the biochar itself could be pressed into stable pellets without a binding agent. This was probably due to the fact that the remaining lignin acts as a binder (Reza et al., 2012), which is only completely destroyed after pyrolysis. Coating a metallic current collector (dimensionally stable anode) with biochar was chosen for electrode production, as contacting the pressed electrodes proved to be inconvenient. By pressing the electrodes for example into an elongated cylindrical or rectangular shape, the metallic carrier could be eliminated if this enables reproducible contacting. Also, the advantage of pressing is that the electrical conductivity is increased by compacting the previously powdery material (Adinaveen et al., 2016). Melting a binder, mixing it with the biochar, and allowing the mixture to harden in the desired geometry was also investigated. However, this turned out to be impractical in the laboratory. The alternative of dissolving the binder in a solvent and then mixing it with the biochar is a preferable alternative, as it does not require as much energy. Polylactic acid (PLA) might be a promising alternative binder as it is biodegradable and can be made from bio-based raw materials. However, the microorganisms must not be able to break down the PLA during the bioelectrochemical process. Granulation of the biochar might be interesting for use in a fluidized bed reactor as a fluidized bed electrode. Ren et al. have previously demonstrated the granulation of biochar (Ren et al., 2015). A fluidized bed reactor can be designed with granulate as the electrode material, enabling improved mass transfer (Dong et al., 2018). However, there is still a lot of research to be done in this field. Individual shapes of the electrodes could also be realized by additive manufacturing as previously shown

for biochar (Idrees et al., 2018). Additive manufacturing is a practical method for testing new electrode geometries in the laboratory. Rocha et al. have shown how the production of such a conductive 3D printing filament could work (Rocha et al., 2020). However, this is not (yet) practical on an industrial scale. The most optimal processing of the biochar into an electrode for primary use in the bioelectrochemical system must be investigated further. Finally, a manufacturing process should be selected that can also be implemented semi-automatically on a large scale.

The application of the produced carbonized grass electrodes in microbial electrosynthesis for water electrolysis was investigated. However, as expected, the performance of the electrodes produced could not keep up with conventional metal electrodes (e.g. Pt). The dimensionally stable anodes are designed for oxygen formation in water electrolysis. The metal also has a higher electrical conductivity, which means that ultimately less energy is required. As a result, the microbial growth of *C. necator* was slower using the carbonized grass electrode. The carbonized grass electrodes have shown different electrochemical properties than the dimensionally stable anode as demonstrated via cyclic voltammetry as well as linear sweep voltammetry. It appears that unspecific side reactions take place or the electrodes show an ohmic behavior. The electrochemical behavior of the electrodes should be investigated further to improve the electron transfer. However, the use of electrodes in microbial electrosynthesis is not discussed any further.

It was shown that electrodes made from carbonized green waste have potential for application in the MFC. In the experiments, the biochar and the carbonized grass electrodes showed some advantageous properties compared to commercial graphite electrodes. When using the carbonized grass electrodes in the MFC with *G. sulfurreducens*, the acetate was consumed faster than with graphite electrodes, resulting in an earlier current response. However, it should also be noted that due to the design of the MFC and the different geometries of the electrodes, the surface area of the carbonized grass electrode is almost 30% larger in the experiments. Accordingly, a possible faster biofilm formation and faster substrate consumption may be due to the larger electrode surface area. On the other hand, advantageous surface properties of the carbonized grass electrode may also be responsible for faster biofilm formation. For example, microscopic observation revealed a considerably more porous surface structure of the carbonized grass electrodes compared to graphite. Overall, however, the integral of the current curve was larger for the graphite electrode. This could be due to the better electrochemical properties of the graphite electrode. The graphite has a higher conductivity and thus favors electron transport, while patterns of ohmic resistances or unspecified side reactions were detected in the electrochemical characterization of the carbonized grass electrodes. It was shown that the carbonized grass electrodes are suitable for use in the MFC over a longer period of more than six weeks. A substantially longer running time would probably have been possible. In future experiments, the carbonized grass electrodes should be tested in real MFC applications in municipal or industrial wastewater treatment plants.

5.4 Production of PHB from flue gas by microbial electrosynthesis

This work demonstrates for the first time the production of PHB with *C. necator* in microbial electrosynthesis using flue gas as a CO_2 source. Additionally, this was the first time that microbial electrosynthesis using flue gas as a source of CO_2 was demonstrated with

continuous gassing with flue gas directly on-site at the power plant. The use of flue gas has shown no detectable inhibitory effect on microbial growth and product formation compared to a clean gas mixture. In MES experiments with *C. necator* and a similar flue gas composition by Wu et al., also no inhibition of microbial growth was observed (Wu et al., 2022). However, they gassed every 24 h for 30 min with the flue gas. With the present results, it can therefore be concluded that even continuous gassing with flue gas over several days has no adverse effect on the microorganism. This is an important next step towards a pilot plant in an industrial environment. The PHB concentrations from the microbial electrosynthesis are far below the previously reported values, especially as the experiments only serve as a proof of concept. The experimental setup can be optimized, particularly with regard to process mode, gas pressure, gas residence time, or electrode-to-volume ratio. A comparison of the experiments in the laboratory and the cogeneration plant shows that a component of the flue gas causes the pH value to drop. The exact cause is still unclear and needs to be investigated in further experiments. However, a component of the flue gas could influence the pH value either directly or as a result of electrochemical or biological conversion. Therefore, pH control must be implemented in experiments with continuous gassing with flue gas. The electrochemical process can be optimized, primarily by reducing the energy input. This can be achieved by reducing the internal resistance, e.g. by increasing the medium conductivity or by adjusting the hydrogen production to the consumption of the microorganisms. Due to the advances in electrochemical hydrogen production, the fermentative use of CO_2 in combination with electrochemically generated hydrogen as an electron donor possesses a high technology readiness level compared to similar processes (Stöckl et al., 2022a). However, this also shows that the limitation lies on the biotechnological side of the process. This means that the technical process parameters in particular must be optimized, but genetic modifications to the organism can also provide a decisive advantage. It was recently shown that using a genetically modified *C. necator* strain, an 11% increase in biomass accumulation and a 28% increase in PHB formation could be achieved (Kim et al., 2022). In addition to the production of PHB by *C. necator*, genetic engineering of the microorganism allows the production of further valuable products such as fuels and solvents (Teetz et al., 2022) or different terpenes (Krieg et al., 2018; Milker and Holtmann, 2021; Wu et al., 2022). All microbial electrosynthesis experiments were carried out at ambient pressure. The gas transfer in the reactor can be improved by applying an overpressure (Yu and Munasinghe, 2018). PHB production can be limited by poor oxygen transfer at high cell densities (Garcia-Gonzalez et al., 2015). It can be assumed that the low gas solubility of carbon dioxide and hydrogen also influences PHB production and microbial growth during autotrophic cultivation. The optimal use of the gases should be ensured. In order to obtain a balance of CO_2 consumption and PHB production, the CO_2 concentration of gas entering and leaving the reactor should be analyzed in the future. The analysis can also provide information on hydrogen and oxygen consumption. Ideally, the ratio of the individual gases is adapted to the consumption of the microorganism. On a larger scale, continuous gas monitoring is essential anyway to control the risk of explosion. Overall, the residence time of H_2 must be increased. The gas residence time and gas transfer in microbial electrosynthesis can be optimized through a suitable design of the bioreactor by using a bubble column or an airlift reactor (Pan et al., 2024). When setting up a larger bioreactor, safety aspects play a particularly important role due to the risk of explosion with hydrogen and oxygen. Accordingly, an explosion-proof reactor must be designed. What such a reactor could look like has already been demonstrated on a laboratory scale for autotrophic PHB production with *C. necator* (Lambauer and Kratzer, 2022). As shown by Lim et al., a continuous process for PHB

production can be realized by the simultaneous extraction of PHB and the feeding of fresh cells (Lim et al., 2023).

In theory, microbial electrosynthesis can also be made more economically efficient by working with the highest possible current density. For this purpose, the electrode surface area needs to be kept as small as possible. Under optimal conditions, investment costs can be compensated through higher productivity despite lower energy efficiency. The cultivation of *C. necator* at higher current densities of about 100 mA cm^{-2} was investigated in the laboratory. However, the high current density also causes the voltage to rise considerably, which can lead to undesirable side reactions. The internal resistance must therefore be kept as low as possible in order to keep the voltage low and generally reduce energy consumption. In order to increase the conductivity of the electrolyte or growth medium, the buffer concentration in the medium was increased. A higher buffer concentration ensures higher conductivity and therefore a lower internal resistance. Up to a tenfold buffer concentration in the growth medium, similar growth of *C. necator* as in the standard medium was possible. With the help of adaptive laboratory evolution, the salt tolerance of microorganisms could be enhanced to further increase the conductivity of the growth medium. Applying high current densities intensifies the effects near the electrode surface such as concentration differences, extreme pH values, or extreme temperatures. In this case, there are new requirements for the electrolyte. Alternating the polarity of the working and counter electrodes can help to counteract adverse effects. The distance between the electrodes can be minimized using a special zero-gap electrode design, which greatly reduces the internal resistance. For this purpose, the working and counter electrode can be cut into a carrier material or be pressed onto a membrane.

The efficiency of the individual processes can also be increased by separating water electrolysis and fermentation. Hydrogen and oxygen can be fed into the bioreactor from the separate water hydrolysis in addition to CO_2. This means that there is no need to compromise on the reaction conditions such as the salt concentration. A similar strategy would be the prior electrochemical conversion of CO_2 into another substrate for the microorganism, which is then utilized as a substrate in the fermentation. One-carbon compounds such as methanol, formic acid, or carbon monoxide are promising substances in this case (Stöckl et al., 2022a). Before the microbial electrosynthesis was tested with *in situ* water electrolysis in the cogeneration plant, the electrochemical conversion of CO_2 in the flue gas to formate was investigated. A plate reactor with a gas diffusion electrode was used for this purpose. In a subsequent step, the formate was to serve as a simultaneous carbon and electron source in fermentation with *C. necator*. Compared to laboratory tests with pure CO_2, no formate could be produced with the flue gas. This was probably due to the fact that the catalyst used favored a different electrochemical reaction, presumably the reaction of oxygen. Perhaps there is a more suitable catalyst for this particular application. However, this shows the advantage of the much more specific biocatalysis, enabling the use of CO_2 from flue gas. Furthermore, the advantage of microbial electrosynthesis is that it combines all steps in one process. With regard to the fermentative PHB production, the first and second phases can also be separated in such a way that the first phase is carried out heterotrophically on a different carbon substrate and is only switched to CO_2 in the second phase. Tanaka and Ishizaki, for example, cultivated *C. necator* in a mineral medium with fructose as a carbon and energy source in the first phase and switched to autotrophic cultivation in the same medium but without fructose for PHB formation (Tanaka and Ishizaki, 1994). By carrying out the first phase heterotrophically, faster microbial growth and consequently a higher cell density can probably be achieved. However, the microorganism must first become adapted to the autotrophic metabolism in the second

phase. In such a case, the heterotrophic growth phase can also be carried out with biogenic residues as substrates, as it was demonstrated with the growth medium from grass clippings (Langsdorf et al., 2022) to keep the feedstock costs to a minimum.

Costs are initially the decisive factor in the market launch of innovative processes or products. Within the publication, the electricity costs for one kilogram of PHB have already been calculated. This resulted in a price of 75 € or 81 € per kilogram of PHB in the laboratory or cogeneration plant due to the electricity costs alone. The global average price of a ton of the similar fossil polymer polypropylene in 2022 was 1133 US$ (Statista, 2024). At the time of writing, this would correspond to a price of 1.04 € per kilogram of polypropylene. Accordingly, a competitive price is still a long way off. However, it has been shown that biotechnological processes are already getting closer to these values. Price estimates of 24 € kg^{-1} (Panuschka et al., 2019), 11.8 US$ kg^{-1} (Mudliar et al., 2008), and 11.50 - 14.00 € kg^{-1} (Listewnik et al., 2007) were reported for the biotechnological production of PHB. The prices calculated depend heavily on the size of the plant, the assumed PHB yield, and the desired purity of the polymer. However, the impact on the climate and the environment is also a decisive factor. The emissions for polypropylene resin pellets were estimated at 1.34 kg CO_2 equivalents per kg of polymer (Greene, 2011). A more competitive value can be achieved for PHB by using residual materials. Alissa Kendall modeled the PHB production based on organics from municipal solid waste to lead to emissions of 3.4 to 5.0 kg CO_2 equivalents per kg of polymer (Kendall, 2012). A similar estimate should be made for the production of PHB from off-gas CO_2 in microbial electrosynthesis using renewable energy for water electrolysis. In the case of PHB production using CO_2 from flue gas as a substrate, the introduction can be accelerated by pricing CO_2 emissions or pricing the production of non-degradable polymers. In this way, such new sustainable products and technologies can be made more attractive. Nonetheless, the productivity and efficiency of the process must continue to be improved. As already described, the setup is a proof of concept and the costs can be greatly reduced through optimization and scaling.

6 Conclusions and future perspectives

In this work, alternative material recycling routes for waste materials and options for the future design of an economy based on biogenic resources, residual materials, and renewable energy were examined. The use of waste materials as raw materials can help to make the chemical industry more sustainable by reducing the generation of waste and emissions, as well as the consumption of fossil resources.

It was shown that *C. necator* was able to grow on juice from grass clippings as a complete growth medium without any other additives. Using the grass juice medium, the terpene α-humulene could be produced. Due to the variability in products from *C. necator*, the cultivation on grass juice has a platform character. Successful fermentation with grass press juice as a complete growth medium was also demonstrated, e.g. for ethanol production with *S. cerevisiae* (Volkmar et al., 2023). This shows the potential of green waste as a feedstock for growth media. There is a need for optimization in the preparation of the growth medium as well as the product formation since the extraction agent *n*-dodecane proved to be unsuitable. Furthermore, the ingredients of the grass juice that can be used by the microorganism must be identified and the influence of the varying starting material on the fermentation must be analyzed. The fermentation should also be carried out in a controlled bioreactor as a fed-batch process. The development of a consolidated bioprocess in which enzymatic hydrolysis and product formation are combined has great potential for the comprehensive use of green waste. With respect to the transition to a vegetarian food culture, the cultivation of edible mushrooms, which possess the necessary enzymes for the degradation of lignocellulose, might be another option for the utilization of lignocellulosic waste. One such example was demonstrated for the commonly used oyster mushroom (Amirta et al., 2016). The UN Sustainable Development Goals (SDG) provide guidelines for shaping a sustainable future. The use of green waste as a fermentation substrate contributes to SDG 12 “Responsible Consumption and Production”. Depending on the product, a further contribution can be made to the UN SDGs.

The successful use of peroxidases from grasses for the conversion of phenolic substances for wastewater treatment was demonstrated. In particular, the grass peroxidases could be used for wastewater with a high content of 2,4-dichlorophenol, since the enzymes showed the highest activity towards 2,4-dichlorophenol out of the tested substrates. As far as possible, peroxidases from grass clippings or green waste should be used as a crude extract for wastewater treatment, as purification of the enzymes would be too costly. Since peroxidases from green waste might not be able to cover the enzyme need for wastewater treatment on its own, the peroxidase crude extracts could be used as a “green” supplement. In the future, further phenolic substrates and real wastewater in particular are to be investigated and compared with the performance of the established HRP. Additionally, wastewater treatment with peroxidases can be further intensified by integrating electrochemical *in situ* H_2O_2 generation. The extraction of peroxidases from green waste contributes to SDG 2 “Zero hunger”, as the raw material does not compete with food for land. It also contributes to SDG 6 “Clean water and sanitation”, SDG 12 “Responsible consumption and production”, SDG 14 "Life below water", and SDG 15 “Life on land” by the removal of toxic phenolic substances.

It was shown that carbonized grass can be an alternative to fossil graphite as an electrode material for microbial fuel cells. A microbial fuel cell with carbonized grass electrodes was successfully operated for more than six weeks. It should be investigated at what time of year carbonization for electrode production is most profitable since a high energy input is required

for pyrolysis. An alternative green waste processing method can be considered for the months in which carbonization of green waste has the least potential. Once an optimal manufacturing process for electrodes specifically for the MFC has been established, a DoE-based experimental setup can be used to produce biochar that is optimal for use in the MFC. Furthermore, the application of carbonized grass electrodes in the microbial fuel cell should be used in a real environment, on a municipal and/or industrial wastewater treatment plant. The production of biochar from biomass as an alternative to fossil graphite for electrodes contributes to SDG 12 “Responsible Consumption and Production” and SDG 13 “Climate Action”. By advancing the microbial fuel cell as an energy generation technology, a contribution is made to SDG 7 “Affordable and Clean Energy”.

Finally, a microbial electrosynthesis for the production of the bioplastic PHB from flue gas as a CO_2 source was successfully realized in a cogeneration plant. It should be further investigated how the growth and product formation phases can be designed separately from each other and how the electrochemical and microbial processes can be optimized by possibly decoupling them. In addition, a pilot plant in the form of a controlled bioelectrochemical reactor should be developed for integration in a cogeneration plant. A modular system, which is relatively simple to install in any power plant, could generate added value from the exhaust gas CO_2. The implementation of a semi-continuous process would be interesting. Even if no significant influence of the flue gas on microbial growth and PHB production was seen in the experiments, new limitations may arise due to the scaling and continuous operation of the experiments. The main aspects that need to be further investigated are the development of an efficient zero-gap electrode design, the optimization of the growth medium with regard to the reaction conditions, the design of a suitable reactor that allows a high gas residence time, and the adaptation of the microorganism to the process conditions. Another approach for future work on microbial electrosyntheses is the introduction of extracellular electron transfer pathways into established microorganisms such as *E. coli*. The utilization of CO_2 emissions contributes primarily to SDG 13 "Climate Action". The production and use of biodegradable polymers contribute in particular to SDG 12 "Responsible Consumption and Production", SDG 14 "Life below Water" and SDG 15 "Life on Land".

In order to achieve a higher added value as well as a lower CO_2 footprint from green waste compared to previous recycling methods, only an all-encompassing utilization of the waste material in the form of a biorefinery can be considered. Currently, there is a lack of data for a reliable assessment of which technology is most suitable in terms of economic and ecological factors as part of a green waste biorefinery. In the future, a green waste biorefinery should be modeled in order to be able to perform a reliable ecological and economic evaluation. For example, Patterson et al. have modeled the microbial production of polyhydroxyalkanoates from waste grass and cultivated grass using green biorefining (Patterson et al., 2021). According to their calculations, over 400 tons of dried biopolymer can be produced from 30,000 tons of fresh grass with cumulative energy demand in the same order of magnitude as for fossil-based polymers. In addition to an economic assessment, the sustainability aspect of the biorefinery must also be considered. Useful criteria for assessing the sustainability of a biorefinery were proposed by Sacramento-Rivero (Sacramento-Rivero, 2012). A biorefinery pilot plant for green waste has to be built from the most promising recycling methods, which also interact and build on each other most efficiently. In pilot plants, the profitability of the processes can also be assessed more accurately. A first concept of what such a green waste biorefinery could look like was previously shown (Kircher and Bayer, 2022) and is reproduced in Figure 1. In the biorefinery, solid and liquid components of the green waste must be

separated from each other by pressing. For the utilization of the solid fraction, only the carbonization of green waste for electrode production was investigated in this work. Leftover dried solids could also be used as lignocellulosic fillers in biocomposites used as building materials (Viretto et al., 2021). However, woody waste is currently preferred for these applications. With regard to the liquid fraction, utilization as fermentation feedstock is currently more likely than the use as peroxidase crude extract. The use as a growth medium has provided promising results compared to the varying phenol conversion rates and the low enzyme yields. Such a green waste biorefinery can be integrated into existing recycling processes such as composting or utilization in biogas plants.

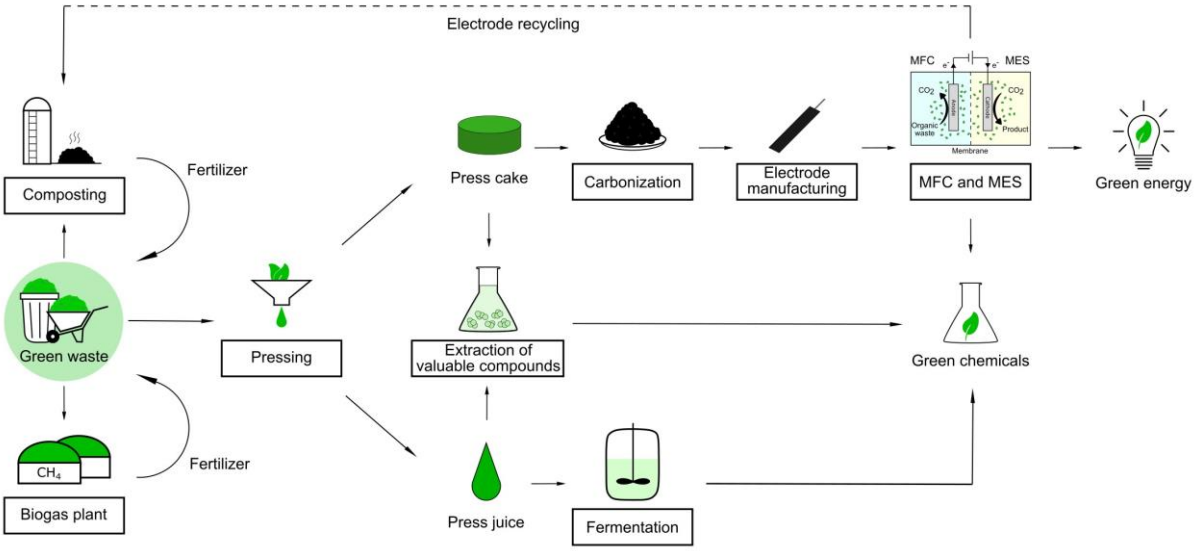

Figure 1: Concept of a green waste biorefinery integrated into existing recycling infrastructure (reproduced with permission from Springer Nature under license number 5785970275332 from May 11th, 2024).

The coupling of biorefineries with bioelectrochemical systems offers great potential for a future sustainable bio-based economy (Harnisch and Urban, 2018). Electrobiorefineries describe a future scenario in which bioelectrosyntheses are implemented in biorefinery concepts in order to convert renewable raw materials into material products with the help of renewable energy. This enables the chemical industry to be linked with the energy sector allowing renewable energy to be used for chemical syntheses. For the future use of bioelectrochemical systems, there is still a need for research into electron transfer mechanisms, the development of suitable bioelectrochemical systems, and reactor design. The strong heterogeneity of green waste, caused primarily by local and seasonal differences, remains the major problem hindering the development of alternative recycling methods. Consequently, the recycling solution must be highly robust. As the composition of green waste varies strongly over the year due to seasonal variabilities, the application of different methods over the course of the year might be advantageous. In the winter months, green waste does not contain much liquid, which is why the production of a growth medium might not be possible. Regarding recycling methods that use the solid fraction of green waste, a lower proportion of liquid can be an advantage. For example, foliage has a low lignin content and little liquid, so in theory, it would be well suited for carbonization.

The advantage of new technologies for off-gas CO_2 utilization over new technologies for green waste utilization is that off-gas has a manageable heterogeneity compared to green waste.

Furthermore, they do not compete with existing recycling methods such as composting. The burden of CO_2 emissions is also a more pressing challenge. For these reasons, it is more likely that CO_2 utilization technologies will be implemented more quickly than alternative recycling processes for green waste. As part of the Rheticus project by the companies Siemens Energy and Evonik, a novel process for CO_2 utilization consisting of electrochemical methods and fermentation has made it into a pilot plant (German Federal Ministry of Education and Research, 2019). In the first step, CO_2 and water are converted into carbon monoxide and hydrogen in a co-electrolysis process to produce syngas. In the second step, the syngas is used as a substrate for clostridia to produce alcohols. Ultimately, suitable products need to be found for the valorization of the respective residual material. The products must be produced efficiently and as conveniently as possible within the existing infrastructure, while achieving the highest possible added value and contributing to a sustainable economy. Using only the microorganism *C. necator*, the production of the terpene α-humulene from grass clippings and the production of polyhydroxybutyrate from flue gas as a source of CO_2 was demonstrated. This shows the potential of biotransforming waste materials into valuable products. Further work should aim to find the most suitable biocatalyst for the respective applications, to use genetic modifications to tailor this organism to the application, and to optimize the process to suit the organism. Likely, this would promote the development of attractive processes.

Ultimately, however, alternative processes must have a superior cost balance compared to the current production or recycling processes. As long as more sustainable alternatives cannot compete with established processes and products in terms of cost, this will remain the primary obstacle to commercial introduction. In the case of green waste recycling, this process has to compete with composting in particular. With off-gas CO_2, it is somewhat more complicated, as the CO_2 is currently simply released into the atmosphere. The pricing of CO_2 emissions or the generation of non-biodegradable plastic waste can help pave the way for the commercialization of more sustainable products and technologies. The 1.5 and 2 °C target by 2030 of the Paris Agreement was mentioned at the beginning. However, it is also important to look beyond this period. Most technologies may not be ready for commercial use by 2030. Particularly in Germany, a country with few fossil resources and a strong technological base, a bio-based economy and the use of bioelectrosynthesis to utilize renewable electrical energy have great potential. Bringing together interdisciplinary scientists from disciplines such as bioprocess engineering, molecular biology, electrochemistry, materials science, etc. is important for the further development of these novel technologies. This also means that related projects must continue to be supported by public authorities and industry, especially with regard to the transfer of laboratory results into pilot plants and commercial products. The infrastructure of the fossil-based economy must be gradually transformed towards a bio-based economy and the use of renewable energies. Even in the laboratory tests carried out in this work, it was not possible to use publicly collected waste material for experiments due to legal hurdles. This means that the legal framework for new technologies that utilize residual and waste streams must be created in the future. Legal restrictions must not stand in the way of the introduction of new technologies. Increasing awareness of sustainability issues in society can accelerate the shift towards more sustainable technology and products. However, with regard to climate change and environmental pollution, this transition is currently progressing nowhere near fast enough. Only through a comprehensive approach to the above-mentioned points a shift to a circular bioeconomy can be achieved in the near future.

List of publications

Antichronologically listed scientific publications in the course of the doctorate:

Langsdorf, A., Halim, M., Volkmar, M., Stöckl, M., Harnisch, R., Hahn, P., Ulber, R., Holtmann, D., 2024. Electrodes from carbonized grass clippings for bioelectrochemical systems. Cleaner Chemical Engineering 9, 100118. https://doi.org/10.1016/j.clce.2024.100118.

Langsdorf, A., Schütz, J.P., Ulber, R., Stöckl, M., Holtmann, D., 2024. Production of polyhydroxybutyrate from industrial flue gas by microbial electrosynthesis. Journal of CO2 Utilization 83, 102800. https://doi.org/10.1016/j.jcou.2024.102800.

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

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

Langsdorf, A., Volkmar, M., Ulber, R., Hollmann, F., Holtmann, D., 2023. Peroxidases from grass clippings for the removal of phenolic compounds from wastewater. Bioresource Technology Reports 22, 101471. https://doi.org/10.1016/j.biteb.2023.101471.

Langsdorf, A., Drommershausen, A.-L., Volkmar, M., Ulber, R., Holtmann, D., 2022. Fermentative α-humulene production from homogenized grass clippings as a growth medium. Molecules 27, 8684. https://doi.org/10.3390/molecules27248684.

Langsdorf, A., Volkmar, M., Ulber, R., Holtmann, D., 2022. The green waste biorefinery, in: Jacob-Lopes, E., Queiroz Zepka, L., Costa Deprá, M. (Eds.), Handbook of waste biorefinery: circular economy of renewable energy. Springer International Publishing, Cham, pp. 906–907.

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

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