Microbial production of *cis,cis*-muconic acid from hydrothermally converted lignocellulose

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Summary

Cis,cis-muconic acid, a chemical of recognized commercial value, receives increasing interest to be produced from renewables. Catabolic microbial pathways can be tailored to accumulate *cis,cis*-muconic acid from a range of aromatic compounds. A renewable, sustainable and under-valued resource for aromatics is lignin. In this work, using hydrothermal conversion, several lignin types were depolymerized into hydrolysates with up to 615 mM aromatic monomer content. Catechol-rich hydrolysates were generated for bioconversion with the previously developed *cis,cis*-muconic acid producers *P. putida* MA-9 and *C. glutamicum* MA-2, whereas hydrolysates were guaiacol-rich for *Amycolatopsis sp.* MA-2. When grown with glucose as a co-substrate, *C. glutamicum* MA-2 yielded 2.6 g L^{-1} (100 % yield) *cis,cis*-muconic acid from catechol. Towards an even more sustainable process, glucose was then replaced by hemicellulose, a non-food renewable. Hemicellulose, a co-constituent of lignin in lignocellulose, was hydrothermally converted into a mixture of C_5 and C_6 sugars. As hemicellulose was mainly converted into xylose (91 % yield), *C. glutamicum* MA-2 was engineered to utilize this pentose. Fed-batch bioconversion on a catechol-rich Kraft lignin hydrolysate as well as a hemicellulose hydrolysate using *C. glutamicum* MA-4 yielded 4 g L^{-1} muconic acids. As the developed process was non-competitive to feed and food, it is a promising starting point for future application in bio-based industrial settings.

Zusammenfassung

Für *Cis,cis*-Mukonsäure, eine wichtige Chemikalie, gibt es großes Interesse diese auf Basis nachwachsender Rohstoffe herzustellen. Mikrobielle Stoffwechselwege können mittels biotechnologischer Methoden so angepasst werden, dass *cis,cis*-Mukonsäure auf Basis von aromatischen Verbindungen akkumuliert. Eine erneuerbare, nachhaltige und kaum genutzte Quelle für Aromaten ist Lignin. Mittels hydrothermaler Umsetzung wurden verschiedene Lignintypen depolymerisiert, um Hydrolysate mit bis zu 615 mM aromatischem Inhalt zu erzeugen. Hydrolysate mit hohem Catecholgehalt wurden für Umsetzungen mit vorher entwickelten Mukonsäureproduzenten *P. putida* MA-9 und *C. glutamicum* MA-2 erstellt. Hydrolysate mit hohem Guaiacolgehalt wurden mit *Amycolatopsis sp.* MA-2 getestet. *C. glutamicum* MA-2 erreichte 2,6 g L^{-1} (100 % Ausbeute) *cis,cis*-Mukonsäure mit Glucose als Co-Substrat. Um einen vollständig erneuerbaren Prozess zu gestalten, wurde Glucose durch Hemicellulose ersetzt. Hemicellulose, wurde mittels hydrothermaler Umsetzung zu C_5 und C_6 Zuckern depolymerisiert. Da als Hauptprodukt Xylose (91 % Ausbeute) gewonnen wurde, wurde *C. glutamicum* MA-2 weiterentwickelt um diesen C_5 Zucker zu nutzen. Bei Umsetzung auf IndulinAT Hydrolysat und Hemicellulose Hydrolysat mit *C. glutamicum* MA-4 wurden 4 g L^{-1} Mukonsäuren erreicht. Da dieser Prozess nicht mit der Futter- und Nahrungsindustrie konkurriert, ist er daher ein vielversprechender Startpunkt für zukünftige Anwendungen in einer bio-basierten Industrie.

1 Introduction

1.1 General introduction

The use of fossil resources poses serious environmental risks and hazards [36], which has initiated a strong interest in more sustainable alternatives. The pursuit of sustainable alternatives needs to consider, among others, environmental friendliness, CO_2-neutrality, renewability and non-competitiveness to feed- and food-industry. A particularly attractive resource that meets these criteria is lignocellulose [145], a highly oxygenated, heterogeneous solid that constitutes the inedible plant biomass. It consists of 3 different polymers: cellulose, hemicellulose and lignin [145]. Cellulose is a linear β-D-glucose polymer [86]. Hemicellulose, on the other hand, is a branched heteropolymeric carbohydrate, containing mainly C_5 and C_6 sugars [140]. Lignin is an aromatic polymer with an undefined primary structure of cross-linked phenolic rings [145]. Due to the exceptional recalcitrance of lignin, its valorization requires an aggressive fractionation and depolymerization to derive aromatic monomers, i.e. high temperature and pressure, often in combination with solvents and catalysts [145]. Hereby, the resulting monomer yield and product spectrum depend on the source of the lignocellulosic biomass, its degree of polymerization as well as the chosen fractionation and depolymerization method [145].

Notably, the derived aromatic monomers can fuel microbial processes to generate valuable chemical intermediates such as vanillin, vanillate, ferulate, pyruvate, PHA and *cis,cis*-muconic acid [20]. To exploit the bioconversion of lignin-derived aromatic compounds, the recalcitrance and diverse composition of lignin pose particular challenges [14, 16, 87, 88, 94,

96, 110, 149, 151, 164, 166, 172]. Most microorganisms are sensitive towards the aromatic compounds and residues from the used solvents and catalysts [20]. Therefore, the fractionation and depolymerization strategy, as well as the microbial factory, need to be chosen carefully. Tolerance towards the aromatic compounds, as well as metabolic engineering of the catabolic aromatic pathways to streamline product accumulation, are a necessity [20]. Despite its attractiveness, lignin valorization has not yet reached industrial relevance [20].

1.2 Objectives

The aim of this work was to develop a process for the production of *cis,cis*-muconic acid, entirely from lignocellulosic biomass. To this end, different combinations of lignocellulose fractionation, lignin depolymerization and microbial host should be evaluated. Hydrothermal depolymerization was tested on multiple technical lignins, originating from different wood species and fractionation methods. Once a promising combination of lignin type and depolymerization strategy was found, hydrothermal conversion should be fine-tuned to the needs of selected microbial hosts, i.e. previously developed *cis,cis*-muconic acid producers *P. putida*, *C. glutamicum* and *Amycolatopsis sp.* [14, 16, 87]. The cascaded process from lignin into *cis,cis*-muconic acid should first be demonstrated for glucose as the co-substrate. Subsequently, the process should be extended to use C_5 sugars from hemicellulose instead of glucose. In this way, a *cis,cis*-muconic acid production process, entirely lignocellulose-based, and thus non-competitive to feed and food industry, should be developed.

2   Theoretical Background

2.1   Biomass as a resource

2.1.1   <u>Economic perspectives</u>

The mass that makes up all plants and animals is called biomass. Plant biomass constitutes approximately 450 Gt of carbon on earth [11], with an annual production of about 100 billion metric tons of carbon [54]. According to a recent study [15], the most effective counter act to climate change, is the restoration of trees around the world. The study showed that only 80 % of the potential 4.4 billion hectares are covered with trees. If the remaining 20 % were to be reforested, they could store an additional 205 Gt of carbon [15]. The importance of plant biomass is additionally highlighted by the 3×10^{21} J of energy from sunlight that are stored as chemical bonds each year [107].

Although only 3 % of the annually produced biomass is used economically, this resource has an enormous potential [107]. In the US, up to 1.3 billion tons biomass could be produced sustainably from agriculture and forestry annually [128]. Pending future development, Europe could produce up to 300 million tons oil equivalent by 2030, which would be sufficient to feed its entire chemical industry [171]. Today more than 66 % of the renewable energy in the EU stems from biomass, surpassing the combined contribution of solar, wind, geothermal and hydro-power energy [171]. Additionally, biomass is the only renewable source that yields liquid transportation fuel [171]. In 2007, the US declared that by 2022, 79 billion

liters of biofuel should be produced each year [128], which would be CO_2-neutral, widely available and not competing with food production.

Disadvantages in contrast to oil are that plant biomass is solid, very heterogeneous and highly oxygenated [145], which requires extensive research efforts towards industrial solutions. The final goal of such developments are biorefineries, which convert biomass into fuel and chemicals in an integrated process. As of 2017, 224 biorefineries are operating in Europe [23]. First generation biorefineries use food crops, such as sugarcane or corn, for the production of bioethanol. Second generation biorefineries use non-food materials, whereas third generation biorefineries meanwhile aim for the use of aquatic biomass [7]. A major challenge for biorefineries is deriving energy and value added compounds at an economically viable efficiency [155]. Typically, several process steps have to be conducted, including harvesting of biomass, its fractionation and depolymerization into smaller units and finally the conversion into high value chemicals and fuels [13]. Of course, the exploitation of biomass as a resource should be managed such that a sustainable use is possible in the foreseeable future.

2.1.2 Lignin - a highly underexploited renewable

A major part of plant biomass is called lignocellulose, a mixture of cellulose, hemicellulose and lignin (Figure 2.1). About 90 % of the plant dry weight can be accounted to lignocellulose [165]. Cellulose makes up about 45 % of the plant dry weight. It is a linear polymer, composed of D-glucose

subunits, linked by β-1,4-glycosidic bonds, forming cellobiose molecules [118]. Hemicellulose, on the other hand, is a branched polymer of C_5 and C_6 sugar monomers and can constitute up to 30 % of plant dry weight [145].
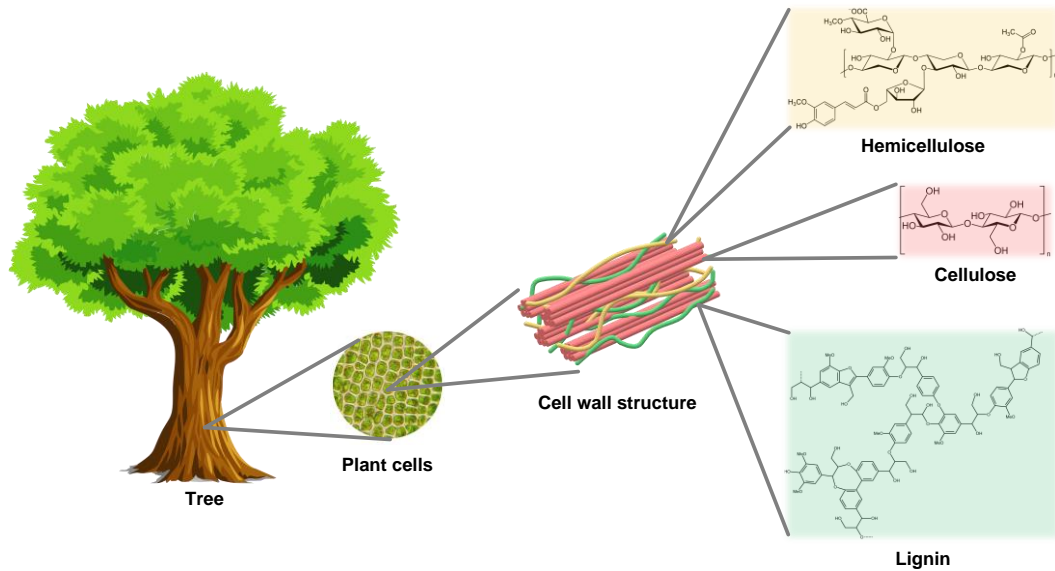
Figure 2.1: Composition of plant biomass. The wall of each individual plant cell is made of macrofibrils in which cellulose, hemicellulose and lignin are intertwined. The lignin structure is an exemplary molecular structure.

Lignin can make up over 30 % of plant biomass dry weight. Together with cellulose and hemicellulose, it is the prime building block for the cell wall [165]. Lignin provides structural support, rigidity and stability [145]. Furthermore, it provides impermeability and resistance against microbial attack and oxidative stress [122]. Softwood has the highest lignin content (25 - 30 %), followed by hardwood (18 - 20 %) (Figure 2.2). Certain species can even reach more than 48 % of lignin content. In comparison, grass contains less lignin (8 - 20 %). In moss and algae, lignin is almost completely missing, because these plants do not require the structural stability required by vascular plants [1].
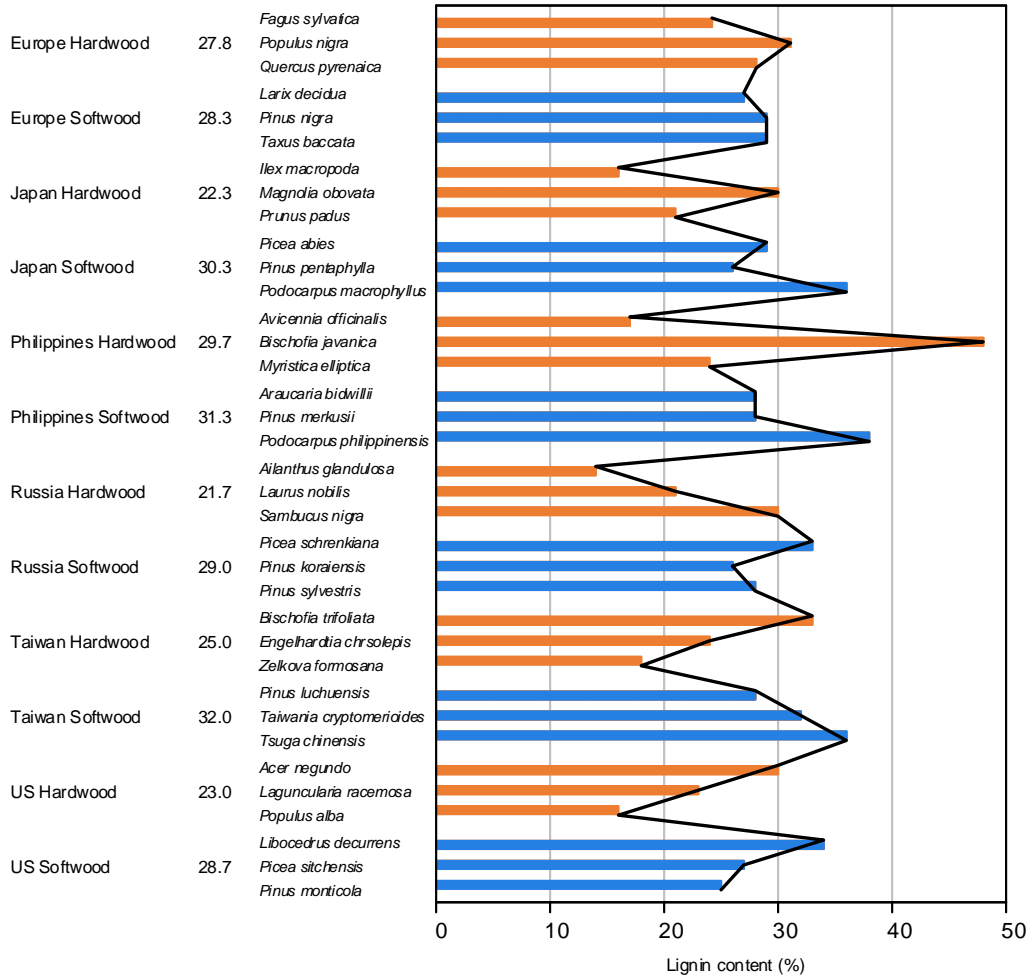
Figure 2.2: Lignin content [%] of soft- and hardwoods from different countries. The values next to the country are the average of the given species. The data are taken from literature, where more species can be found [2, 4, 50, 119].

Naturally, lignin is synthesized by radical polymerization from substituted phenyl propylene units [1], for which the involvement of enzymatic pathways to direct the polymerization is suggested [171]. Lignin biosynthesis is fueled by the amino acid L-phenylalanine, which is first converted to cinnamic acid via the shikimate pathway before forming the aromatic subunits of lignin [1].

Lignin is an amorphous heteropolymer and displays a three-dimensional randomized complex network of interlinked aromatic monomers [118]. Lignin consists of three types of monomer subunits, also known as

monolignols: *para*-coumaryl alcohol, coniferyl alcohol, and sinapyl alcohol [20] (Figure 2.3).
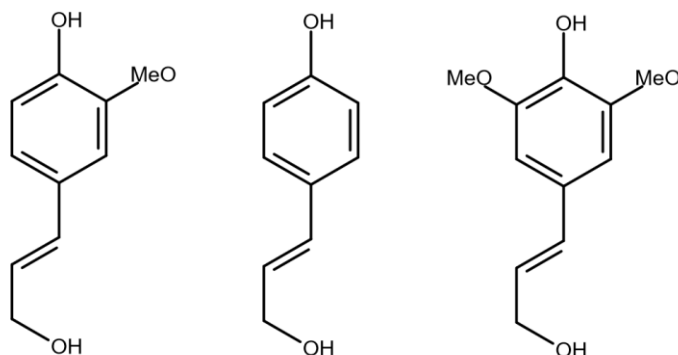
Figure 2.3: The three typical lignin monomers (monolignols) are coniferyl alcohol, *p*-coumaryl alcohol and sinapyl alcohol (from left to right).

They are based on *para*-hydroxyphenyl-, guaiacyl- and syringyl-units, respectively, and differ from each other by the number of methoxy substituents. In lignin, the monolignols are connected by ether (β-O-4) and carbon-carbon bonds [20]. While softwood has a high guaiacyl content of up to 90 %, hardwood exhibits an equal amount of guaiacyl and syringyl units [116]. The composition of lignin varies from plant species to plant species and can also differ within one species or even individual plants depending on season, region and climate [171] (Table 2.1). The exact structure of lignin is still unknown, due to difficulties in isolation of intact lignin for analysis [171]. Therefore the exact molecular weight remains to be determined. Currently, it is assumed to be in the range of approximately 2,500 to 10,000 g mol^{-1} [145].

Lignin is the second most abundant polymer in the world and the only renewable resource for aromatic compounds [1]. Approximately, 300 billion

tons of lignin are present in plants worldwide. Each year, about 20 billion tons are formed [20]. So far, lignin is considered a low value side-stream and even a waste, because it is non-edible and has to be separated from the product cellulose during pulp and paper manufacturing [20]. The removal is conducted mechanically and/or chemically. The three major routes of lignin fractionation are the Kraft process, the Sulfite process and the Organosolv process [157]. The Kraft process is the most widely applied method [145]. It uses a solvent, typically NaOH, for dissolution of lignocellulose and removal of lignin. Afterwards the lignin is recovered. Kraft-lignin, also known as alkali-lignin, is relatively free of sulfur, in contrast to the lignin produced by sulfite pulping, which relies on salts of sulfurous acid. In comparison, the Organosolv process uses a mixture of ethanol and water as the solvent, which results in a more unaltered and possibly purer lignin. Due to its relatively high carbon and low oxygen content, the energetic value of lignin is higher than that of carbohydrates [122]. Lignin is therefore typically burned to generate heat and electric power. However, this use appears highly inefficient, considering the inherent valorization potential and furthermore generates massive amounts of the climate gas CO_2. To some extent, lignin is applied as a low cost dispersant in cement and gypsum blends, and as an emulsifier or chelating agent for the removal of heavy metals [156]. Other uses include the use as carbon fibers, polymer resins and dispersants. A niche product in lignin valorization is the production of vanillin, a flavoring component for the food industry, by the company Borregaard (Sarpsborg, Norway). The worldwide vanillin market in 2011 comprised 16,000 tons (230 million US Dollar) with 3,000 tons produced from lignin [122]. In addition, aromatics, such as benzene, toluene

and xylene (BTX), can be derived from lignin [122]. In 2010, approximately 100 million tons of BTX were produced in oil refineries, showing the great potential of lignin valorization. To this date, the production of BTX from lignin is still under development, but expected to become a commercially viable process within the next two decades [122].

In 2010, the global production of chemical pulp was about 130 million tons, resulting in 170 million tons black liquor, a mixture of lignin and inorganic chemicals [142]. So far, less than 100,000 tons of lignin are commercially exploited per year [9]. In addition, a typical bioethanol plant produces approximately 70,000 tons of lignin each year [22], which is about 60 % more than the plant would need for complete coverage of its heat and power demand [128]. In the US alone, more than 165 bioethanol plants were operated in 2020 [131]. Even if these plants would be self-sufficient in heat and power, nearly 7 million tons of lignin are left unused, indicating a huge potential for a new lignin-based industry.

Table 2.1: Composition of different species of woods [117]

Wood species	Wood type	Lignin [%]	Polysaccharide [%]	C_6 [%]	C_5 [%]	Glc [%]	Man [%]	Gal [%]	Ara [%]	Xyl [%]
Beech	Hardwood	23.00	73.93	55.70	18.23	71.91	2.62	0.82	0.54	24.11
Birch	Hardwood	22.00	74.61	54.80	19.81	70.07	2.49	0.90	0.40	26.14
Chestnut	Hardwood	23.70	60.16	46.79	13.37	71.03	4.40	2.34	0.70	21.53
Eucalyptus globulus	Hardwood	15.94	80.41	62.80	17.61	74.50	2.28	1.32	0.60	21.31
Eucalyptus urograndis	Hardwood	27.43	70.11	59.37	10.74	82.08	1.50	1.10	0.23	15.09
Oak	Hardwood	23.50	63.24	49.48	13.76	74.73	1.53	1.98	0.90	20.86
Poplar	Hardwood	26.00	70.04	56.79	13.25	76.68	3.64	0.76	0.37	18.55
Aleppo Pine	Softwood	25.90	70.35	61.98	8.37	69.94	15.29	2.87	1.81	10.09
Black Pine	Softwood	26.10	68.62	63.40	5.22	69.57	20.64	2.19	1.47	6.14
Douglas-fir	Softwood	26.10	70.59	64.00	6.59	73.79	13.62	3.27	1.53	7.79
Maritime Pine	Softwood	27.20	68.17	60.57	7.60	69.44	15.33	4.09	1.95	9.18
Norway Spruce	Softwood	27.30	68.95	62.55	6.40	71.28	16.82	2.61	1.38	7.90
Scots Pine	Softwood	26.90	68.28	62.32	5.96	69.48	18.79	3.00	.158	7.15

2.1.3 <u>Hemicellulose - the second most abundant polymer on earth</u>

As mentioned above, hemicellulose is, next to cellulose and lignin, a part of plant cell walls and displays up to 30 % of biomass [145] (Figure 2.1). Similar to lignin, it consists of a complex network of monomers. Different to lignin and more similar to cellulose, hemicellulose is composed of C_5 and C_6 sugar monomers and corresponding sugar acids [135]. Typical examples are D-xylose, D-mannose, D-galactose, D-glucose, and L-arabinose. The individual monomers are mostly linked by β-1,4-glycosidic bonds. Different to cellulose, which is a rather linear molecule, hemicellulose exhibits branches. Hardwood hemicellulose contains mostly xylans, whereas softwood hemicellulose contains mostly glucomannans [135]. The degree of polymerization of hardwood hemicellulose is about 150 to 200. In softwood it is only 70 to 130 [135]. The monomer composition differs between species. For example, birch wood contains 89 % xylose and only 1 % arabinose, whereas rice bran contains equal shares of xylose (46 %) and arabinose (45 %) [135]. Unlike cellulose, which is mostly found in a crystalline structure, hemicellulose is rather amorphous and therefore more easily solubilizable [135]. Applications of hemicellulose in industry involve the use as a food packaging and food coating agent. Due to its high stability and biocompatibility, hemicellulose is also used in pharmaceutical applications [61].

2.2 Depolymerization of biomass

2.2.1 The pulping process

The vast majority of technical lignin is produced in the pulp and paper industry, where the Kraft process is the dominant method for delignification, the separation of lignin from the plant carbohydrates [145]. Over 90 % of all chemical pulps are produced in this way [145]. The term Kraft stems from the German word "Kraft", meaning strength. The process involves cooking of the biomass at a temperature between 155 and 175 °C in an aqueous solution of NaOH and Na_2S, which is termed white liquor. The mixture provides hydroxide and hydrogensulfide ions as reactive reactants, which then break the β-O-4 ether bonds of lignin, as well as the bonds, that connect lignin, hemicellulose, and cellulose [1]. After several hours of cooking, a solid pulp, containing cellulose is separated from the liquid. The liquid, termed black liquor, contains the lignin and hemicellulose fraction. It is further processed to recover the reactants and to separate the lignin using precipitation at neutral pH [157]. This process forms condensed and water-insoluble lignin with a low sulfur content of about 1 to 3 % [157]. As mentioned above, most Kraft lignin is incinerated to generate heat and power [145]. Methods to completely gasify the black liquor into syngas, have so far not reached commercial scale [1]. Advantages of the Kraft process are, a high quality cellulose pulp, the possibility of reactant recovery and a self-covered energy demand [145]. Because of these advantages, Kraft pulping is likely to remain the most important delignification process. Therefore the further valorization of Kraft lignin is of particular importance, considering the economics of future biorefineries. A disadvantage of the

Kraft process, to be mentioned from the view point of lignin valorization, is the fact that the lignin structure is severely changed from its native form and that repolymerization reactions occur, making it harder to later depolymerize it into smaller monomers [145].

An alternative delignification method is the sulfite pulping process. It predates the Kraft process, but because of the above mentioned advantages of Kraft pulping, sulfite pulping only accounts for small amounts of produced lignin. During sulfite pulping, the biomass is cooked at 140 to 170 °C in aqueous solutions, with salts of sulfurous acids [20]. The pH during cooking can be acidic, neutral or alkaline. In this process, the lignin becomes fully water soluble, but is highly degraded and has an even higher sulfur content (4 to 7 %) [20]. The high sulfur content causes potential problems for further processing, due to elevated risks of catalyst poisoning [157].

A variation of the sulfite process is called soda pulping [20]. It is similar to the Kraft process: the biomass is cooked at 160 to 170 °C in an aqueous solution of NaOH, but without Na_2S [20]. Therefore, the obtained lignin is sulfur free. However, the cellulose fibers are less strong, as compared to the Kraft process. The efficiency of soda pulping, can be improved by adding anthraquinone, which decreases the degradation of the carbohydrates [145].

A third process for delignification is called Organosolv process. Here, the cooking is conducted in an organic solvent, such as acetone, methanol, and ethanol [171]. The solvent solubilizes lignin and hemicellulose during the cooking. This method is considered more environmentally friendly than

other pulping processes, because the solvents can be easily recovered by distillation [137]. Additionally the lignin is considered of better quality, as it is of higher purity and has a lower molecular weight [26]. However, the costs for the solvents and waste treatment can be rather high, reducing the efficiency of the Organosolv process.

All three process concepts mentioned above have in common that the lignin fraction is separated from the cellulose by solubilization. In contrast to that, there are also methods that hydrolyze the plant carbohydrates in acid media, while the lignin remains as an insoluble solid, e.g. the Klason process [145]. This method is used for the determination of the lignin content in plants [64], but has no industrial market.

In addition, several other processes for lignin extraction from biomass are under development and have reached at least a viable proof of concept stage. A method to extract native-like lignin from lignocellulose uses ionic liquids, salts with a melting point below 100 °C [171]. These salts are known as excellent solvents and can be tailored to certain specifications by choosing the right cation/anion pair [171]. Due to the low temperature and mild conditions, the structure of lignin remains largely unchanged during the fractionation. Ionic liquids are considered green solvents because of their low environmental impact and non-toxic character [122]. They are also recyclable, however expensive and require a high purity to work efficiently [157]. At present, such expensive processes are not commercially viable. Lignin gained from ball milling also has a native-like structure. After milling, the lignin needs to be extracted using organic solutions. As the milling

process can last for days to even weeks and the delignification degree is usually below 35 % [157], it is a rather unattractive method.

After fractionating the biomass into three streams, cellulose, hemicellulose, and lignin, further processing is needed to convert them into their useful forms. The cellulose is used in the paper industry and is partly degraded into its sugar monomers, which in turn can be used as substrate in fermentation [46, 56]. Similarly, hemicellulose is processed [25, 46]. The complex nature and the degraded state of the lignin, however, requires other methods for technical depolymerization.

2.2.2 Biochemical lignin depolymerization in nature

In nature, lignin is mainly degraded by white-rot fungi, e.g. *Phanerochaete chrysosporium* and *Coriolus versicolor* [85]. Additionally, certain bacteria show ligninolytic behavior, e.g. *Streptomyces viridosporus* and *S. barius* [60]. Lignin degradation is much slower than that of the carbohydrates. Therefore, the largest organic fraction in soil is lignin [1]. Since lignocellulose polymers are quite large, the degradation enzymes are mostly secreted. During lignin degradation, enzymes from different families act together, e.g. laccases, peroxidases, esterases, and cellulases [60]. Advantages in using microorganisms and enzymes for technical lignin breakdown are that they are highly specific, environmentally friendly and work under mild conditions [9]. However, the accessibility of the enzymes to natural lignocellulose is limited and therefore a pretreatment step is necessary. Furthermore the production of the enzymes is still expensive and

in particular, depolymerization rates are very low [1]. Moreover, technical lignin, such as Kraft lignin, structurally differs from natural lignin. Consequently, the enzymes evolved to deal with natural lignin might be limited in their ability to convert technical lignin [9].

2.2.3 Technical depolymerization of lignin

Technically, lignin is depolymerized via reductive and oxidative processes and with acid and base catalysts (Figure 2.4). Reductive depolymerization uses a redox catalyst and a reducing agent, mostly hydrogen [145], which only cleaves the ether bonds in lignin. Different to that, oxidative processing allows to also break C-C bonds [157]. Oxidative treatment uses alkaline media plus oxygen or hydrogen peroxide as additional reagents. The production of vanillin from lignin is one example of such an oxidative valorization [116]. Alternatively, lignin can be depolymerized in a redox-neutral environment, using acid or base catalysts. The bonds of the aromatic rings are not cleaved during such treatment, resulting in a mixture of phenolic compounds [157]. A variety of metal, salt, acid or base catalysts are often employed to improve the chemical depolymerization and to narrow the product spectrum [77, 145]. Disadvantages of the chemical processes are sometimes the high costs, the formation of unwanted by-products and the partly corrosive character of the used acid media [9, 122].

In addition, lignin is technically depolymerized by applying heat and irradiation. The latter uses microwaves in the presence of hydrogen peroxide to destroy the ether bonds and partly also the aromatic ring

structure of lignin [115]. By using microwaves, the obtained depolymerized lignin has a narrower molecular weight distribution and a lower degree of condensation [115].

In the field of depolymerization with heat, two major methods have been investigated: pyrolysis and hydrolysis. Pyrolysis heats up lignin in the absence of oxygen to break down its molecular structure [45]. The lack of oxygen prevents an unwanted combustion into carbon dioxide. Temperatures range from 400 to 600 °C [45]. The formed gaseous product stream is typically condensed into a lignin-rich oil. High heating rates (300 to 1000 °C min^{-1}) and short residence times (few seconds) are required to obtain high yield [145]. However, the gained oil is rather unstable and needs further processing. Additionally, the lignin for pyrolysis has to be dried before the pyrolytic process, which limits efficiency [48].

Hydrolysis, also known as hydrothermolysis, aqueous liquefaction or hydrothermal conversion (HTC), uses hot compressed water for the depolymerization [77, 89, 121, 138]. The reaction temperature ranges from 200 to 500 °C and the pressure can reach values of more than 300 bar [48]. Due to the fact that the critical point of water exists at 374 °C and 221 bar [12], the conversion may involve sub- or supercritical water. Water reveals interesting physical properties close to its critical point. Its dielectric constant drops to 20 (from a value of 80 at room temperature) [12], which is in the range of organic solvents like acetone or methanol, while the density remains at a value of a liquid. As a consequence, water becomes a powerful solvent for organic compounds under these conditions [12]. Additionally, due to an ionization constant of about 10^{-11}, near-critical water provides

more hydroxide and hydronium ions [12]. With rising temperature, the viscosity decreases, resulting in better heat and mass transfer properties [107]. All these changes drive the hydrolysis process. Generally, water is cheap, environmentally friendly and the selectivity for keeping functional groups is higher than in organic solvents [144]. Obviously, no drying of the substrate is necessary before the hydrolysis. While nitrogen oxides and sulfur oxides are released by other depolymerization methods, these compounds remain dissolved in water [77]. The dilution of educts and products also reduces unwanted cross reactions [107].

After hydrothermal conversion of lignin, the derived product stream is an aqueous liquid with phenolic and methoxylated phenolic compounds [48]. Similar to pyrolysis, fast heating rates and the avoidance of temperature gradients in the reaction media are beneficial for high yield [77, 107]. One possibility to achieve both requirements, is the operation in continuous mode; however this requires sophisticated equipment [1]. A potential disadvantage is a certain tendency for corrosion, while using near- or supercritical water, although, studies have shown that the corrosion-liberated metal ions from the equipment serve as catalysts, even improving the depolymerization [107]. A range of other catalysts can also be added and some studies even used organic solvents instead of water, because of their lower critical point [77, 98, 116]. However, such catalysts need to be recovered and can be expensive, while organic solvents may have a negative impact on the environment, which makes the use of water attractive.

A drawback of thermal depolymerization of lignin is the formation of char [77, 116]. The many reactive groups present in lignin cause the repolymerization of monomers and oligomers into unsoluble fragments and thus diminish the yield for aromatic monomers [1, 145]. The char also tends to block reactors and pipes [77]. Several suggestions have been published to reduce the formation of char. The addition of capping agents like phenol, cresol, methanol or ethanol reduces the amount of repolymerization and cross linkage, by increasing lignin solubility in the medium [1, 98, 136]. Other additives that help in the dissolution and stabilization are formaldehyde [145] and γ-valerolactone (GVL) [97]. Also, increasing the pressure at a constant temperature has been shown to decrease char formation [170].
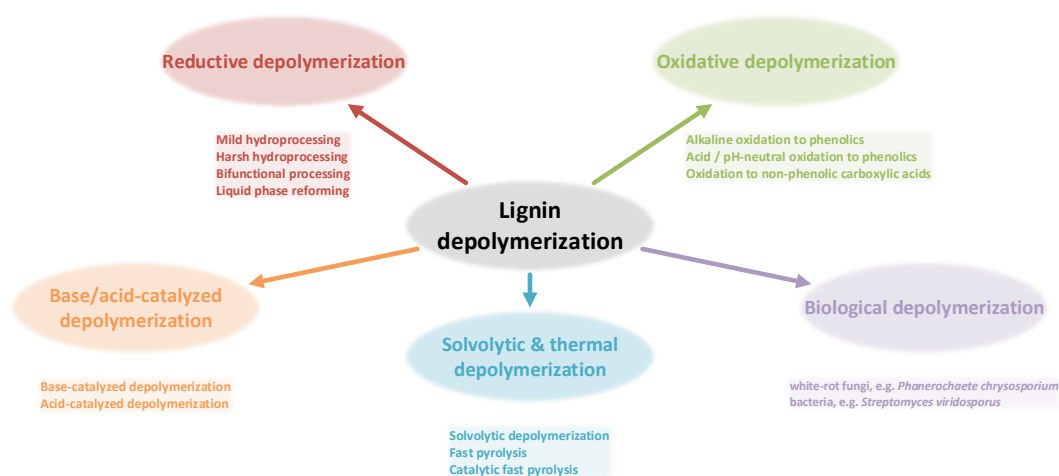

Figure 2.4: Different ways for the depolymerization of lignin, with several exemplary methods [1, 145].

2.2.4 Use of genetically engineered plants

A completely different approach to drive the valorization of lignin, is the use of genetically modified plants [128, 145]. As shown in a proof-of-principle,

such plants exhibit more customized lignin with increased solubility in water and an altered degree of polymerization [109, 163]. This technology is still in an early phase and economic, political and ethical issues still have to be resolved. Nevertheless, a modified lignin could provide a higher yield and easier access to aromatic monomers, which then can be further upgraded using biotechnological methods.

2.3 Bioconversion of biomass-derived carbon

2.3.1 *Pseudomonas putida*

Pseudomonas putida is a Gram-negative soil bacterium [87]. Most industrial strains are based on *P. putida* KT2440, a plasmid-free variant of *P. putida* mt-2, which is known to be stress resistant and easily genetically manipulated [72]. Also, it was the first Gram-negative bacterium to be classified as a safety strain by the Recombinant DNA Advisory Committee [159]. Strains of this bacterium can grow on a variety of aromatic compounds, e.g. benzoate or catechol [159]. Like many other degraders, *P. putida* uses converging catabolic pathways for the degradation of aromatic compounds. Hereby, the different aromatic compounds are metabolized into a few central intermediates, the major ones being catechol and protocatechuate [20]. The latter is, in many microbes, converted into catechol as well. Typical aromatic compounds channeled into protocatechuate are *p*-coumarate and vanillic acid [87]. Phenol, *m*-cresol and benzoate, as example, are channeled into catechol [20, 62]. Typically, further decomposition of catechol leads to β-ketoadipate, which is then

metabolized into tricarboxylic acid (TCA) cycle intermediates. An important step of catabolic breakdown is the cleavage of the aromatic ring. Catechol and protocatechuate have two neighboring hydroxyl substitutes on the aromatic ring. In this regard, the ring fission is termed *ortho* (or intradiol), when it occurs between the two hydroxyl groups, and *meta* (or extradiol), when it occurs outside, next to one of them [47, 62]. In more rare cases, the central intermediate has the two hydroxyl groups in *para* position, e.g. as found in the gentisate pathway [62].

The enzymes, responsible for ring cleavage, are dioxygenases. Dioxygenases are oxidoreductases, which add one or two oxygen atoms from molecular oxygen into the substrate. They often require metal ions, e.g. iron, as cofactor [57]. Dioxygenases, which catalyze *meta*-cleavage, are able to also convert methylated catecholic compounds [62]. In *P. putida*, catechol is *ortho*-cleaved into *cis,cis*-muconic acid (*cc*MA) by catechol-1,2-dioxygenase [158]. Two genes, *catA* in the *cat* operon and *catA2* in the *ben* operon, code for this enzyme [159]. The well-studied regulator *catR* regulates the expression of *catA*, with *cis,cis*-muconic acid as inducer [62]. Naturally, c*is,cis*-muconic acid is metabolized via the TCA cycle [20]. By deactivating the enzyme responsible for further degrading the intermediate, *cis,cis*-muconic acid is typically accumulated, which forms the basis of producing cell factories [159] (Table 2.2). Chemically, *cis,cis*-muconic acid can be converted into adipic acid, the precursor for nylon-6.6, an important industrial plastic [20]. The aromatic ring is chemically very stable. This stability exists because the alternating single and double carbon-carbon bonds create an evenly distributed electron density throughout the ring

structure. A C_6 carbon ring with alternating single and double carbon bonds was first proposed in 1865 by August Kekulé as the structure of benzene [57]. Because of the stability of the aromatic ring, the ring opening during the conversion of catechol is known to be slow and can cause accumulation of catechol [159]. Catechol is a highly toxic compound, causing inhibition of the bacterial cells, which has to be considered for strain engineering [159]. By disrupting the pathway downstream of *cis,cis*-muconic acid, the bacterium can no longer grow solely on aromatics. Another carbon source (e.g. glucose) is typically needed for biomass growth during *cis,cis*-muconic acid production by engineered microbes.

2.3.2 *Corynebacterium glutamicum*

The actinomycete *Corynebacterium glutamicum* is a Gram-positive, non-pathogenic soil microorganism [133], first isolated in Japan in 1957 [84]. Its name stems from the fact that it is able to secrete the amino acid L-glutamate [152]. This ability enabled the biotechnological production of L-glutamate by *C. glutamicum*, which has reached the million tons scale [27]. Over the last years, strains of *C. glutamicum* have been metabolically engineered to also produce other amino acids, e.g. L-lysine [21] and L-methionine [24, 125], organic acids [63, 167], proteins [91] and other value added products (e.g. ectoine [18, 59], 1,5-diaminopentane [29, 81, 82, 83] and 5-aminovalerate [133]) [17]. The fact that the microbe is regarded as safe (GRAS status), its physiology is very well understood, a variety of genetic tools are available and that it has the ability to produce in large scale fermentations, makes it an ideal organism in biotechnological production of

value added products [133]. In addition, its whole genome sequence is available [126]. *C. glutamicum* has also been described to be able to degrade a variety of aromatic compounds, while also showing a high toxicity tolerance towards these compounds [20]. It uses similar pathways as *P. putida* for the degradation of aromatic compounds into TCA cycle intermediates, via catechol and protocatechuate (Figure 2.5).
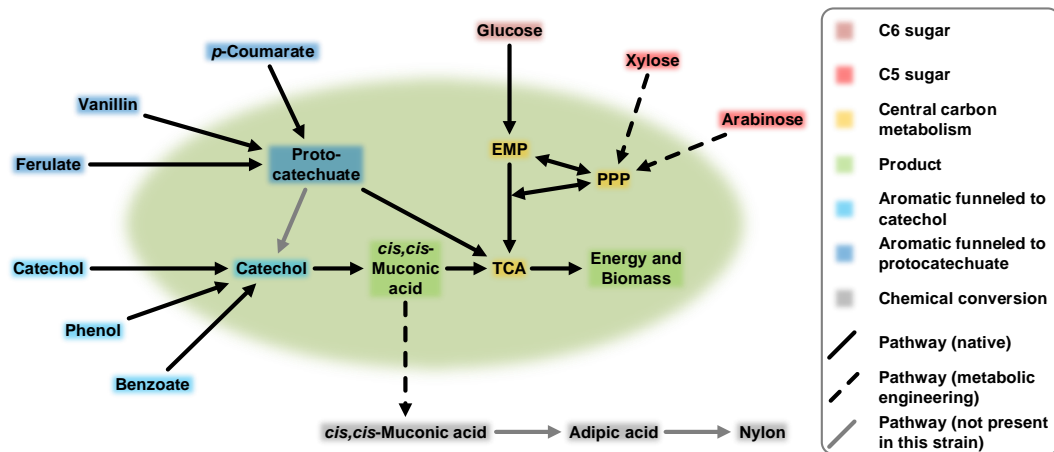
Figure 2.5: Uptake and metabolic conversion of sugars and aromatic compounds in *C. glutamicum* [29, 75, 78, 124, 147].

Compared to other frequently used industrial strains, like *Escherichia coli* and *Saccharomyces cerevisiae*, *C. glutamicum* has the advantage of showing only weak carbon catabolite repression [13]. This means it can co-utilize different sugars during fermentation. However, complex sugar polymers, for example cellulose, starch or xylan, cannot be directly used as carbon source [13]. As a consequence, most industrial fermentations of *C. glutamicum* rely on glucose from hydrolysates of starch or fructose from molasses [27]. Also, pentoses cannot be naturally used as carbon source [28], but through metabolic engineering the substrate spectrum of *C. glutamicum* can be broadened such that xylose, arabinose or ribose are

also utilized [13]. Two well studied pathways exist for the utilization of xylose in *C. glutamicum*: the isomerase pathway via the pentose phosphate pathway (PPP) and the Weimberg pathway [34]. For the first one, xylose isomerase (e.g. *xylA* from *Escherichia coli*) must be heterologously expressed. It converts xylose into xylulose, which is further phosphorylated into xylulose 5-phosphate by *xylB*. XylB is a native enzyme in *C. glutamicum*, but its activity is very low [78], which is why it is heterologously expressed in most cases. Xylulose 5-phosphate is further metabolized in the PPP. A drawback of this pathway is the loss of carbon into CO_2 [127]. A more efficient way seems the five step oxidative Weimberg pathway, where xylose is converted into α-ketogluterate, which then enters the TCA cycle [34]. By introducing a single operon, *xylABCD*, from *Caulobacter crescentus*, *C. glutamicum* is able to utilize xylose without loss of carbon [27]. Since the growth rate on a new substrate is often rather low, adaptive laboratory evolution is frequently employed, to gain more insight into metabolism and to improve uptake and growth rates [27, 152]. Uptake of sugar into the cell is, in most cases, done by transporter proteins. The co-expression of such transporters can greatly enhance the growth rate during cultivation [34]. The native xylose transporter of *C. glutamicum* has not been discovered yet [28], but AraE, an arabinose importer, also has the ability to transport xylose into the cell [34]. In *E. coli*, XylE is the transporter for xylose and its expression in *C. glutamicum* improves the uptake and growth rate significantly [34].

2.3.3 *Amycolatopsis sp.*

About 30 years ago, the soil actinomycete *Amycolatopsis sp.* ATCC 39116 (previously known as *Streptomyces setonii*) was reported to depolymerize lignin [5, 44]. The depolymerization involved the utilization of several aromatic compounds, such as catechol, guaiacol, benzoate and vanillin [44]. Especially the capability to convert guaiacol is remarkable, because it is a rare ability among bacterial strains [14]. Given the choice between sugars and aromatics, it even prefers the latter as carbon source [20]. *Amycolatopsis sp.* strains are employed in industry as efficient producers of vanillin, but very little is known about genetic optimization of this organism [104].

2.4   *Cis,cis*-muconic acid - an important platform chemical

Muconic acid, also referred to as 2,4-Hexadienedioic acid, is a dicarboxylic acid with two conjugated double bonds and a C/O ratio of 1.5 [79]. Three isomeric forms can be chemically synthesized: *cis,cis*-muconic acid, *cis,trans*-muconic acid and *trans,trans*-muconic acid [79]. In 2018, muconic acid accounted for a global market size of 79 million USD, which is expected to rise to more than 110 million USD in 2024 [79]. The compound is mainly used as pivotal precursor for adipic acid, terephtalic acid and caprolactam monomer production, which are polymerized into commercial polyamides and polyesters [19, 20, 79] (Figure 2.6). As example, nylon-6.6 can be produced from adipic acid [20], a drop-in product for the petrochemical

industry with an average market price of 1,650 euro ton^{-1} and a turnover of 3,737.8 kilotons in 2016 [113, 160].
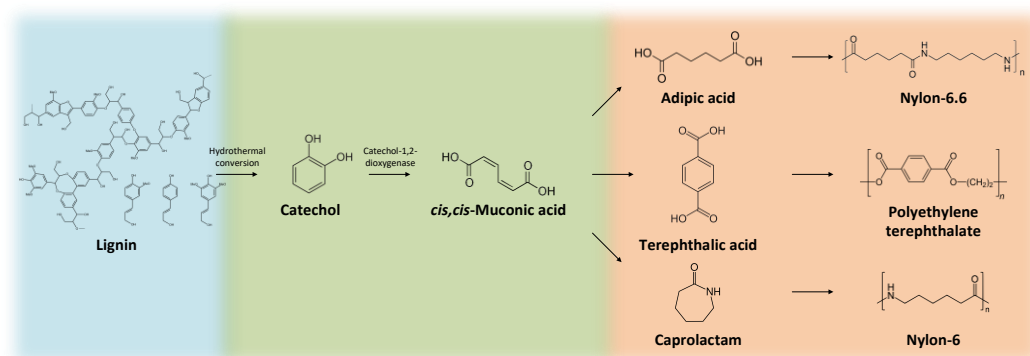

Figure 2.6: Lignin-based synthesis of value added precursors to commercial polymers such as adipic acid, terephthalic acid and caprolactam. This is a three-phased cascaded process starting with depolymerization of lignin (blue) e.g. through hydrothermal conversion, followed by bioconversion of aromatic monomers into *cis,cis*-muconic acid by a microbial host (green) e.g. *C. glutamicum* and finally the chemical synthesis of value added products such as nylon-6.6, Polyethylene terephthalate and nylon-6 (red). The shown process is exemplary and by no means represents all possible valorization strategies of lignin via *cis,cis*-muconic acid.

The high energy demand and CO_2 emission of petrochemical synthesis drive the development of bio-based industrial processes [20, 42, 160]. The biotechnological production of *cis,cis*-muconic acid from aromatic monomers has been demonstrated for benzoate [10, 16, 35, 71, 95, 106, 141, 161, 164, 168, 169], catechol [16, 76, 87, 100, 101, 102], toluene [37, 66, 67, 68, 99], phenol [16, 164], 4-hydroxybenzoate [151, 164], *p*-coumarate [73, 164], ferulate [73], vanillate [151], vanillin [151] and guaiacol [14]. For example, *P. putida* [87, 164], *C. glutamicum* [16] and *E. coli* [100, 101, 102], have been genetically engineered to convert catechol and phenol into *cis,cis*-muconic acid, whereas metabolically engineered *Amycolatopsis sp.* was reported to produce *cis,cis*-muconic acid from guaiacol [14]. In recent years, developments towards bio-based *cis,cis*-

muconic acid production have been impressive with reported titers of up to 85 g L^{-1} [16]. This show-cases the potential of an even greener bio-process, based on the renewable aromatic resource lignin [20].

Table 2.2: Conversion of lignin and lignin hydrolysates into value-added products using different production hosts

Strain	Aromatic source	Product	Cultivation type	Titer [g L^{-1}]	Ref.
Pseudomonas putida IDPC/pTS110	Lignin hydrolysate	cc-Muconic acid	Fed-batch	0.02	[151]
Sphingomonas paucimobilis SME257/pTS084	Lignin hydrolysate	cc-Muconic acid	Fed-batch	0.03	[151]
Amycolatopsis sp. ATCC 39116 MA-2	Lignin hydrolysate	cc-Muconic acid	Fed-batch	0.2	[14]
Pseudomonas putida KT2440-CJ103	Lignin hydrolysate	cc-Muconic acid	Batch	0.7	[164]
Corynebacterium glutamicum MA-2	Lignin hydrolysate	cc-Muconic acid	Fed-batch	1.8	[16]
Pseudomonas putida KT2440 MA-9	Lignin hydrolysate	cc-Muconic acid	Fed-batch	13	[87]
Phanerochaete chrysosporium	Lignin	Succinic acid	Batch	0.02	[65]
Pandoraea sp. ISTKB	Kraft lignin	PHA	Batch	0.02	[88]
Pseudomonas putida	Lignin hydrolysate	PHA	Batch	0.25	[94]
Cupriavidus basilensis	Kraft lignin	PHA	Fed-batch	0.32	[149]
Rhodococcus opacus DSM1069	Kraft lignin	Lipid	Batch	0.07	[166]
Rhodococcus opacus PD630	Kraft lignin	Lipid	Batch	0.15	[172]
Rhodococcus opacus PD630	Lignin	Lipid	Batch	1.83	[96]

3 Material and Methods

3.1 Lignin and hemicellulose

Kraft lignin from spruce (*Picea abies*) was obtained from Sigma Aldrich (St. Louis, MO, USA) and from TCI Deutschland (Eschborn, Germany). In addition, Kraft lignin (IndulinAT) from pine (*Pinus sylvestris*) was obtained from S3Chemicals (Bad Oeynhausen, Germany). Organosolv lignin from beech (*Fagus sylvatica*) originated from the Fraunhofer Center for Chemical and Biotechnological Processes (CBP) (Leuna, Germany). Additionally, Kraft lignin from pine and organosolv lignin from spruce were provided by the Energy Research Centre of the Netherlands (ECN, Petten, Netherlands). Hemicellulose was obtained from Bolise Co., Ltd. (Xiamen, China). In addition, purified xylan from Carl Roth (Karlsruhe, Germany) was used as a model compound.

As an example, lignin powder from Sigma Aldrich was analyzed in more detail, including elemental analysis (DIN 51732, DIN EN ISO 11885, ERGO Umweltinstitut GmbH, Dresden, Germany) (Table 3.1) and estimation of the particle size distribution (DIN ISO 14887:2010-03, Fraunhofer IKTS, Dresden, Germany).

Table 3.1: Elemental analysis of Kraft lignin from Sigma Aldrich

Component	Amount [% (w/w)]
Carbon	61.5
Oxygen	30.4
Hydrogen	4.5
Nitrogen	1.3
Ash	2.3

The analysis of the particle size distribution showed, that 10 % of the particles were smaller than 3.9 µm, 50 % were smaller than 19.5 µm and 90 % were smaller than 59 µm.

3.2 Hydrothermal lignin conversion

Hydrothermal conversion (HTC) experiments were conducted in a stirred pressure vessel (Type 4575A, Parr Instruments, Moline, IL, USA). The vessel (500 mL) was made of stainless steel (T316) and had a fixed head. Prior to conversion, lignin powder was suspended in deionized water at a concentration specified below. The suspension was filled into the vessel, after which the reactor was sealed. The reactor was then stirred at 400 rpm, while gaseous nitrogen (99.999 %) was used to purge the reactor three times. Afterwards, the heating process to the desired reaction temperature was initiated. In order to keep the heating phase short, while avoiding an overshoot of the temperature, a value of 450 °C was chosen as initial set point. Once the reactor had reached a temperature of approximately 10 °C below the desired value, the set point was reduced to that desired value. The start point of the reaction was defined as the time point, when the temperature had reached the set point (± 1 °C). At the end of the chosen reaction time, heating was stopped and the reactor was cooled down, using the internal cooling coil and tap water (6 °C) as the coolant. In addition, cooling was supported by an external fan. Once the temperature had reached 85 °C, the reactor was opened. The content of the vessel was then poured into a glass bottle for further processing. The reactor was cleaned by heating the empty vessel up to a temperature of 395 °C, while flushing

with synthetic air over night. After the reactor was cooled down, the inside was cleaned with a wire brush, a tooth brush and compressed air.

3.3 Hydrothermal lignin conversion at miniaturized scale

Small-scale HTC experiments were conducted in tubular batch reactors (length 100 mm, inner diameter 8 mm, outer diameter 12 mm), made of stainless steel 1.4571 and closed with a top and a bottom cap (Swagelok, Solon, OH, USA). The volume of each reactor was 5 mL. After filling with lignin powder and deionized water, the reactor was purged with gaseous nitrogen (99.999 %) for 10 seconds, sealed, and placed in a sand bath in an oven (Nabertherm, Lilienthal, Germany). The oven was preheated to the desired reaction temperature. An initial experiment, using a thermocouple placed inside one reactor, revealed a heating rate of 25 °C min^{-1}. The start point of the reaction time was the time point, when the desired reaction temperature (± 1 °C) had been reached. At the end of the conversion, the reactor was quenched to room temperature in a water bath. After cooling, the content of each reactor was collected by rinsing with methanol to a total volume of 10 mL. The obtained solution was centrifuged (10,000 x g, 5 minutes, room temperature). The supernatant was stored at -20 °C until further processing. The pellet represented the char formed during the conversion. Its amount was determined gravimetrically, after drying of the tubes for 24 hours. All experiments were done in triplicate.

3.4 Hydrothermal hemicellulose conversion

Hydrothermal hemicellulose conversion was conducted in a stirred pressure vessel (Type 4575A, Parr Instruments, Moline, IL, USA). Hemicellulose (or xylan) was suspended in deionized water and filled into the vessel. After sealing and purging three times with gaseous nitrogen (99.999 %) under stirring at 400 rpm, hemicellulose was hydrolyzed at 200 °C. At the end of the conversion the reactor was cooled down, using the internal cooling coil with tap water (6 °C) as the coolant and an additional fan to cool from the outside. The reactor was opened and its content collected once it had reached 85 °C.

For small scale experiments, miniaturized reactors were used as described above. Prior to the conversion, a suspension of hemicellulose (or xylan) in deionized water was prepared and filled into the reactor. Each reactor was purged with nitrogen (99.999 %) for 10 seconds, sealed, and placed inside an oven at the desired reaction temperature. After the desired reaction time, the reactors were quenched in a water bath to room temperature. Their contents were centrifuged (10,000 x *g*, 5 minutes, room temperature). The supernatant was stored at -20 °C until further processing. All experiments were conducted in triplicate.

3.5 Fractionation of depolymerized hemicellulose by distillation

Water steam distillation was used to remove furfural, formed during the HTC process, from the product solution. For this purpose, approximately 4 L deionized water were boiled in a 5 L glass flask and the water steam was

flushed through a round bottomed glass flask (500 mL), containing the raw hydrolysate of hemicellulose (250 mL), for 30 minutes. The flask was incubated in an oil bath (130 °C) and was connected to a distillation column, using tap water (6 °C) as coolant. The obtained distillate (containing furfural) was collected.

3.6   Distillation of lignin hydrolysate

Conventional distillation was employed to enrich guaiacol from lignin hydrolysates (250 mL) for later cultivation of *Amycolatopsis sp.*. For this purpose, the hydrolysate was filled into a round bottomed glass flask (500 mL), which was placed in an oil bath (120 °C). The flask was connected to a distillation column, using tap water (6 °C) as coolant. Distillation lasted for 3 hours. The obtained distillate (containing guaiacol) was collected.

3.7   Concentration of lignin and hemicellulose hydrolysates

A vacuum concentrator (AVC 2-33 IP, Christ, Osterode, Germany) was used for the concentration of different hydrolysates, obtained from HTC and possibly already treated using distillation. Depolymerized lignin was concentrated at 15 mbar and 40 °C for 2.5 hours. Afterwards, the obtained liquids were collected and clarified (10,000 x *g*, 5 minutes, room temperature). Hemicellulose hydrolysates were concentrated in the same manner.

3.8 Decolorization of hemicellulose hydrolysates and removal of toxic ingredients

Hemicellulose hydrolysates were decolorized after the preceding distillation and concentration steps. First, the pH value was adjusted to 2.0 (5 M HCl). Afterwards, 10 % (w/w) of powdered activated charcoal (Grüssing GmbH, Filsum, Germany) was added. The suspension was stirred for 10 minutes at room temperature and then centrifuged (10,000 x g, 10 minutes, room temperature). The pH value of the supernatant was adjusted to 6.5 (5 M NaOH). The solution was filtrated (0.2 µm, VWR, Radnor, PA, USA) and the obtained filtrate was collected.

3.9 Microorganisms and plasmids

Pseudomonas putida MA-9 was taken from previous work [87].

Corynebacterium glutamicum MA-1 and MA-2 were also obtained from previous work [16]. *C. glutamicum* MA-3 and MA-4, which additionally carried an episomal plasmid [29], were provided by Sarah Pauli and Lukas Jungmann (Institute of Systems Biotechnology, Saarland University, Germany). *C. glutamicum* MA-3 was based on *C. glutamicum* MA-1 and *C. glutamicum* MA-4 was based on *C. glutamicum* MA-2.

Amycolatopsis sp. MA-2 was taken from a recent study [14].

All strains were maintained as kryo stock at -80 °C.

3.10 Media

The minimal defined media for the cultivation of strains of *P. putida, C. glutamicum* and *Amycolatopsis sp.* are given in Table 3.2, Table 3.3 and

Table 3.4. In all cases, media and media components were sterilized by autoclaving (121 °C, 1 bar, 15 minutes) or filtration (0.2 μm, VWR, Radnor, PA, USA), as specified. Each medium was prepared from concentrated stock solutions, whereby sterile deionized water was added, where needed. Prior to mixing, autoclaved stock solutions were cooled down to room temperature. For the preparation of agar plates, liquid medium was mixed with 20 g L^{-1} agar (Carl Roth, Karlsruhe, Germany).

For the production of *cis,cis*-muconic acid, aromatics of choice were added to the minimal medium. Aromatics were either pure compounds, mixtures thereof or lignin hydrolysates as described below.

Table 3.2: Composition of glucose minimal medium for cultivation of *Pseudomonas putida*

#	Compound	Concentration	Sterilization	Stock Conc.
I	Glucose·H_2O	550 g L^{-1}	Autoclaving	100x
II	K_2HPO_4	38.75 g L^{-1}	Autoclaving (pH adjusted to 7.0)	5x
	$NaH_2PO_4·H_2O$	18.80 g L^{-1}		
	$(NH_4)_2SO_4$	10 g L^{-1}		
III	$MgCl_2·6H_2O$	100 g L^{-1}	Filtration	1000x
IV	$Na_2H_2EDTA·2H_2O$	12.75 g L^{-1}	Filtration	1000x
V	$FeSO_4·7H_2O$	5 g L^{-1}	Filtration	1000x
VI	$CaCl_2·2H_2O$	1 g L^{-1}	Filtration	1000x
VII	$ZnSO_4·7H_2O$	200 mg L^{-1}	Filtration	100x
	$MnCl_2·4H_2O$	120 mg L^{-1}		
	$CoCl_2·6H_2O$	40 mg L^{-1}		
	$Na_2MoO_4·2H_2O$	20 mg L^{-1}		
	$CuSO_4·5H_2O$	20 mg L^{-1}		

Table 3.3: Composition of glucose minimal medium for cultivation of *Corynebacterium glutamicum*

#	Compound	Concentration	Sterilization	Stock Conc.
I	Glucose·H_2O	550 g L^{-1}	Autoclaving	100x
II	K_2HPO_4	348 g L^{-1}	Autoclaving (mixed together to adjusted pH to 7.8)	10x
	KH_2PO_4	272 g L^{-1}		
III	$(NH_4)_2SO_4$	150 g L^{-1}	Autoclaving (pH adjusted to 7.0)	10x
IV	NaCl	20 g L^{-1}	Autoclaving	20x
	$MgSO_4 \cdot 7H_2O$	8.2 g L^{-1}		
	$CaCl_2 \cdot 2H_2O$	1.5 g L^{-1}		
V	DHB	30 g L^{-1}	Filtration	1000x
VI	$FeSO_4 \cdot 7H_2O$	2 g L^{-1}	Filtration (pH adjusted to 1.0)	100x
VII	$MnSO_4 \cdot H_2O$	200 mg L^{-1}	Filtration	100x
	$FeCl_3$	120 mg L^{-1}		
	$ZnSO_4 \cdot 7H_2O$	80 mg L^{-1}		
	$CuCl_2$	16 mg L^{-1}		
	$Na_2B_4O_7$	11 mg L^{-1}		
	$(NH_4)_6Mo_7O_{24} \cdot 4H_2O$	10 mg L^{-1}		
VIII	Thiamine-HCl	50 mg L^{-1}	Filtration	50x
	Ca-Pantotheat	50 mg L^{-1}		
	Biotin	25 mg L^{-1}		

For cultivation of *C. glutamicum* on xylose, instead of glucose, a stock solution (100x) containing 500 g L^{-1} xylose was used. Sterilization was done by autoclaving. In the case of cultivations using hemicellulose hydrolysate, the processed hydrolysate was used instead of the xylose stock. Because the genes for xylose utilization were on a plasmid, kanamycin (50 mg L^{-1}) was added to the culture.

Table 3.4: Composition of glucose minimal medium for cultivation of *Amycolatopsis sp.*

#	Compound	Concentration	Sterilization	Stock Conc.
I	Glucose·H_2O	550 g L^{-1}	Autoclaving	100x
II	K_2HPO_4	38.75 g L^{-1}	Autoclaving (pH adjusted to 7.0)	5x
	NaH_2PO_4·H_2O	18.80 g L^{-1}		
	$(NH_4)_2SO_4$	10 g L^{-1}		
III	NaCl	200 g L^{-1}	Autoclaving	1000x
IV	$MgSO_4$·$7H_2O$	100 g L^{-1}	Filtration	1000x
V	$CaCl_2$·$2H_2O$	25 g L^{-1}	Filtration	1000x
VI	Na_2H_2-EDTA·$2H_2O$	12.5 g L^{-1}	Filtration	1000x
VII	$FeSO_4$·$7H_2O$	1.25 g L^{-1}	Filtration	4000x
VIII	$ZnSO_4$·$7H_2O$	200 mg L^{-1}	Filtration	100x
	$MnCl_2$·$4H_2O$	120 mg L^{-1}		
	$CoCl_2$·$6H_2O$	40 mg L^{-1}		
	Na_2MoO_4·$2H_2O$	20 mg L^{-1}		
	$CuSO_4$·$5H_2O$	20 mg L^{-1}		

In all cultures of *Amycolatopsis sp.* glass beads (5 mm diameter) were used to control cell morphology and 2 mM guaiacol was added before each inoculation [14].

3.11 Cultivation of *Pseudomonas putida*

A minimal agar plate was inoculated from a kryo culture, incubated overnight at 30 °C, and used to inoculate the preculture. The preculture was grown in a 500 mL baffled shake flask, filled with 50 mL glucose minimal medium (Table 3.2) and incubated overnight on a rotary shaker at 30 °C and 230 rpm (Infors Multitron, Infors HT, Bottmingen, Switzerland).

Afterwards, cells were collected (10,000 x *g*, 5 minutes, room temperature), resuspended in 5 mL sterile 0.9 % NaCl solution, and used to inoculate the main culture to a starting cell concentration of 0.2 OD_{600nm} units. Three 500 mL baffled shake flasks, each filled with 50 mL glucose-based medium were inoculated and incubated at 30 °C and 230 rpm.

3.12 Cultivation of *Corynebacterium glutamicum* strains

A minimal agar plate was inoculated from a kryo culture, incubated overnight at 30 °C, and used to inoculate the preculture. The preculture was grown in a 500 mL baffled shake flask, filled with 50 mL glucose minimal medium (Table 3.3) and incubated overnight on a rotary shaker at 30 °C and 230 rpm (Infors Multitron, Infors HT, Bottmingen, Switzerland). Afterwards, cells were collected (10,000 x *g*, 5 minutes, room temperature), resuspended in 5 mL sterile 0.9 % NaCl solution, and used to inoculate the main culture to a starting cell concentration of 0.2 OD_{660nm} units. Three 500 mL baffled shake flasks, each filled with 50 mL glucose-based medium were inoculated and incubated at 30 °C and 230 rpm.

In addition to glucose, *C. glutamicum* MA-3 and MA-4 were also cultivated on xylose and hemicellulose hydrolysates. For this purpose, 50 mL xylose minimal medium was inoculated from a minimal glucose agar plate in a 500 mL baffled shake flask. The preculture was incubated overnight at 30 °C and 230 rpm and used to inoculate a second xylose-based preculture to an initial optical density (OD_{660nm}) of 2. After four hours, cells were collected and used to inoculate the main culture, containing hemicellulose

hydrolysate as carbon source (three 500 mL baffled shake flasks, each filled with 50 mL medium). The cultures were incubated at 30 °C and 230 rpm.

3.13 Cultivation of *Amycolatopsis sp.*

A 500 mL unbaffled shake flask, filled with 50 mL medium (Table 3.4) was inoculated from a kryo culture. This first preculture was incubated overnight at 37 °C and 230 rpm (Infors Multitron, Infors HT, Bottmingen, Switzerland). A second preculture (same conditions as above) was then inoculated to an initial optical density (OD_{600nm}) of 0.5 and also grown overnight. Afterwards, cells were collected (10,000 x *g*, 5 minutes, room temperature), resuspended in 5 mL sterile 0.9 % NaCl solution and used to inoculate the main culture to a starting cell concentration of 0.2 (OD_{600nm}). Three unbaffled shake flasks, each filled with 50 mL medium were inoculated and incubated at 37 °C and 230 rpm.

3.14 Fed-batch production of *cis,cis*-muconic acid

A fed-batch set-up was used for the production of *cis,cis*-muconic acid from hemicellulose- and lignin-derived hydrolysates. The fermentation was operated in a 250 mL bioreactor system (DASGIP, Eppendorf, Hamburg, Germany). The medium was as described above and the reactor was filled with 70 mL batch medium, containing 10 g L^{-1} xylose from hemicellulose. The temperature was set to 30 ± 0.1 °C. The pH was kept at 7.15 ± 0.05 by automated addition of 6 M NaOH. The reactor was aerated with air (18 sL h^{-1}) and the stirrer speed was set to 400 rpm. The dissolved oxygen

level and the pH value were measured online. Xylose level was kept above zero by adding processed hemicellulose hydrolysate based on external monitoring and lignin hydrolysate was added every 2 hours.

3.15 Quantification of glucose and xylose

Quantification of xylose and glucose was conducted using isocratic HPLC (1260 Infinity Series, Agilent Technologies). The two sugars were separated on an Aminex HPX-87H column (Bio-Rad, Hercules, CA, USA) at 85 °C, using deionized water as mobile phase at a flow rate of 0.5 mL min^{-1}. The analytes were detected using refraction index measurement at 55 °C. Their quantification was based on external standards.

3.16 Quantification of mono- and disaccharides

Quantification of different monosaccharides (arabinose, galactose, glucose, ribose, and xylose) and disaccharides (cellobiose and xylobiose) was conducted using GC-MS [80]. For this purpose, 25 µL of a sample was dried under a nitrogen stream. Subsequently, 50 µL methoxylamine (2 % in pyridine) was added. The mixture was incubated for 25 minutes at 80 °C. Afterwards, 50 µL N,O-bis(trimethylsilyl)trifluoroacetamide (BSTFA) was added and the incubation was continued at 80 °C for another 30 minutes. Subsequent analysis used a GC-MS system (Agilent Technologies 7890B GC, 5977A MSD System, Agilent Technologies, Santa Clara, CA, USA) with helium as mobile phase at a flow rate of 1 mL min^{-1} and a split ratio of 20:1. An HP-5-MS (30 m x 0.25 mm x 0.25 µm (Agilent Technologies)) was used

as stationary phase. The temperature at the inlet was 250 °C, at the transfer line 280 °C, at the quadrupole 250 °C and at the detector 230 °C. Electron energy was 70 eV. The temperature gradient was as follows: 150 °C to 192°C at a rate of 2 °C min^{-1} and 192-325 °C at a rate of 27 °C min^{-1}. The total run time was 41 minutes. Quantification was done by using trehalose as internal standard [80].

3.17 Quantification of aromatics and muconic acid derivatives

Catechol, phenol, guaiacol, *cis,cis*-muconic acid, and *cis-trans*-muconic acid (as well as (m)ethylated forms of catechol and *cis,cis*-muconic acid) were separated by HPLC (1260 Infinity Series, Agilent Technologies, Santa Clara, CA, USA), using a reversed phase column (Nucleodur E100/3 C18 Isis, 3 µm, Macherey-Nagel, Düren, Germany) and a gradient of eluent A (0.025 % H_3PO_4) and eluent B (acetonitrile) at 25 °C and a flow rate of 1 mL min^{-1}. The ratio of A and B over time was as follows: 99:1 from 0 to 13.8 min, 67:33 from 13.8 to 14.3 min, 0:100 from 14.3 to 17.8 min and 99:1 from 17.8 to 24.3 min. The analytes were detected using UV absorption at 210 nm (aromatics) and 260 nm (*cis,cis*- and *cis,trans*-muconic acid derivatives). Quantification was based on external standards.

3.18 Fingerprinting of aromatics in lignin hydrolysates

The aromatic composition of lignin hydrolysates was analyzed using GC-MS. First, the analytes of interest were extracted from the sample using dichloromethane. The organic phase was then dried with sodium sulfate.

Afterwards, 30 µL of the dried extract was mixed with 30 µL DMF (0.1 % pyridine) and 30 µL MBDSTFA (N-methyl-N-tert-butyldimethylsilyl-trifluoroacetamide). The mixture was incubated for 1 hour at 80 °C and then analyzed using GC-MS. An Agilent Technologies 7890B GC and 5977A MSD System (Agilent Technologies, Santa Clara, CA, USA) was used with helium as mobile phase at a flow rate of 1 mL min^{-1} and a split ratio of 50:1. An ultra-inert HP-5-MS column (30 m x 0.25 mm x 0.25 µm (Agilent Technologies)) was used as stationary phase. The temperature at the inlet was 250 °C, at the transfer line 280 °C, at the quadrupole 250 °C and at the detector 230 °C. Electron energy was 70 eV. The temperature gradient was as follows: 2 min at 100 °C and 100-300 °C at a rate of 20 °C min^{-1}. Total run time was 14 minutes.

3.19 Quantification of organic acids and alcohols

Formic acid, acetic acid, furfural, and 5-hydroxymethylfurfural were quantified using isocratic HPLC (1260 Infinity Series, Agilent Technologies) with 12 mM H_2SO_4 as mobile phase at a flow rate of 0.5 mL min^{-1} and 45 °C and an Aminex HPX-87H column (Bio-Rad, Hercules, CA, USA) as stationary phase. The detection was conducted using refraction index measurement at 55 °C. Quantification was based on external standards.

3.20 Quantification of cell concentration

The cell concentration was measured as optical density (UV-1600PC, VWR, Radnor, PA, USA). The detection wavelength was 600 nm for strains of *P. putida* and *Amycolatopsis sp.* and 660 nm for strains of *C. glutamicum*.

4 Results and Discussion

4.1 Microbial production of *cis,cis*-muconic acid from aromatics

Several microorganisms were tested for their capability to convert aromatic compounds, the expected depolymerization products of lignin, into *cis,cis*-muconic acid. This included recently developed strains of *P. putida* MA-9 [87], *C. glutamicum* MA-2 [16] and *Amycolatopsis sp.* MA-2 [14].

Catechol, phenol and guaiacol were selected for the experiments, because those three aromatics were likely candidates to be expected from lignin through the planned hydrothermal conversion [145]. Cultures of *P. putida* MA-9 and *C. glutamicum* MA-2 were tested on a mixture of catechol and phenol, using glucose as a co-substrate. Two hours after the inoculation, the aromatics were added to a concentration of 2.0 mM (catechol) and 0.5 mM (phenol). Pre-tests had shown that an immediate addition of the aromatics at the beginning of the process potentially caused an inhibition of the cells, at least of *P. putida* MA-9. As both strains were not capable to metabolize guaiacol [75], this methoxylated substrate was not tested. *Amycolatopsis sp.* MA-2, however, was able to metabolize guaiacol [14, 75]. Therefore, guaiacol (2.2 mM) was tested. The results of the cultivations are shown in Figure 4.1.
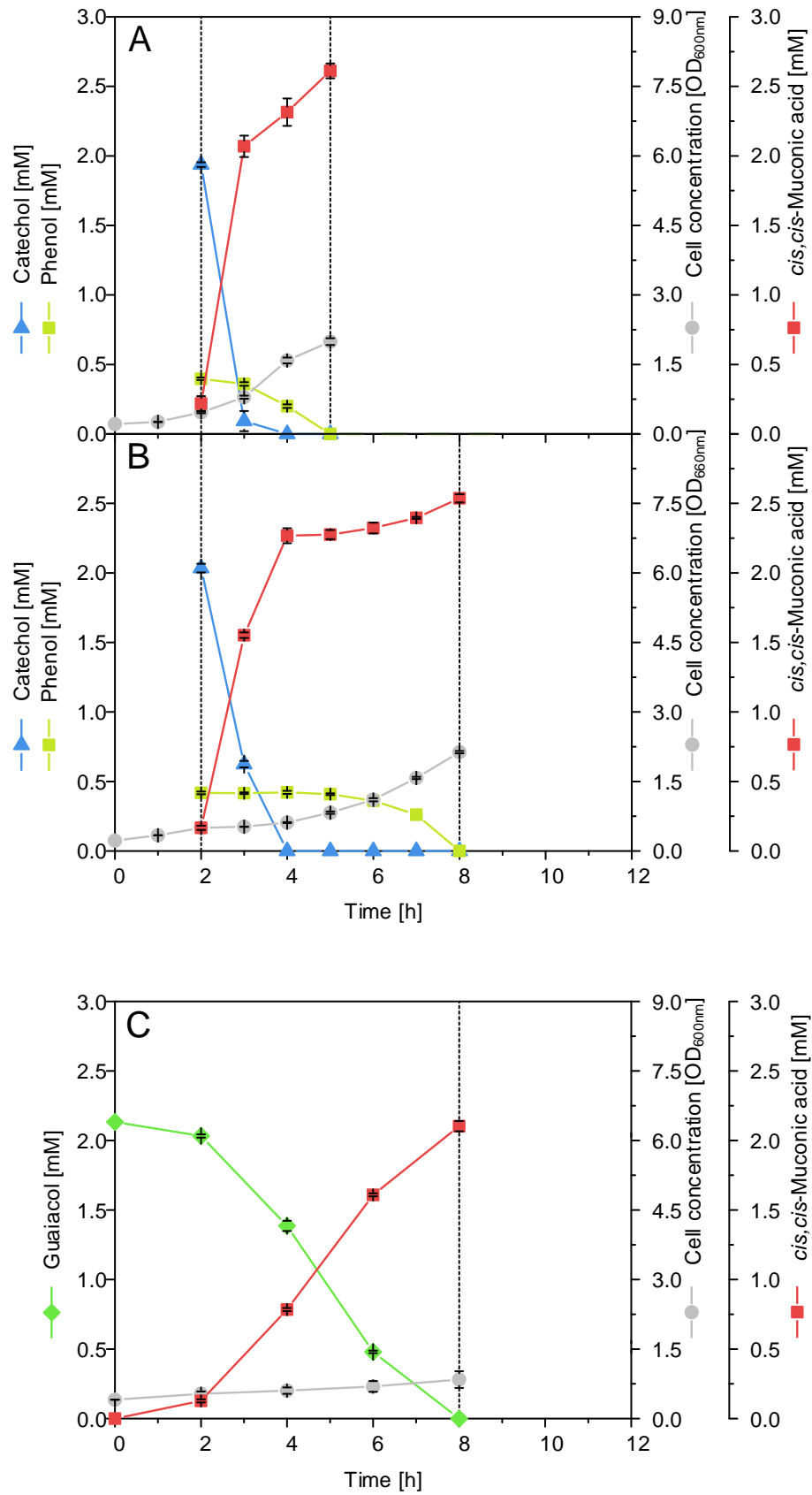
Figure 4.1: Production of *cis,cis*-muconic acid from different aromatics using metabolically engineered strains *Pseudomonas putida* MA-9 [87] (A), *Corynebacterium glutamicum* MA-2 [16] (B) and *Amycolatopsis sp.* MA-2 [14] (C) at 30 °C. To each cultivation, glucose (5 g L^{-1}) was added as a co-substrate to support growth. (n=3)

P. putida MA-9 first converted catechol into *cis,cis*-muconic acid, while the conversion of phenol started afterwards (Figure 4.1 A). The overall yield of *cis,cis*-muconic acid from the two aromatics was 102 ± 2 %, matching previously reported values for *P. putida* and the expected value based on pathway stoichiometry [87]. The selected strain grew in the presence of the aromatics, indicating a certain tolerance to the aromatics [87, 111]. The observed titer (0.4 g L^{-1}) was comparable to previously reported values [87, 164] (Table 4.1).

Likewise, *C. glutamicum* MA-2 stoichiometrically converted catechol and phenol into the target product (Figure 4.1 B). The yield was 97 ± 2 %, confirming the previously reported performance of *C. glutamicum* MA-2 [16]. Due to the disrupted β-ketoadipate pathway, *cis,cis*-muconic acid was not degraded further. Growth was rather unaffected by the aromatics. Again, catechol was the preferred substrate. Phenol was utilized only after depletion of the latter, leading a process with two distinct production phases (Table 4.1).

Next, *Amycolatopsis sp.* MA-2 was tested for its capability to utilize guaiacol for the production of *cis,cis*-muconic acid. Within 6.5 hours, the initially added guaiacol was entirely converted into *cis,cis*-muconic acid (Figure 4.1 C). The yield was 99 ± 2 % (Table 4.1). Growth was rather slow, as compared to the other two strains.

Taken together, all three strains produced *cis,cis*-muconic acid from the supplied aromatic monomers (Table 4.1). Conversion of catechol was the

fastest. This was likely related to the fact that this aromatic compound was the direct precursor of the product [16]. In none of the experiments, catechol accumulated. The slightly slower conversion of phenol and guaiacol indicated a limited capacity in the upstream catabolic pathway. The enzyme catalyzing the conversion of phenol into catechol is a phenol 2-monooxygenase, while aromatic O-demethylase (cytochrome P450) is known to demethylate guaiacol into catechol [75]. For the intended application, i.e. high level *cis,cis*-muconic acid production, the preferred use of catechol over the other aromatic appeared promising, because it indicated a lower risk of catechol overflow. Beneficially, a yield of approximately 100 % was reached and no by-products were formed. Whereas the growth of *C. glutamicum* MA-2 and *Amycolatopsis sp.* MA-2 appeared unaffected by the toxic aromatics, *P. putida* MA-9 showed a certain growth retardation. This indicated a certain sensitivity to catechol and/or phenol [87]. Given the obvious robustness and also the fast growth, *C. glutamicum* MA-2 appeared particularly suitable among the different strains and was therefore selected for further studies.

Table 4.1: Microbial production of *cis,cis*-muconic acid from aromatics during batch processes, using metabolically engineered microbes

Strain	Aromatic substrate	Time [h]	Titer [g L^{-1}]	Yield [%]	Ref.
Amycolatopsis sp. MA-2	Guaiacol	8	0.3	99 ± 2	this work
P. putida MA-9	Catechol/ Phenol	5	0.4	102 ± 2	this work
C. glutamicum MA-2	Catechol/ Phenol	8	0.4	97 ± 2	this work
P. putida MA-9	Catechol/ Phenol	5	0.5	100	[87]
P. putida CJ103	Phenol	24[a]	0.6[a]	64	[164]
C. glutamicum MA-1	Phenol	22.5	0.7	100	[16]
C. glutamicum MA-1	Catechol	24	1.4	100	[16]
C. glutamicum MA-2	Catechol	10	1.4	100	[16]

[a] estimated from reference

4.2 Cascaded production of *cis,cis*-muconic acid from Kraft lignin

4.2.1 Hydrothermal conversion of Kraft lignin into aromatic monomers

To assess whether Kraft lignin, the most abundant industrially produced lignin [145], can be used as a raw material to derive *cis,cis*-muconic acid, its hydrothermal conversion was studied next.

As catechol was the direct precursor of *cis,cis*-muconic acid and was preferably converted into the target product [159], a main goal of the HTC process to be developed was a high catechol yield. Towards such an optimized process, different parameters (reaction time and temperature, additives such as NaCl, MgCl$_2$ and CaCl$_2$, loading of the reactor and initial lignin concentration) were studied. In a first set of experiments, Kraft lignin (Sigma Aldrich) was hydrothermally converted and process dynamics were studied (Figure 4.2 A).
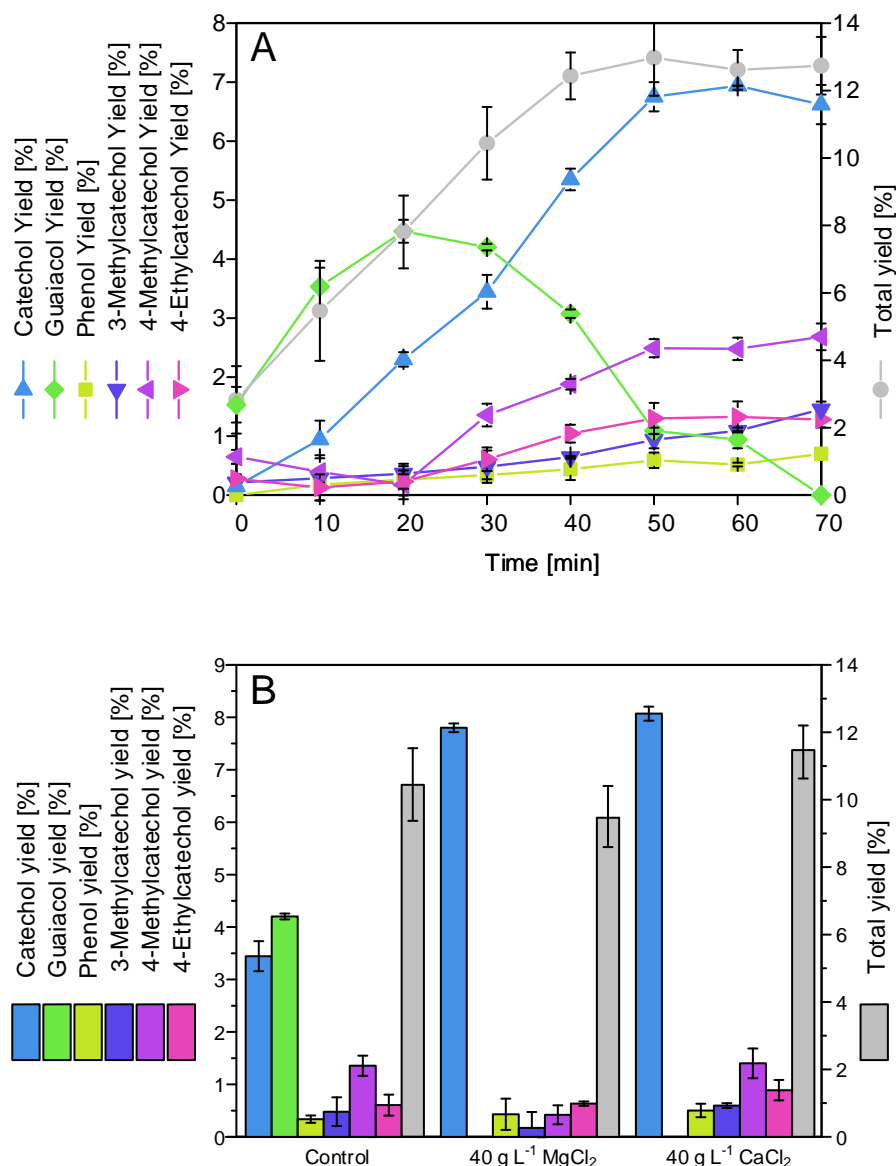
Figure 4.2: Yield of aromatic monomers during hydrothermal conversion of Kraft lignin (Sigma Aldrich) at 400 °C in pure water (A). Performance with the addition of salt conducted for 30 minutes (B). The yield was determined by dividing the mass of each produced monomer by the initial mass of lignin. (n=3)

During the initial phase (20 minutes), guaiacol was the main product formed and even occurred during the heating phase. Catechol accumulated slightly delayed, when the level of guaiacol was reduced, suggesting that it, at least partly, originated from guaiacol [138]. In a similar fashion, the sequential occurrence of guaiacol and catechol was previously observed, e.g. during hydrothermal treatment of alkali lignin [121] and IndulinAT lignin [8]. In

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addition to catechol, 3- and 4-methylcatechol, as well as 4-ethylcatechol were detected. However, these compounds occurred at lower level. The amount of phenol rose in a linear fashion during the whole experiment, but reached only small concentrations. Phenol was even the lowest of all aromatics. Phenol and the (m)ethylated catechol derivatives obviously stemmed from the complex aromatic network of lignin, which consisted also of methylated and ethylated aromatic rings, as well as different phenolic compounds [145]. The major monomer was catechol (7 %). The total aromatic monomer yield increased linearly during the first 40 minutes and remained stable at 13 % until the end. A slight decrease in total yield at the very end pointed to a certain degree of decomposition of the aromatic monomers and underlined the impact of stopping the reaction at the right moment.

Subsequently, hydrothermal lignin conversion was performed under addition of 40 g L^{-1} salts ($MgCl_2$ and $CaCl_2$) (Figure 4.2 B). These additives were supposed to enhance the conversion process [70]. Here, the conversion was conducted for 30 minutes. Indeed, the catechol yield was more than doubled, when adding the salt. At the same time, the level of guaiacol was reduced from 5 % to 0 %, indicating that the conversion was strongly accelerated. The use of 20 g L^{-1} NaCl resulted in a comparable catechol yield of 7.6 % (data not shown). The yields of (m)ethylated catechol were also found increased, especially when using $CaCl_2$. It should, however, be noted that the overall yield of all aromatics was the same for all conditions, suggesting that salts mainly accelerated the process, but did not lead to higher yield.

When evaluating reaction temperature and pressure (Figure 4.3 A), the highest yield in catechol was achieved at 400 °C, the highest temperature feasible with the used reaction vessel. This characteristic appeared transferable to other lignin types, such as IndulinAT Kraft lignin and alkali lignin [8, 121]. The highest guaiacol yield was found at a subcritical temperature of 350 °C (Figure 4.3 C).

Furthermore, addition of NaCl was beneficial for catechol formation (Figure 4.3 B), whereas it decreased the guaiacol yield (Figure 4.3 D). Similar to the other tested salts, NaCl accelerated the reaction.
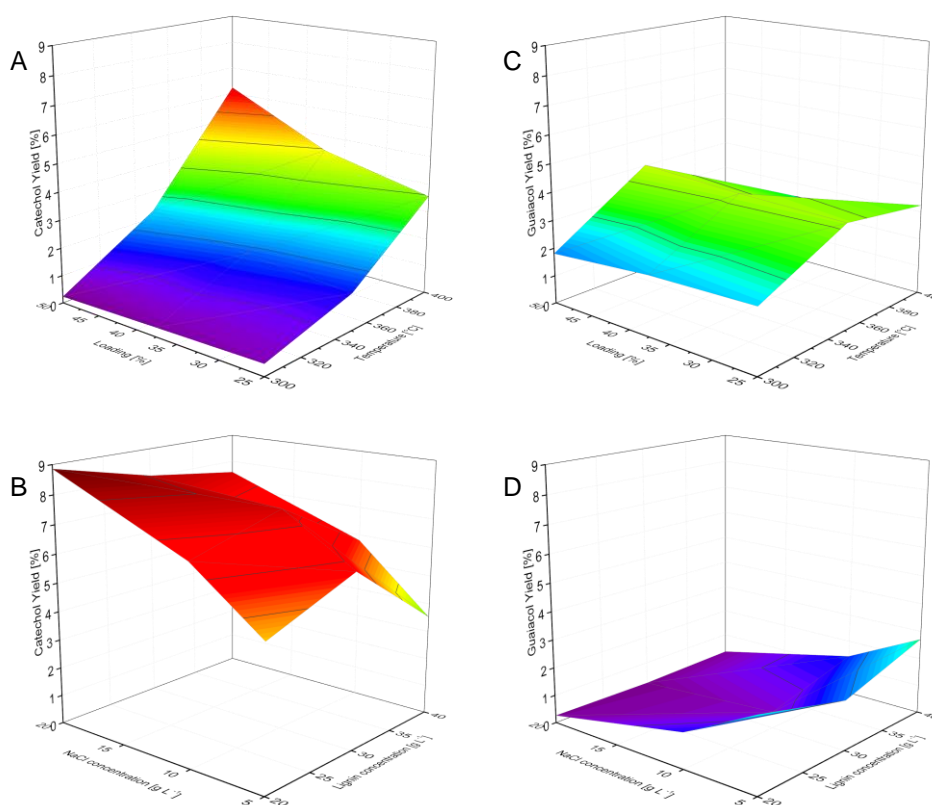

Figure 4.3: Yield of catechol and guaiacol after hydrothermal conversion of Kraft lignin (Sigma Aldrich) (30 minutes) at different reactor loading and reaction temperature (A, C) and for different levels of lignin and NaCl (B, D). The yield was determined by dividing the mass of the produced catechol by the initial mass of lignin. (n=3)

The lignin concentration showed an anti-proportional behavior to the catechol yield (Figure 4.3 B). This could be due to the low solubility of lignin in water [142]. Apparently, undissolved lignin remained in solid state during the conversion and thus was not fully available for depolymerization [173]. The loading of the reactor had only a minor influence.

In order not to overload the subsequent bioconversion with salt, potentially inhibiting microbial activity [130], the process set-up without additives was chosen for further experiments. Beneficially, HTC in pure water formed catechol as major product, which could be efficiently converted into *cis,cis*-muconic acid (Figure 4.1 A and B). Considering equipment limitations, chosen process conditions for catechol-rich hydrolysates were 395 °C and 1 hour. Shorter incubation times (20 minutes) and a subcritical reaction temperature (370 °C) enabled the generation of guaiacol-rich hydrolysates.

4.2.2   Production of *cis,cis*-muconic acid from lignin hydrolysate

Next, *P. putida* MA-9, *C. glutamicum* MA-2 and *Amycolatopsis sp.* MA-2, were tested for their production performance on real-case hydrolysates from the hydrothermal conversion of Kraft lignin. For *P. putida* and *C. glutamicum* a catechol-rich hydrolysate (395 °C and 1 hour) was applied, whereas a guaiacol-rich hydrolysate (370 °C and 20 minutes) was obtained for *Amycolatopsis sp.*. Both hydrolysates were concentrated as given below (chapter 4.3.3). In short, the catechol-rich hydrolysate was vacuum-concentrated and the guaiacol-rich hydrolysate was distilled. The production of *cis,cis*-muconic acid from the different hydrolysates was then investigated (Figure 4.4).
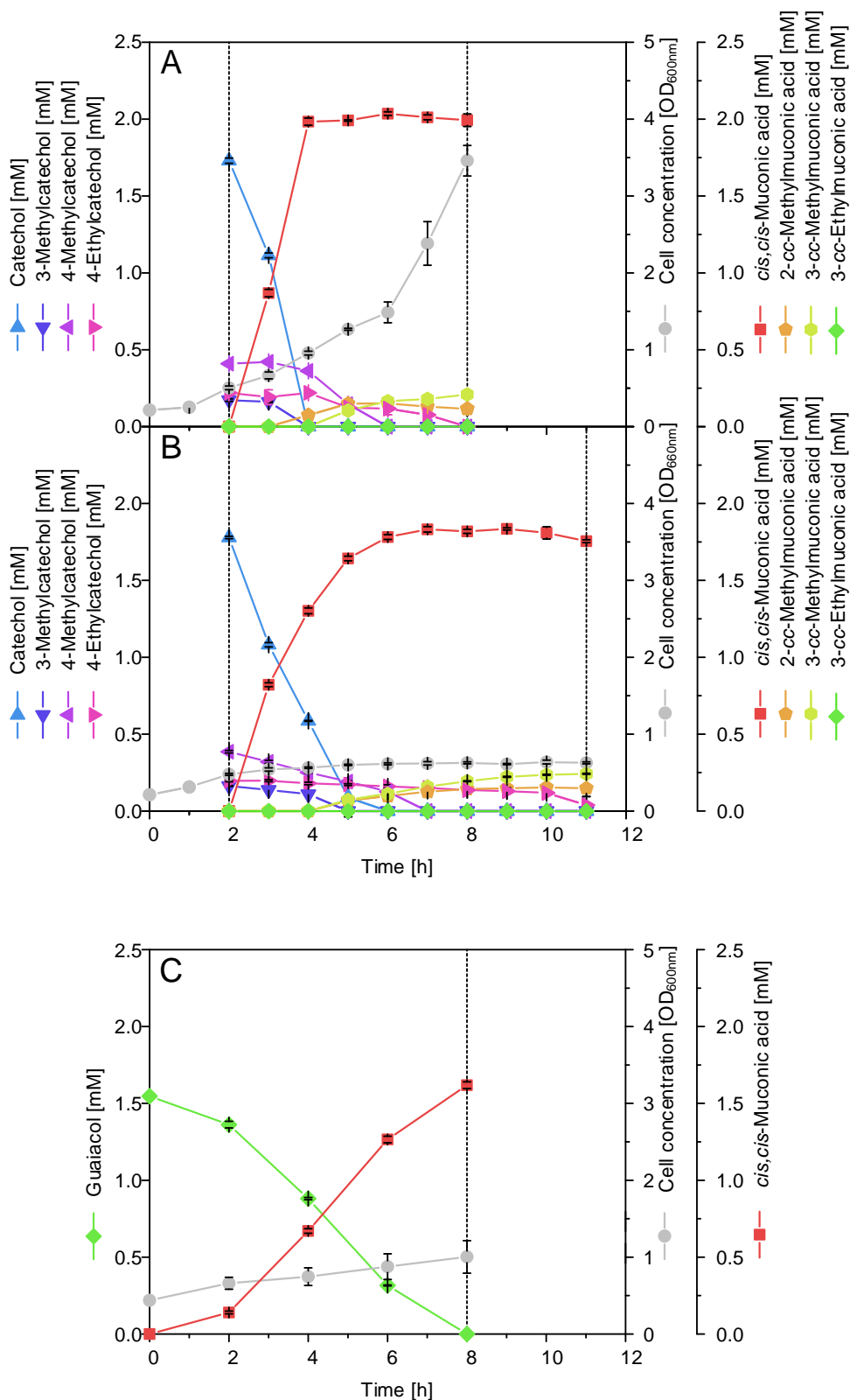
Figure 4.4: Production of *cis,cis*-muconic acid from Kraft lignin hydrolysate using metabolically engineered strains *Pseudomonas putida* MA-9 [87] (A), *Corynebacterium glutamicum* MA-2 [16] (B) and *Amycolatopsis sp.* MA-2 [14] (C) at 30 °C. To each cultivation, glucose (5 g L^{-1}) was added as a co-substrate to support growth. Lignin hydrolysates were obtained from hydrothermal conversion of Kraft lignin (Sigma Aldrich) at 395 °C for 1 hour (A and B) and 370 °C for 20 minutes (C), followed by vacuum-concentration (catechol-rich) or distillation (guaiacol-rich). No phenol was detected. (n=3)

Similar to the experiment with pure aromatics, catechol was converted fastest by *P. putida* MA-9 (Figure 4.4 A). Interestingly, methylated *cis,cis-*muconic acids were produced from the corresponding methylated forms of catechol. Reaction chemistry suggested that 2-*cis,cis*-methylmuconic acid likely originated from 3-methylcatechol and 3-*cis,cis*-methylmuconic acid from 4-methylcatechol [30, 31]. Similarly, it could have been expected that 4-ethylcatechol would yield 3-*cis,cis*-ethylmuconic acid. Indeed, the latter was detected in trace amounts.

C. glutamicum MA-2 successfully converted the catechol from the hydrolysate into *cis,cis*-muconic acid (Figure 4.4 B). As observed for *P. putida* MA-9, methylcatechols were converted into the corresponding *cis,cis*-methylmuconic acids. Here, ethylated *cis,cis*-muconic acid was not quantifiable despite the consumption of 4-ethylcatechol. This was probably due to the limited sensitivity of the measurement of 3-*cis,cis*-ethylmuconic acid.

In less than 8 hours, guaiacol from the corresponding hydrolysate was completely converted into *cis,cis*-muconic acid by *Amycolatopsis sp.* MA-2 (Figure 4.4 C). Since practically no (m)ethylcatechol was present in this hydrolysate, *cis,cis-* (m)ethylmuconic acid was not found.

All three strains were able to utilize aromatics, derived from hydrolyzed Kraft lignin. They produced *cis,cis*-muconic acid from catechol and guaiacol, respectively, at a yield of 100 % and even converted derivatives of catechol

from the hydrothermal conversion into (m)ethylated forms of *cis,cis*-muconic acid.

In addition to HTC, other depolymerization methods have been applied to process lignin [8, 90, 121, 174]. Some of these methods, however, prohibit straightforward bioconversion. As example, lignin from sulfite pulping contains high sulfur contents [20, 157] and is thus not well suited for subsequent bioconversion [41, 129, 143]. HTC hydrolysate, however, was converted in a very efficient way, with different microbes. In this regard, HTC appeared to be a superior lignin processing strategy, with further beneficial characteristics such as environmental friendliness, simplicity and low production cost [145].

4.3 Impact of the lignin-type on depolymerization and bioconversion efficiency

4.3.1 Screening of hydrothermal conversion efficiency

Lignin structure and composition varies with biomass origin and pre-processing [154]. To evaluate this aspect, four different types of Kraft lignin and two different types of organosolv lignin were compared (Figure 4.5). The Kraft process is the most common method for lignin production and results in lignin pulp containing sulfur [145]. Sulfur-free pulp can be obtained through the less aggressive organosolv process [137]. Kraft-processed wood species, tested here, included Norway spruce (*Picea abies*) and Scots pine (*Pinus sylvestris*), which are both softwood species with lignin percentages of 27.3 % and 26.9 %, respectively [117] (Table 4.2). The

organosolv process resulted in lignin pulp from Norway spruce (*Picea abies*) and beech (*Fagus sylvatica*), the latter being a hardwood species with a lignin percentages of 23.0 % [117]. Softwood nearly exclusively consists of guaiacyl units, whereas hardwood contains both guaiacyl and syringyl units. The latter has a lower fraction of carbon-carbon bonds, potentially influencing hydrothermal conversion efficiency [145]. All six lignin powders were processed in tailor-made miniaturized reactors that mimicked the larger scale reactor and allowed for higher throughput screening with less starting material. Better than the existing high-throughput miniaturization systems developed for hydrothermal conversion [58, 108, 146, 153], the designed tube reactors were capable to withstand temperatures of up to 400 °C.

Table 4.2: Impact of lignin type and wood species on the efficiency of hydrothermal conversion into aromatic monomer hydrolysates. In all cases, the hydrothermal conversion was conducted at 400 °C for 30 minutes in pure water as solvent. The lignin level was 40 g L^{-1}. Sigma Kraft: Kraft lignin from Sigma Aldrich, TCI: Kraft lignin from TCI Deutschland, ECN Kraft: Kraft lignin from the Energy Research Centre of the Netherlands (ECN), ECN OS: organosolv lignin from ECN, FH OS: organosolv lignin from the Fraunhofer CBP. In each case, the yield was calculated by dividing the mass of the produced monomers by the initial mass of lignin. (n=3)

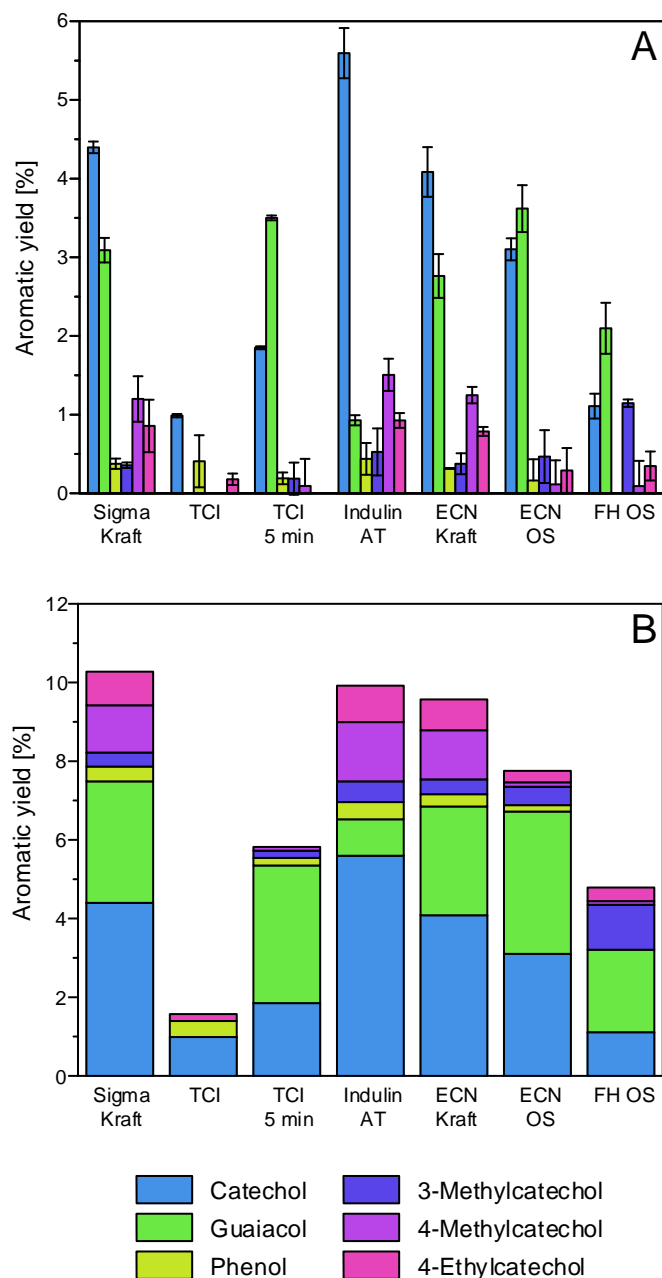| Lignin type	Wood species	Wood type	Lignin [%]	Total aromatic yield [%]	Catechol yield [%]	Guaiacol yield [%]
Sigma Kraft	Spruce	Softwood	27.3	10.3 ± 1.0	4.4 ± 0.1	3.1 ± 0.2
TCI	Spruce	Softwood	27.3	5.8 ± 0.7	1.8 ± 0.0	3.5 ± 0.0
IndulinAT	Pine	Softwood	26.9	9.9 ± 1.2	5.6 ± 0.3	0.9 ± 0.1
ECN Kraft	Pine	Softwood	26.9	9.6 ± 0.9	4.1 ± 0.3	2.8 ± 0.3
ECN OS	Spruce	Softwood	27.3	7.8 ± 1.6	3.1 ± 0.1	3.6 ± 0.3
FH OS	Beech	Hardwood	23.0	4.8 ± 1.0	1.1 ± 0.2	2.1 ± 0.3

Figure 4.5: Yield of aromatic monomers from hydrothermal conversion of different lignin types. A: individual yields of aromatic compounds. B: total yield. Sigma Kraft: Kraft lignin from Sigma Aldrich, TCI: Kraft lignin from TCI Deutschland, ECN Kraft: Kraft lignin from the Energy Research Centre of the Netherlands (ECN), ECN OS: organosolv lignin from ECN, FH OS: organosolv lignin from the Fraunhofer CBP. In each case, the yield was calculated by dividing the mass of the produced monomers by the initial mass of lignin. HTC was performed at 400 °C for 30 minutes for all lignin types, except for TCI which was processed for either 5 (TCI 5 min) or 30 minutes (TCI). (n=3)

As example, the hydrothermal conversion of Kraft lignin (Sigma Aldrich) provided a fairly good yield of catechol (4.4 %) and also rather high amounts

of guaiacol (3.1 %) and 4-methylcatechol (1.2 %). Further monomers (< 1 %) including phenol, 3-methylcatechol and 4-ethylcatechol were detected as well. The other lignin types resulted in substantial differences in the obtained monomer yield. The highest overall yields were achieved for Sigma Kraft lignin (10.3 %), and IndulinAT Kraft lignin (9.9 %) (Figure 4.5). Kraft lignin from ECN behaved similar to that from Sigma Aldrich, although they did not originate from the same wood species. Surprisingly, the third Kraft lignin (TCI Deutschland) performed less efficiently, despite the fact it originated from the same wood species and was produced with a similar lignin pulping method. A subsequent test of TCI lignin at a shorter conversion time of only 5 minutes, performed much better in terms of total yield (5.8 %). This result suggested that the TCI lignin reacted much faster, so that the incubation time of 60 minutes already suffered from aromatic monomer decomposition.

ECN OS lignin delivered a catechol yield of 3.1 %. The latter, however, was coupled with a rather high yield of guaiacol as by-product. In contrast, organosolv lignin from the Fraunhofer CBP showed a rather low catechol yield (1.1 %) so that the Kraft lignin finally emerged as most suitable.

Based on the overall aromatic monomer yield, as well as catechol and guaiacol levels reached, IndulinAT and Sigma Kraft lignin were selected for upscaling experiments. Due to the fact that the Kraft process is by far the most important process in lignocellulose pulping [85], the achieved results have a substantial industrial impact. Additionally, selected experiments were conducted using organosolv lignin (Fraunhofer CBP), to learn more about the optimized processing of hardwood-lignin.

4.3.2 Optimized conversion of Kraft and organosolv lignin

The two best-performing Kraft lignins (Sigma Aldrich, IndulinAT) and organosolv lignin (Fraunhofer CBP) were chosen for further studies in a pressure vessel (0.5 L volume). Each lignin was depolymerized in two different ways to selectively produce streamlined hydrolysates for the cell factories. One setup (395 °C, 1 hour) aimed at catechol as major compound for subsequent cultivation with *P. putida* MA-9 and *C. glutamicum* MA-2 (Figure 4.6 A). A second setup (370 °C, 20 minutes) was conducted such that mainly guaiacol accumulated, to be used later for cultivation with *Amycolatopsis sp.* MA-2 (Figure 4.6 B).

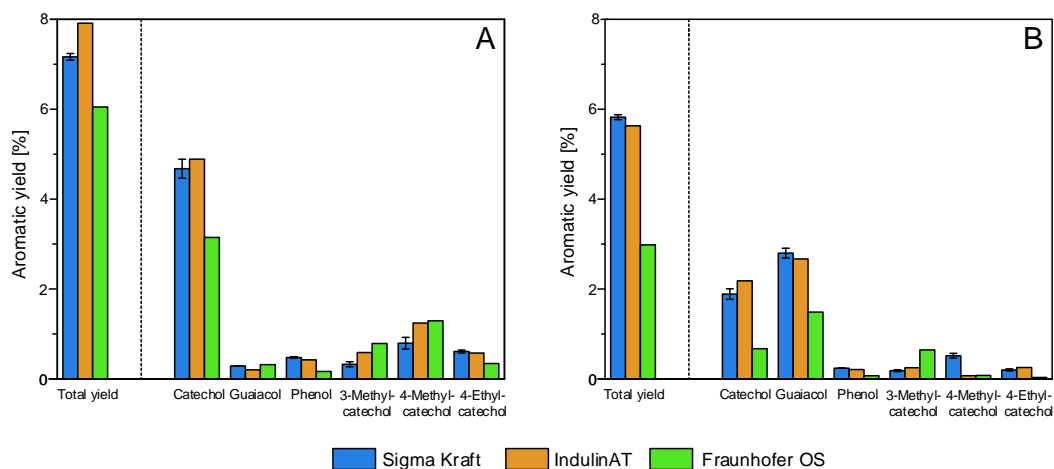Figure 4.6: Yield of aromatic monomers from hydrothermal conversion of different lignin types (Kraft lignin from Sigma Aldrich (n=3), IndulinAT (n=1) and organosolv lignin (Fraunhofer CBP) (n=1)). The process conditions were 395 °C and 1 hour reaction time (A) and 370 °C and 20 minutes reaction time (B), respectively. In each case, the yield was calculated by dividing the mass of the produced monomers by the initial mass of lignin.

Similar to the miniaturized experiments described above, the yield of catechol from Sigma Kraft (4.7 %) and IndulinAT (4.9 %) were higher than that from organosolv lignin (3.1 %), when the production of catechol was targeted. The yield for guaiacol was below 0.5 % for all three lignins. A total yield of up to 8 % aromatics was reached.

When the process was streamlined to produce preferably guaiacol, Sigma Kraft and IndulinAT yielded approximately 3.0 % guaiacol, double the yield of the organosolv lignin (1.5 %). The total aromatic yield (up to 6 %) was slightly lower as compared to the higher temperature processes.

Notably, the results from the miniature reactors were obviously transferable to the lab scale reactor and supported the upscaled production of catechol-rich and guaiacol-rich hydrolysates. The small spectrum of aromatics formed in each case, appeared beneficial for the subsequent bioconversion. In addition, the use of different setups for the hydrothermal conversion allowed to tailor the composition of the mixture for the microbe used afterwards.

4.3.3 Improvement of the aromatic content using vacuum concentration and distillation

In order to increase the content of the aromatics, the hydrolysates were treated by vacuum concentration or distillation, depending on the target compound of interest (Figure 4.7). Obtaining such a post-processed hydrolysate was important to support the efficiency of the subsequently

planned bioconversion [96]. In addition, an increased content of aromatics

reduced the dilution, caused by later feeding the hydrolysate to the cultures

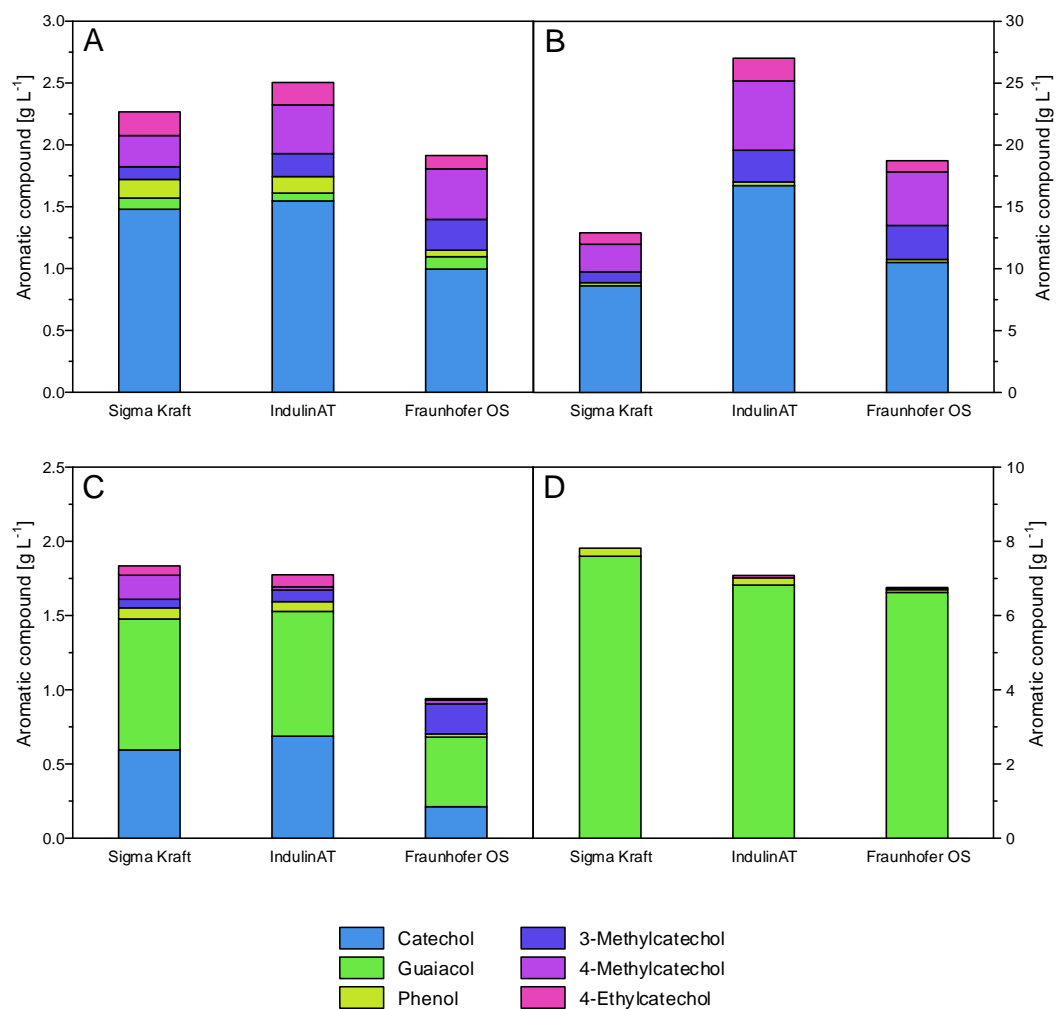and thus facilitated downstream processing [39].

Figure 4.7: Concentration of aromatic monomers after HTC and post-processing of three different lignin types (Kraft lignin form Sigma Aldrich and IndulinAT and organosolv lignin from the Fraunhofer CBP). Catechol-rich hydrolysate was obtained with a reaction temperature of 395 °C and a reaction time of 1 hour, whereas guaiacol-rich hydrolysate was obtained at 370 °C and after 20 minutes reaction time. Catechol-rich hydrolysate was processed by vacuum concentration and guaiacol-rich hydrolysate by distillation. A: catechol-rich hydrolysate, before processing. B: catechol-rich hydrolysate, after processing. C: guaiacol-rich hydrolysate, before processing. D: guaiacol-rich hydrolysate, after processing. (n=1)

Using a vacuum concentrator, the level of the catechol could be increased

6-fold for Sigma Kraft lignin and even 10-fold for IndulinAT and organosolv

lignin. A maximum aromatic content of more than 25 g L^{-1} in the remaining concentrate could be reached. The level of the (m)ethylated forms of catechol was also found increased, whereas guaiacol was completely separated into the gas phase. This promised improved performance, when feeding these hydrolysates to microbes, not capable to use guaiacol. Up to 66 % of the aromatics was present as catechol. About 99 % was either catechol or (m)ethylcatechol. The processed catechol-enriched hydrolysate from IndulinAT was superior to the others, with regard to its high aromatic content and high catechol yield.

A second approach attempted to separate all catechols from guaiacol and phenol in order to later generate pure muconic acid without methylated variants. As shown, all catechols remained in the liquid phase during distillation, whereas guaiacol evaporated and was collected as distillate. Guaiacol was increased about 8-fold for Sigma Kraft and IndulinAT and 14-fold in the case of organosolv lignin. Only trace amounts of (m)ethylcatechol were detectable. This resulted in a purification of guaiacol to over 96 %, with phenol as main by-product. The three lignin types, resulted in comparable final aromatic levels of up to 8 g L^{-1} in the enriched hydrolysate. A similar purity with comparable by-product spectrum was previously only reached after thermolysis in alternative solvents, such as *n*-hexadecane or 1-methylnaphtalene [53]. However, as both solvents were gained from processed crude-oil, hydrothermolysis is clearly to be preferred for its sustainability. Very recently, a 100 % pure guaiacol solution was obtained from lignin through Lewis acid La(OTf)$_3$ catalysis in methanol/water solvent [148]. Possibly, also a pure catechol solution could

be attained from such a pristine guaiacol extract through hydrolysis. Nonetheless, the simplicity of hydrothermal conversion, followed by distillation, appears still more favorable due to the expensiveness and reduced availability of Lewis acid La(OTf)$_3$. Also, the catalyst contains rare metals of which the mining process is potentially polluting and hazardous, thus reducing the attractiveness for a sustainable industrial process [132].

The achieved enrichment of the monomers (catechol by vacuum concentration and guaiacol from distillation) and the high total aromatic level of both hydrolysates displayed a promising starting point for the subsequent bioconversion.

4.3.4 Bioconversion of lignin hydrolysates into *cis,cis*-muconic acid using different microbes

The processed, catechol-rich hydrolysates of Kraft lignin (IndulinAT) and organosolv lignin (Fraunhofer CBP) were now used to produce *cis,cis*-muconic acid. *P. putida* MA-9 and *C. glutamicum* MA-2 were cultivated on the catechol-rich Kraft lignin hydrolysate (Figure 4.8), while feeding glucose as growth-supporting second carbon source.
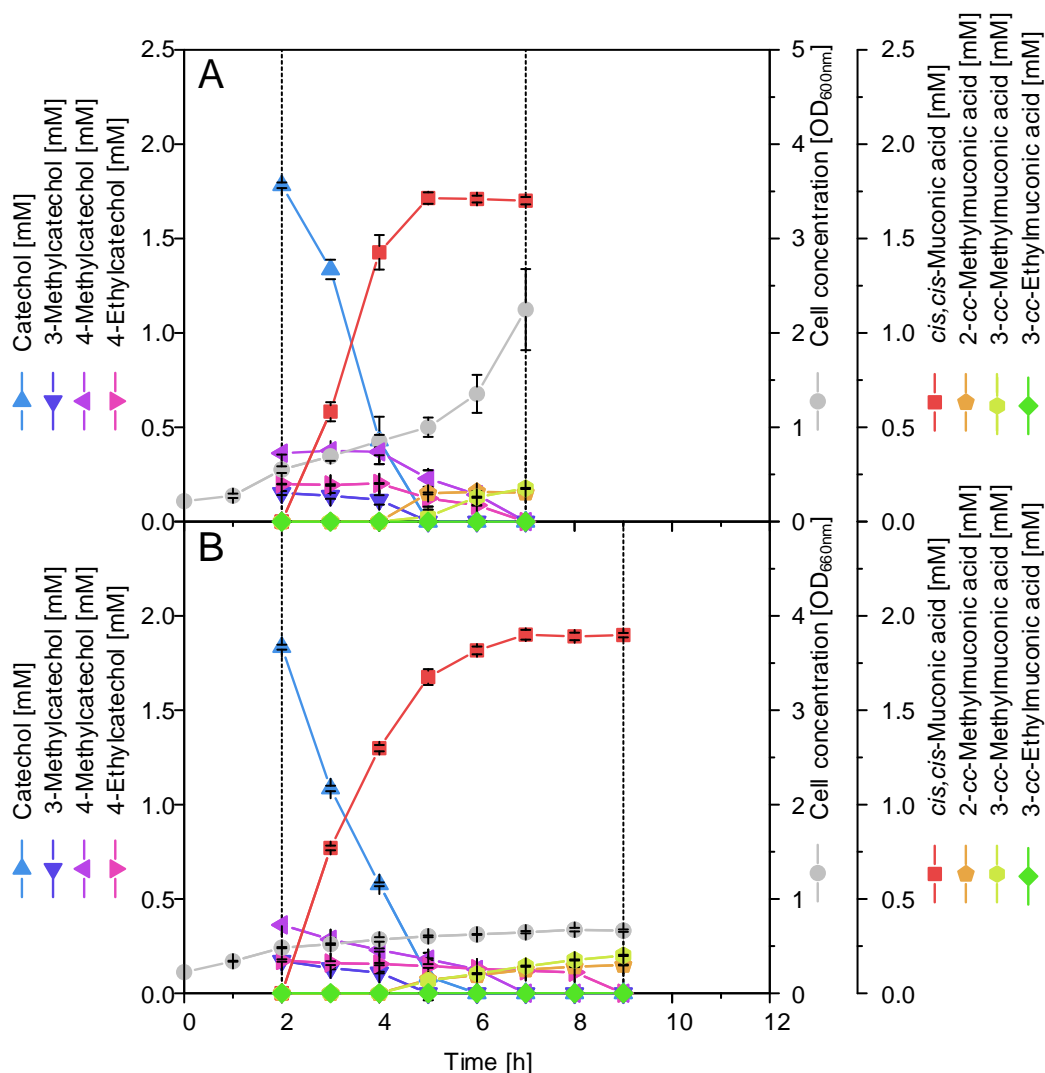
Figure 4.8: Production of *cis,cis*-muconic acid from a catechol-rich Kraft lignin hydrolysate using metabolically engineered strains *Pseudomonas putida* MA-9 [87] (A) and *Corynebacterium glutamicum* MA-2 [16] (B) at 30 °C. To each cultivation, glucose (5 g L⁻¹) was added as a co-substrate to support growth. The lignin hydrolysate was obtained from hydrothermal conversion of IndulinAT at 395 °C for 1 hour, followed by vacuum concentration. No phenol was detected. (n=3)

P. putida MA-9 and *C. glutamicum* MA-2 stoichiometrically converted catechol to *cis,cis*-muconic acid. The conversion was completed in less than 10 hours. Glucose (added at the beginning to a level of 5 g L⁻¹) was available during the entire conversion (data not shown). Methylcatechol was converted into the methylated forms of *cis,cis*-muconic acid. The yields appeared a bit lower, although a precise quantification of (m)ethylmuconic

acid was complicated by its low concentration. *P. putida* MA-9 started to grow after consuming the aromatics, whereas *C. glutamicum* MA-2 did not. Overall, both strains demonstrated the capacity to produce *cis,cis*-muconic acid from hydrolyzed IndulinAT.

In a second set of experiments, the catechol-rich hydrolysate from hydrothermal conversion of organosolv lignin was tested (Figure 4.9).

Figure 4.9: Production of *cis,cis*-muconic acid from a catechol-rich organosolv lignin hydrolysate using metabolically engineered strains *Pseudomonas putida* MA-9 [87] (A) and *Corynebacterium glutamicum* MA-2 [16] (B) at 30 °C. To each cultivation, glucose (5 g L^{-1}) was added as a co-substrate to support growth. The lignin hydrolysate was obtained from hydrothermal conversion of organosolv lignin at 395 °C for 1 hour, followed by vacuum concentration. No phenol was detected. (n=3)

Although the initial level of aromatics was chosen similarly to the cultivation on Kraft lignin hydrolysate, *P. putida* MA-9 did not grow on the processed organosolv lignin hydrolysate (see appendix Figure 6.1). A reduced initial hydrolysate amount, however, was successfully converted (Figure 4.9 A). It appeared that the presence of toxic ingredients specifically present in organosolv lignin, in addition to catechol, caused an inhibition of the strain. In this regard, lignin hydrolysates are known for their inhibitory effects on microbial cells. As example, inhibition is caused by phenolic compounds (e.g. vanillin, syringaldehyde, p-coumaric acid, ferulic acid, 4-hydroxybenzaldehyde), furfural, hydroxyl-methyl furfural, acetic acid and extractives [3, 33, 55, 145]. The most potent inhibitory phenolics during bioconversion with *Saccharomyces cerevisiae* are related in structure to Hibberts ketones [38, 112]. As Hibberts ketones have been primarily found in organosolv lignin types [40, 105], it is possible that Hibberts ketones impaired growth of *P. putida* as well. Notably, *C. glutamicum* MA-2 did not reveal such a sensitivity and converted the processed organosolv lignin (Figure 4.9 B). The performance was similar to the cultivation on IndulinAT.

A third set of experiments investigated the guaiacol-rich hydrolysates from both Kraft lignin (IndulinAT) and organosolv lignin using *Amycolatopsis sp.* MA-2 (Figure 4.10).
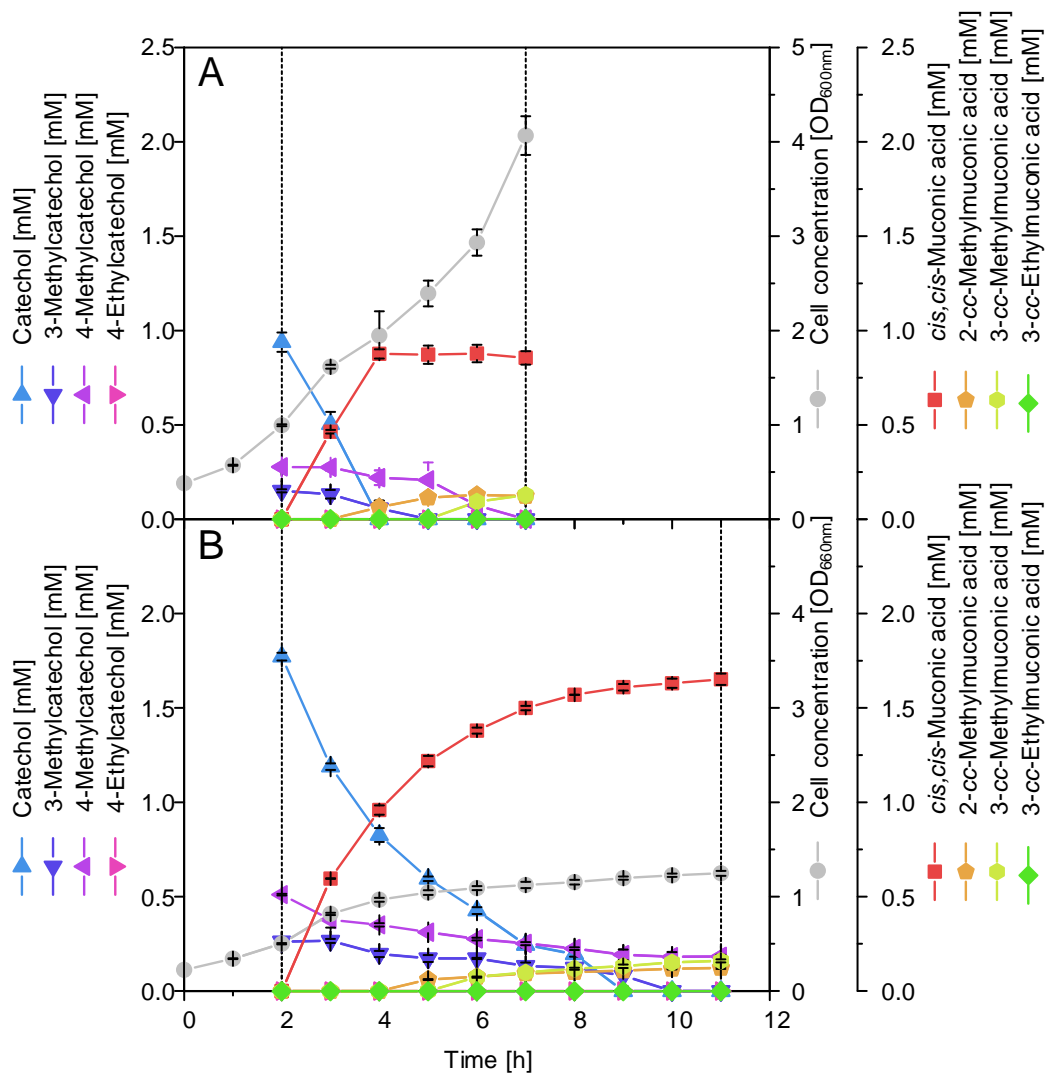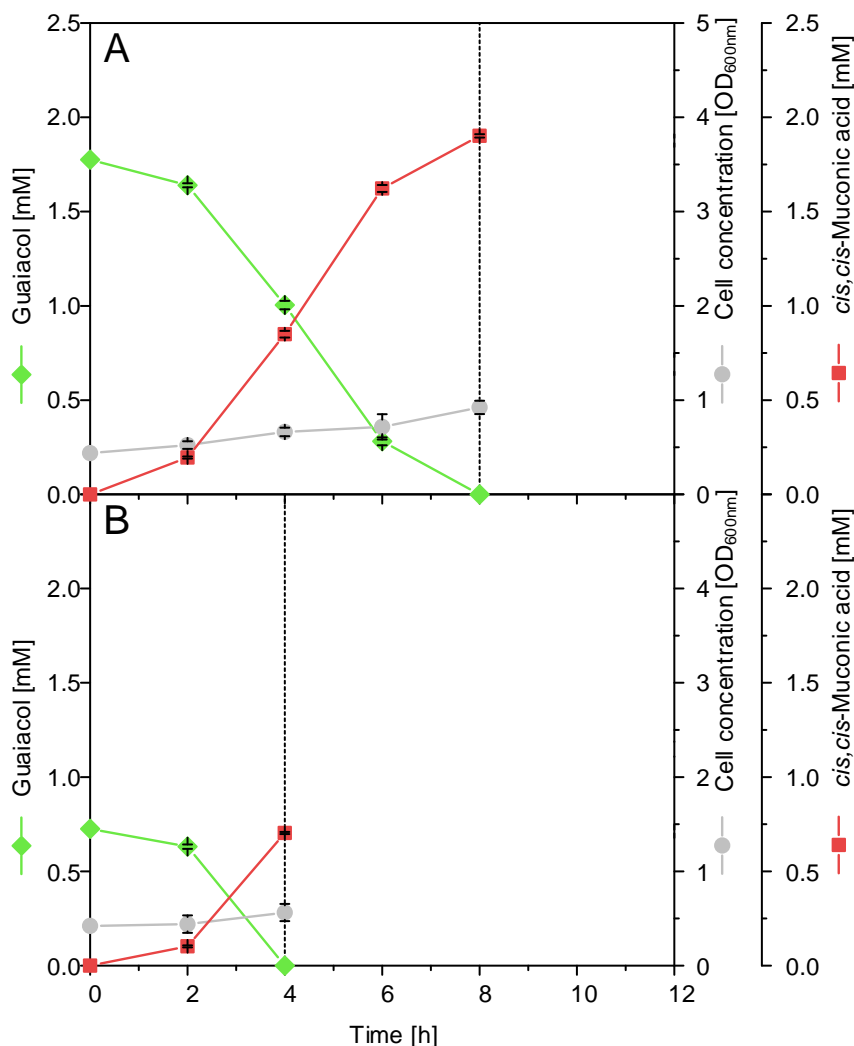
Figure 4.10: Production of *cis,cis*-muconic acid from a guaiacol-rich lignin hydrolysate using metabolically engineered strain *Amycolatopsis sp.* MA-2 [14] at 30 °C. To each cultivation, glucose (5 g L^{-1}) was added as a co-substrate to support growth. The lignin hydrolysate was obtained from hydrothermal conversion of IndulinAT (A) and organosolv lignin (B) at 370 °C for 20 minutes, followed by distillation. No phenol was detected. (n=3)

When grown on hydrolysate from Kraft lignin (IndulinAT), *Amycolatopsis sp.* MA-2 successfully converted guaiacol into *cis,cis*-muconic acid (Figure 4.10 A). Due to a limited availability of the guaiacol-rich organosolv lignin hydrolysate, the starting level of guaiacol (0.75 mM) had to be chosen slightly lower in the cultivation of *Amycolatopsis sp.* MA-2 on this material. The strain efficiently converted guaiacol into *cis,cis*-muconic acid (Figure 4.10 B).

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Table 4.3: Microbial production of *cis,cis*-muconic acid from lignin hydrolysates during batch processes

Strain	Raw material	Time [h]	Titer [g L^{-1}]	Yield [%]	Ref.
Amycolatopsis sp. MA-2	Organosolv hydrolysate	8	0.10	101 ± 3	this work
P. putida MA-9	Organosolv hydrolysate	7	0.13	94 ± 1	this work
Amycolatopsis sp. MA-2	IndulinAT hydrolysate	10	0.20	72	[14]
C. glutamicum MA-2	Organosolv hydrolysate	9	0.23	94 ± 2	this work
Amycolatopsis sp. MA-2	Sigma Kraft hydrolysate	8	0.23	105 ± 2	this work
P. putida MA-9	IndulinAT hydrolysate	5	0.24	96 ± 1	this work
C. glutamicum MA-2	Sigma Kraft hydrolysate	9	0.25	99 ± 1	this work
C. glutamicum MA-2	IndulinAT hydrolysate	7	0.27	103 ± 0	this work
Amycolatopsis sp. MA-2	IndulinAT hydrolysate	8	0.27	107 ± 1	this work
P. putida MA-9	Sigma Kraft hydrolysate	6	0.28	103 ± 2	this work
P. putida CJ103	Alkali lignin hydrolysate	24	0.70	-	[164]

Taken together, the conducted experiments demonstrated several important findings. First, Kraft and organosolv lignin derived hydrolysates could be successfully converted into *cis,cis*-muconic acid. Second, the tailored adjustment of the monomer spectrum led to efficient conversion into the target product. From the comparison of the different lignin types, IndulinAT yielded the best results. It provided the highest catechol yield during the depolymerization. When targeting guaiacol, it behaved at least as good as the other tested lignin types. The bioconversion experiments further showed that Kraft lignin and organosolv lignin hydrolysates could both be converted well, independent of the microorganism (Table 4.3). An exception was *P. putida* MA-9, which tolerated only lower amounts of

organosolv lignin hydrolysate. *C. glutamicum* MA-2 and *Amycolatopsis sp.* MA-2 were found very robust though and reached the best space-time-yields found in literature (Table 4.3).

4.4 Gram-scale production of *cis,cis*-muconic acid from repeated feeding of lignin hydrolysate

Repeated hydrolysis of lignin was conducted using the developed hydrothermal conversion approach to generate sufficient amounts for gram-scale production of *cis,cis*-muconic acid. Altogether, 50 mL of concentrated lignin (IndulinAT) hydrolysate was prepared from 7 consecutive hydrothermal conversion at 500 mL scale. The obtained solution contained 70 g L^{-1} of aromatic monomers, mostly catechol (45.2 g L^{-1}), smaller amounts of methylated (6.2 g L^{-1} 3-methylcatechol and 14.2 g L^{-1} 4-methylcatechol) and ethylated (4.6 g L^{-1} 4-ethylcatechol) forms and trace amounts of phenol (0.5 g L^{-1}) (appendix Figure 6.2). It should be noted that guaiacol and a fraction of phenol were removed by vacuum concentration, as described above.

In addition, IndulinAT was depolymerized and then distilled to produce a hydrolysate, rich in guaiacol. Several batches finally yielded a combined distillate with a volume of 120 mL and a concentration of 5 g L^{-1} aromatics, mainly guaiacol (4.4 g L^{-1}) (appendix Figure 6.3). The distillate did not contain the initially present catechol anymore and only contained traces of 4-ethylcatechol (0.1 g L^{-1}) and phenol (0.2 g L^{-1}) in addition to guaiacol.

The two types of hydrolysate could now be used to test the capacity of the microbes for high-level production in fed-batch mode. All three engineered microorganisms were evaluated.

The production process with *P. putida* MA-9 started with a two hour batch phase on glucose, which resulted in a 5-fold increase of the cell concentration (Figure 4.11). Then, a pulse of the catechol-rich hydrolysate was added, which was immediately converted. Subsequently, additional pulses were given every hour. The sugar level was kept in a range of 1 to 5 g L^{-1} by adding pulses from a glucose feed (500 g L^{-1}), when needed. Linked to the linear feed, *cis,cis*-muconic acid was produced constantly at a rate of 0.7 mM h^{-1}. A maximum titer of 15 mM (2.1 g L^{-1}) was reached after 24 hours. Additionally, 2.5 mM 3-*cis,cis*-methylmuconic acid and 1.5 mM 2-*cis,cis*-methylmuconic acid accumulated as by-products from the methylated catechols. After about 12 hours a low but steady increase in the level of *cis,trans*-muconic acid was observed, which reflected the natural isomerization [32]. It should be noted that the level of the lignin-based aromatics remained constant during almost the entire process, indicating that *P. putida* MA-9 efficiently converted the added raw materials. Towards the end of the process, however, methylated and ethylated variants of catechol accumulated slightly. Taken together, the strain performed well on the hydrolysate and accumulated *cis,cis*-muconic acid and its derivatives at the gram-scale.
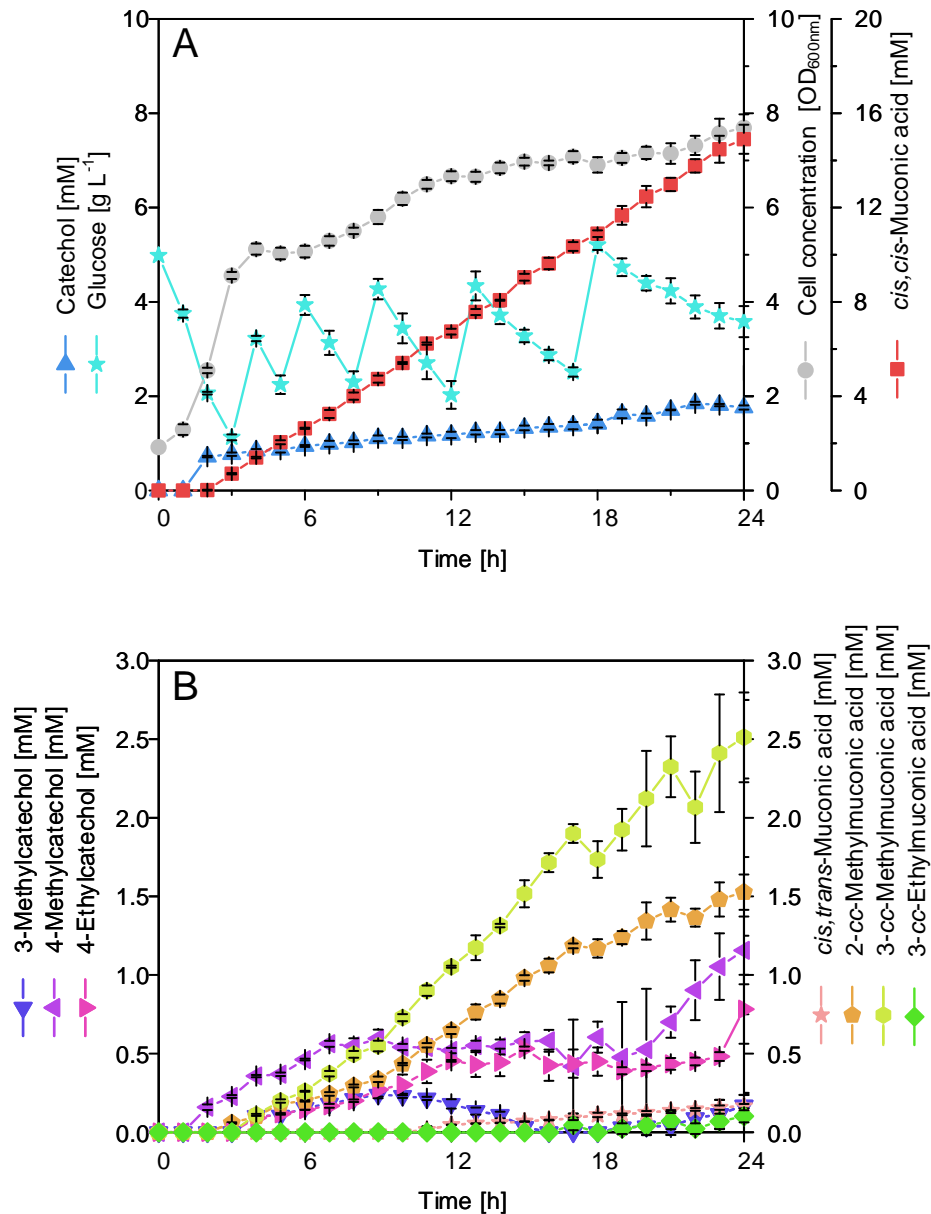
Figure 4.11: Fed-batch production of *cis,cis*-muconic acid from a catechol-rich hydrolysate using metabolically engineered *Pseudomonas putida* MA-9 [87] at 30 °C. Glucose was repeatedly added as a co-substrate to support growth from a concentrated feed (500 g L^{-1}). The lignin hydrolysate was obtained from hydrothermal conversion of IndulinAT at 395 °C for 1 hour, followed by vacuum concentration and was added pulse-wise every hour. The pH was controlled at 7.0 by NaOH. No phenol was detected. (n=3)

Using the same process setup, engineered *C. glutamicum* MA-2 was also tested on the catechol-rich hydrolysate (Figure 4.12). After the initial batch-phase on pure glucose, *C. glutamicum* MA-2 continued to grow, despite the presence of the aromatic substrates. At an optical density of 25 (OD$_{660nm}$)

the supply of glucose was reduced to study the robustness of the cells. In this way, glucose was no longer present in excess, but became limiting. As shown, growth slowed down, but the production of *cis,cis*-muconic acid continued at the same rate (0.7 mM h^{-1}). A maximum titer of 18.5 mM (2.6 g L^{-1}) of *cis,cis*-muconic acid was reached after 30 hours. In addition, methylated *cis,cis*-muconic acid was observed, as was a slight isomerization of the product into *cis,trans*-muconic acid [32]. Ethylated *cis,cis*-muconic acid was not detected. Overall, *C. glutamicum* MA-2 showed an excellent capacity to produce *cis,cis*-muconic acid from the lignin hydrolysate, even better than *P. putida* MA-9. The slight accumulation of aromatics at the end of the process might have been caused by the limited glucose feeding.
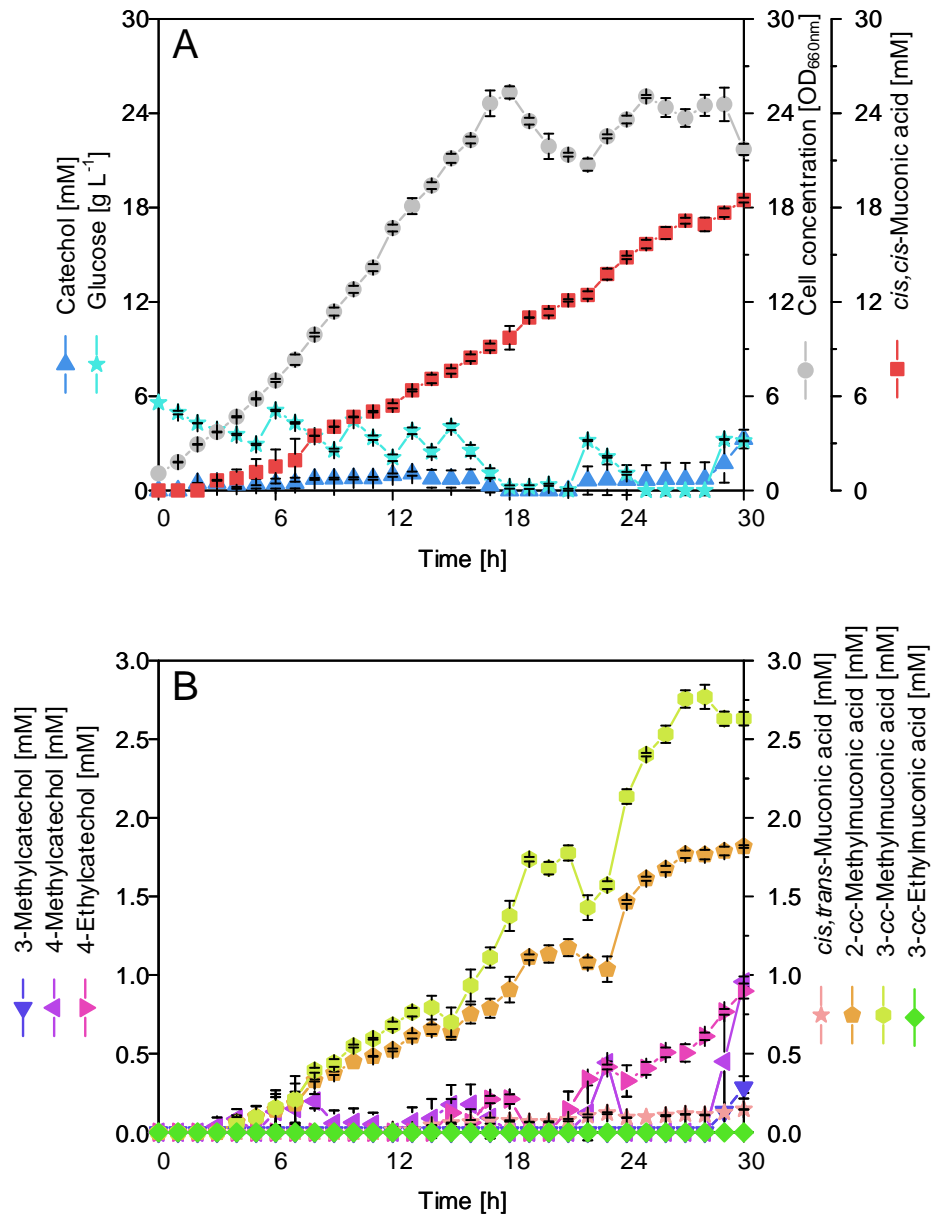
Figure 4.12: Fed-batch production of *cis,cis*-muconic acid from a catechol-rich hydrolysate using metabolically engineered *Corynebacterium glutamicum* MA-2 [16] at 30 °C. Glucose was repeatedly added as a co-substrate to support growth from a concentrated feed (500 g L^{-1}). The lignin hydrolysate was obtained from hydrothermal conversion of IndulinAT at 395 °C for 1 hour, followed by vacuum concentration and was added pulse-wise every hour. The pH was controlled at 7.2 by NaOH. No phenol was detected. (n=3)

In addition, concentrated guaiacol-rich hydrolysate was tested using *Amycolatopsis sp.* MA-2 (Figure 4.13). After six hours of conversion of the initial hydrolysate batch, a first pulse was added. Afterwards, additional pulses were added every six hours, while the sugar level was kept between

1 and 5 g L^{-1}, by adding pulses from a glucose feed (500 g L^{-1}), when necessary. *Amycolatopsis sp.* MA-2 grew during the whole process. *Cis,cis*-muconic acid was produced at a linear rate of approximately 0.32 mM h^{-1}, until a maximum titer of 8.5 mM (1.2 g L^{-1}) was reached after 30 hours. Congruently to the cultivations of *P. putida* MA-9 and *C. glutamicum* MA-2, the natural isomerization into *cis,trans*-muconic acid was observed.
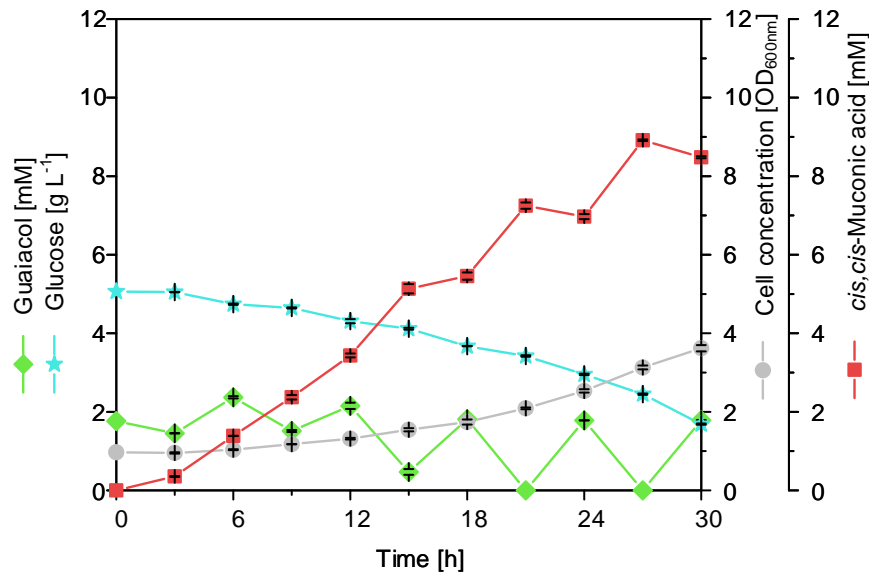

Figure 4.13: Fed-batch production of *cis,cis*-muconic acid from a guaiacol-rich hydrolysate using metabolically engineered *Amycolatopsis sp.* MA-2 [14] at 30 °C. Glucose was repeatedly added as a co-substrate to support growth from a concentrated feed (500 g L^{-1}). The lignin hydrolysate was obtained from hydrothermal conversion of IndulinAT at 370 °C for 20 minutes, followed by distillation and was added pulse-wise every six hours. The pH was controlled at 7.0 by NaOH. No phenol was detected. (n=3)

The cultivations showed the potential of all three strains to produce *cis,cis*-muconic acid (and its *cis,trans* derivative) from hydrolyzed lignin. Using pulse-feeding, higher amounts of the aromatic substrates could be provided, which culminated stoichiometrically in higher titers. *C. glutamicum* MA-2 apparently outperformed *P. putida* MA-9 and *Amycolatopsis sp.* MA-2, when comparing the final titer of *cis,cis*-muconic acid. It was therefore chosen for further studies.

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4.5 The use of hemicellulose-derived sugars as co-substrate enables an entirely lignocellulose-based process

4.5.1 Hemicellulose depolymerization into sugar monomers

Towards a production process, completely based on lignocellulosic biomass, further development aimed to use hemicellulose as co-substrate. Both, lignin and hemicellulose, originate from woody biomass, are noncompetitive to food production, and are even considered waste [61, 145].

To enable utilization of hemicellulose, its depolymerization into the corresponding monomers was attempted. Due to the excellent performance of hydrothermal conversion to obtain highly bio-available aromatic monomer mixtures and the suitability of using it to depolymerize hemicellulose, hydrothermal conversion was chosen as a process strategy [123].

The hydrothermal conversion process was established using xylan as model compound [120]. Xylan is a common hemicellulosic polysaccharide, consisting of a β-(1,4)-linked xylose backbone with α-(1,2)-linked glucuronosyl and 4-O-methyl glucuronosyl residues [140]. Especially hardwood hemicellulose is rich in xylan [135].

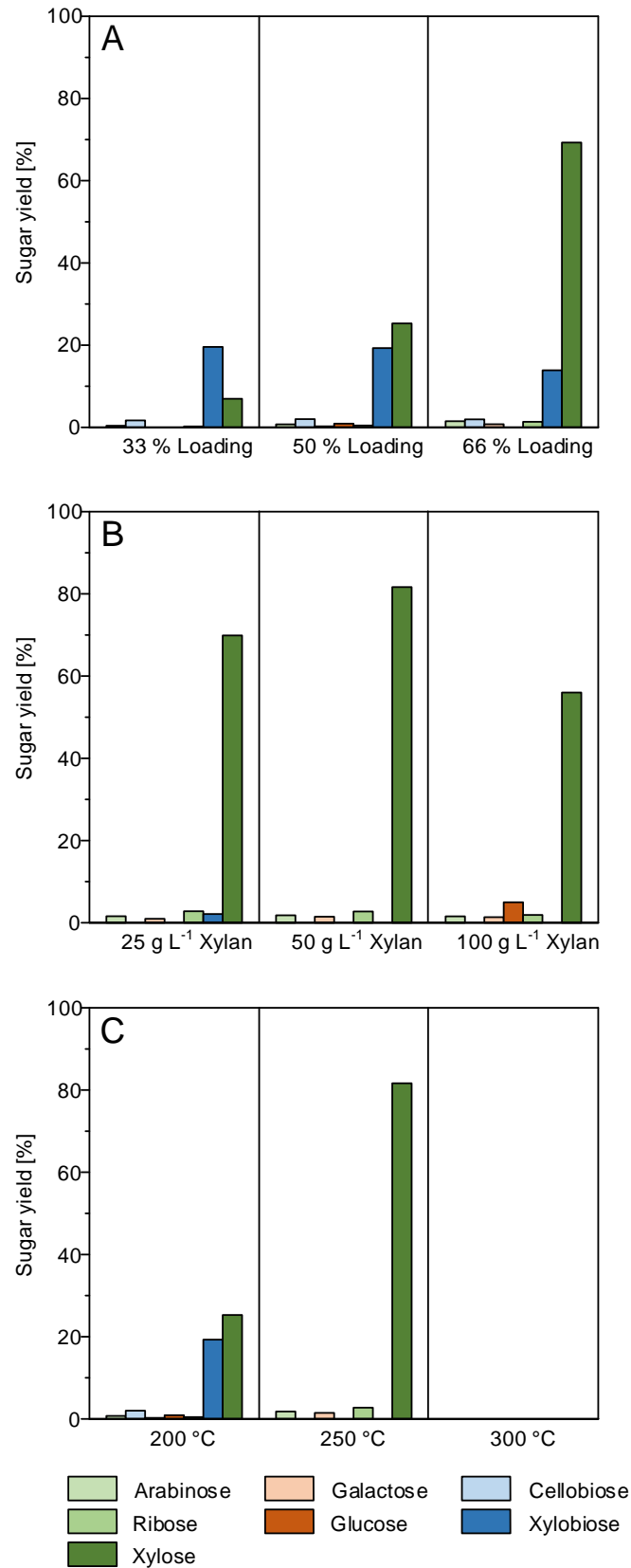Figure 4.14: Recovery of sugars after hydrothermal conversion of xylan under different reaction conditions. 50 g L^{-1} xylan, 200 °C, 25 minutes and different loadings (A), 50 % loading, 250 °C, 25 minutes and different xylan levels (B) and 50 % loading, 50 g L^{-1} xylan, 25 minutes and different temperatures (C). Yields were determined by dividing the mass of the produced sugar by the initial mass of xylan. (n=1)

Using the miniature reactors, described above, four main parameters, i.e. reactor loading, initial substrate concentration, reaction temperature and reaction time, were analyzed (Figure 4.14 and Figure 4.15). Due to the excellent reproducibility of hydrothermal conversion, as demonstrated for this type of setup (see lignin-based studies), the screening was conducted as single experiments for the sake of a broader coverage of conditions. Up to 44 g L^{-1} of total sugar was formed, representing a conversion yield of 90 %. The amount of sugar formed from hemicellulose was much higher than the amount of aromatics formed from lignin. This points to the less recalcitrant and less complex structure of hemicellulose [145]. Xylose was the major product. The optimal reaction temperature was 250 °C. At a temperature of 300 °C all sugars were decomposed [52, 123]. At 200 °C, lower amounts of xylose were formed and higher amounts of xylobiose were still observed, possibly from an incomplete conversion at this time point (25 minutes). Further experiments at a longer reaction time revealed that a high xylose yield (80 % xylose) was also achieved at 200 °C (Figure 4.15). In this case, the maximum yield was observed after 40 to 45 minutes. Small amounts of glucose were produced as well, especially at reaction times longer than 40 minutes. Time-resolved analysis revealed that the conversion was a sequential process (Figure 4.15). First, the disaccharides appeared, which were then further converted into the monomers [123]. The monomers tended to decompose in later stages, underlining the importance of a well-chosen reaction time and reaction temperature.

Figure 4.15: Recovery of sugars after hydrothermal conversion of xylan (200 °C, 50 g L^{-1}, 50 % loading) for different time periods. (n=3 for 20, 35 and 50 minutes). Yields were determined by dividing the mass of the produced sugar by the initial mass of xylan.

From these screening experiments, optimal process conditions were determined as follows: 66 % reactor loading, 50 g L^{-1} xylan, 200 °C and 40 minutes. The next step was to depolymerize real hemicellulose under

the same conditions and to confirm the suitability of the identified parameters (Figure 4.16).
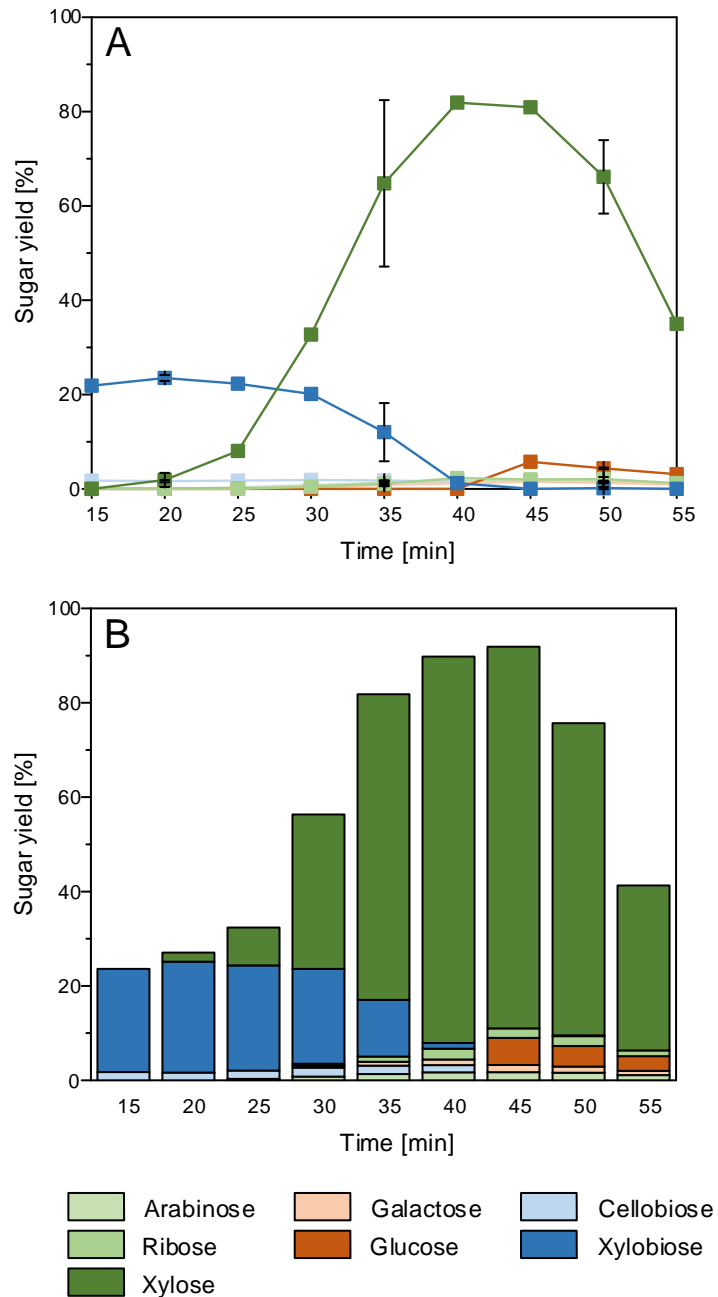

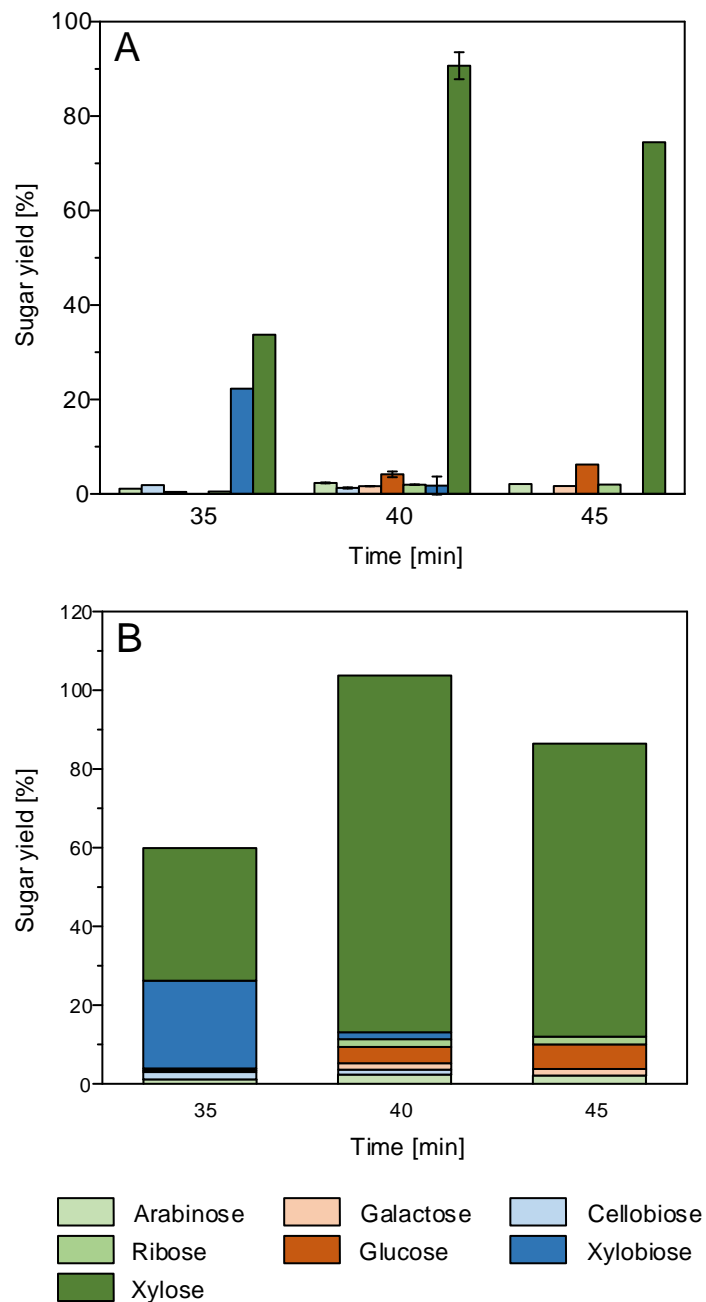Figure 4.16: Recovery of sugars after hydrothermal conversion of hemicellulose (200 °C, 50 g L^{-1}, 66 % loading) for different time periods. (n=3 for 40 minutes). Yields were determined by dividing the mass of the produced sugar by the initial mass of hemicellulose.

As the reaction time seemed to have a high influence on the xylose yield, also 35 and 40 minutes were tested. The highest xylose yield (91 %) was

observed after 40 minutes, with only trace amounts of xylobiose. A slightly shorter reaction time caused high amounts of xylobiose, whereas a slightly longer reaction time showed xylose decomposition. Glucose was the major by-product (6 %). A reaction time of 40 minutes yielded 103 % of total sugar monomers, which appeared slightly too high. However, one should note that water was incorporated during the hydrolysis process, adding between 3 and 6 % to the mass of the products obtained. Therefore, the obtained values appeared fully feasible and indicated a highly efficient conversion.

It is known that heating of sugar-polymers results in the formation of by-products such as furfural [51, 103]. To investigate, whether this was the case here too, HPLC analysis was applied to the hemicellulose hydrolysates (Figure 4.17). Furfural and 5-hydroxymethylfurfural (5-HMF), as well as smaller amounts of formic acid and acetic acid were detected. Furfural obviously accumulated in later stages, because it was formed through dehydration of xylose [52, 69, 123]. Likewise, 5-HMF was formed from hexoses, such as glucose [134, 162]. Formic acid was only detectable for the longest process time. Due to its toxicity [114], furfural was potentially inhibiting the subsequent bio-conversion, so that strategies were developed to remove it from the hydrolysate.
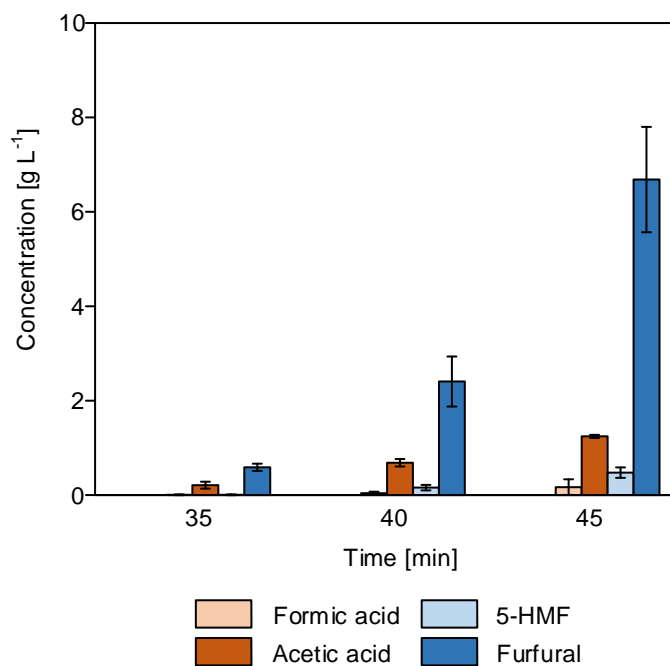
Figure 4.17: Concentration of organic acids and furfurals after hydrothermal conversion of hemicellulose (200 °C, 50 g L^{-1}, 66 % loading). (n=3)

Next, a scale-up of the conversion to 500 mL scale was performed to produce sufficient xylose for bioconversion experiments. As substrate, 16.67 g hemicellulose and 330 mL water were used (66 % loading, 50 g L^{-1} hemicellulose). The reaction temperature was set to 200 °C, resulting in a vessel pressure of 10 bar. In contrast to the miniature reactors, the lab-scale vessel required a much longer time to be heated to the desired temperature. To take this into account, shorter reaction times were considered (Figure 4.18).
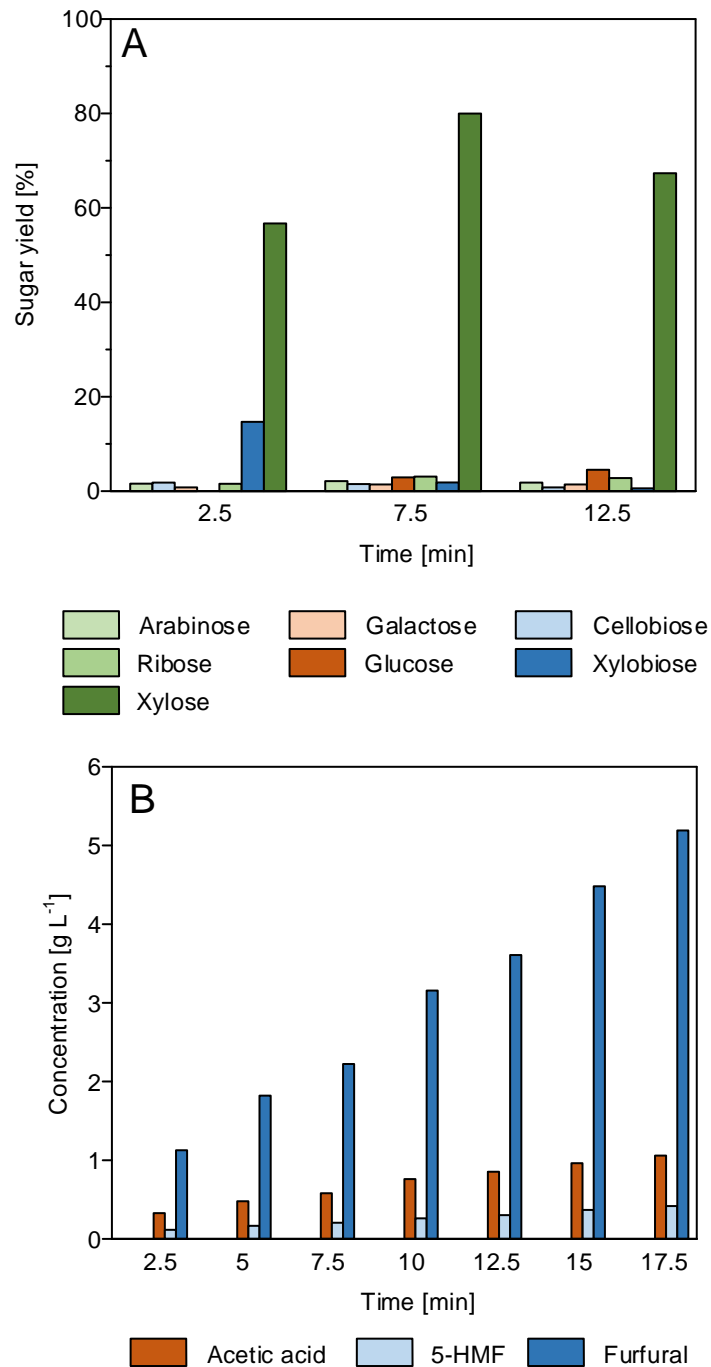
Figure 4.18: Recovery of sugars (A) and concentration of acetic acid and furfurals (B) after hydrothermal conversion of hemicellulose (200 °C, 50 g L^{-1}, 66 % loading) in lab-scale. Yields were determined by dividing the mass of the produced sugar by the initial mass of hemicellulose. No formic acid was detected. (n=1)

A reasonable xylose and glucose yield was observed after 7.5 minutes. Linearly increasing amounts of furfural and other by-products over time underlined the importance of keeping the reaction time low. Notably, formic

acid was not detected. Starting from hemicellulose, several grams of xylose and glucose were produced next to support growth during subsequent lignin-based bioconversion processes.

4.5.2 <u>Purification and vacuum concentration</u>

To reduce the amount of undesired acetic acid and furfurals in the hydrolysate, distillation, vacuum concentration and adsorption with activated charcoal were employed (Figure 4.19). First, the hemicellulose hydrolysate was distilled (20 minutes, 130 °C), which removed most of the furfural. However, 5-HMF and acetic acid remained. Subsequently, the solution was vacuum concentrated. Then, treatment with activated charcoal completely removed the remaining furfural and 5-HMF. The processed hydrolysate had a concentration of more than 90 g L^{-1} xylose, 7 g L^{-1} glucose, minor amounts of other pentoses and hexoses and contained only small amounts of acetic acid.
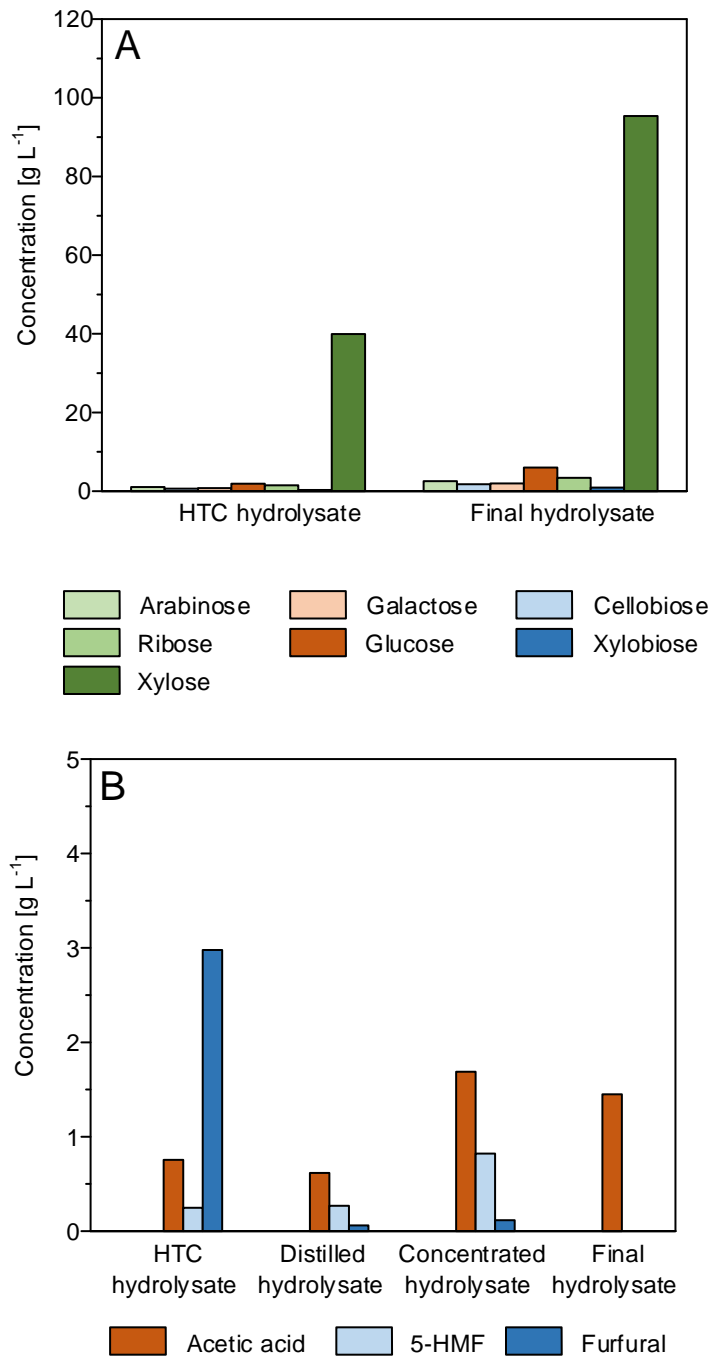
Figure 4.19: Concentration of sugars (A) and acetic acid and furfurals (B) during purification and vacuum concentration of hemicellulose hydrolysate. HTC hydrolysate is the unprocessed hydrolysate. After distillation and subsequent vacuum concentration, the distilled hydrolysate and concentrated hydrolysate were obtained, respectively. The final hydrolysate was additionally treated with activated charcoal. No formic acid was detected. (n=1)

4.5.3 Evaluation of *C. glutamicum* strains for *cis,cis*-muconic acid production using xylose as co-substrate

As hemicellulose was mainly converted into xylose, *C. glutamicum* had to be upgraded to utilize this C_5 monomer. The first tested strain, *C. glutamicum* MA-3, contained the necessary genes *xylA* and *xylB* from *E. coli* on an episomal plasmid [29]. The strain was based on *C. glutamicum* MA-1 on was therefore able to form *cis,cis*-muconic acid from aromatics. *C. glutamicum* MA-3 was used in a fed-batch process (Figure 4.20).

The main culture of *C. glutamicum* MA-3 was inoculated to an optical density of 1 (OD660nm). The medium contained sugars from processed hemicellulose hydrolysate as carbon source. After an optical density of 2.5 was reached (14 hours), a first pulse of lignin hydrolysate was added. Afterwards, additional pulses of the hydrolysate were given every two hours, while the sugar level was kept between 3 and 8 g L^{-1} by adding pulses from the hemicellulose hydrolysate, when necessary. *C. glutamicum* MA-3 efficiently utilized the added xylose and glucose for growth. Glucose was depleted faster, indicating that this sugar was the preferred carbon source for *C. glutamicum* MA-3. The strain was able to produce *cis,cis*-muconic acid from the provided aromatic monomers to a maximum titer of 6.5 mM (0.92 g L^{-1}). Catechol slightly accumulated during the cultivation, likely due to the fast feeding. In a similar manner, (m)ethylated forms of catechol accumulated over time, however at a lower concentration. Approximately, 0.3 mM and 0.6 mM of 2- and 3-*cis,cis*-methylmuconic acid were produced, respectively. Natural isomerization of *cis,cis*-muconic acid was again observed, yielding about 0.2 mM *cis,trans*-muconic acid.

Figure 4.20: Fed-batch production of *cis,cis*-muconic acid from a catechol-rich lignin hydrolysate using metabolically engineered *Corynebacterium glutamicum* MA-3 [16] at 30 °C. Sugars, obtained from HTC of hemicellulose (200 °C, 50 g L⁻¹, 66 % loading, 10 minutes), followed by post-processing (distillation, vacuum concentration and treatment with activated charcoal), was repeatedly added as a co-substrate to support growth. Lignin hydrolysate was obtained from hydrothermal conversion of IndulinAT (395 °C, 30 g L⁻¹, 50 % loading, 1 hour), followed by vacuum concentration and was added pulse-wise every two hours. No phenol was detected. (n=3)

As the cultivation of *C. glutamicum* MA-3 showed a limited conversion rate of catechol, *C. glutamicum* MA-4 was used. This strain was based on *C. glutamicum* MA-2 and contained a constitutive promoter from the *tuf* gene

for the catechol dioxygenase (CatA, NCgl2319) overexpression [16] and additionally expressed the xylose operon on a plasmid [29].

C. glutamicum MA-4 was cultivated on hemicellulose and lignin hydrolysate (Figure 4.21). After inoculation to an optical density of 1 (OD_{660nm}), a first pulse of lignin hydrolysate was added, when an optical density of 2.5 was reached. Subsequent pulses of lignin hydrolysate were given every two hours, while the growth substrate level was kept between 5 and 10 g L^{-1} by adding pulses from the hemicellulose hydrolysate, when necessary.

Remarkably, a final titer of 12.5 mM (1.8 g L^{-1}) of *cis,cis*-muconic acid was achieved. Additionally, 2- and 3-*cis,cis*-methylmuconic acid were produced (1.6 mM and 2.2 mM, respectively), as was a small amount of c*is,trans*-muconic acid from natural isomerization. *C. glutamicum* MA-4 was clearly superior to its parental strain, as it was able to produce double the amount of *cis,cis*-muconic acid in only half of the time with very little accumulation of catechol and (m)ethylcatechol.
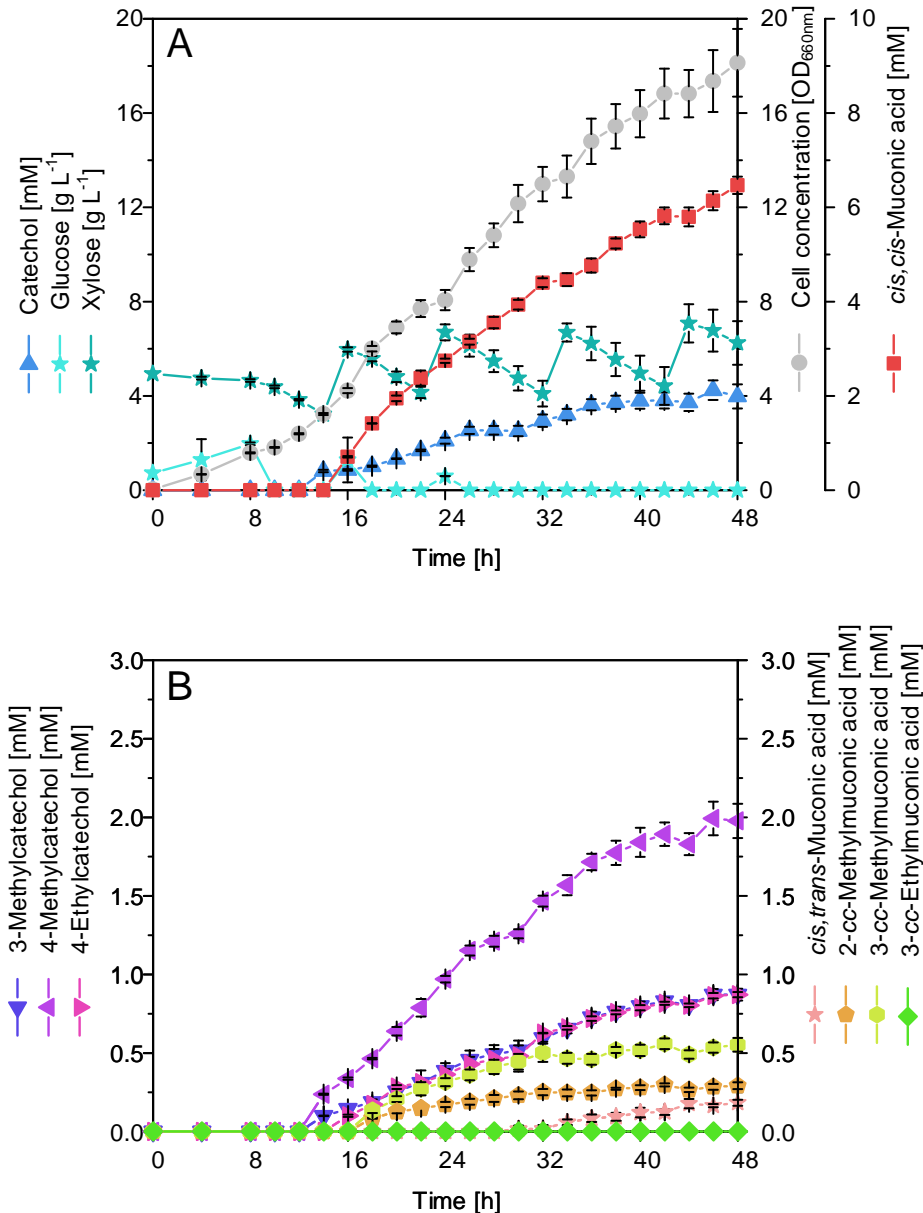
Figure 4.21: Fed-batch production of *cis,cis*-muconic acid from a catechol-rich lignin hydrolysate using metabolically engineered *Corynebacterium glutamicum* MA-4 [16] at 30 °C. Sugars, obtained from HTC of hemicellulose (200 °C, 50 g L^{-1}, 66 % loading, 10 minutes), followed by post-processing (distillation, vacuum concentration and treatment with activated charcoal), was repeatedly added as a co-substrate to support growth. Lignin hydrolysate was obtained from hydrothermal conversion of IndulinAT (395 °C, 30 g L^{-1}, 50 % loading, 1 hour), followed by vacuum concentration and was added pulse-wise every two hours. No phenol was detected. (n=3)

Towards a more industrially relevant setup, the cultivation was scaled up and *C. glutamicum* MA-4 was cultivated in a bioreactor. A similar

bioconversion experiment was performed with *C. glutamicum* MA-3 (see appendix Figure 6.4).

During the initial batch phase, *C. glutamicum* MA-4 grew on the added hemicellulose hydrolysate. After 6 hours, a first pulse of lignin hydrolysate was added to initiate *cis,cis*-muconic acid production. Additional pulses were given every two hours, while the sugar level was maintained by adding pulses from the hemicellulose-hydrolysate, when necessary.

Congruent to the cultivations in shake flasks, *cis,cis*-muconic acid production was faster for *C. glutamicum* MA-4 as compared to *C. glutamicum* MA-3 (Figure 4.24, appendix Figure 6.4). A final titer of 23 mM (3.3 g L^{-1}) was reached within 48 hours. Approximately 2 mM 3-*cis,cis*-methylmuconic acid and 1.5 mM 2-*cis,cis*-methylmuconic acid were produced as well and a concentration of 1 mM *cis,trans*-muconic acid was observed. Compared to the cultivation in shake flasks, *C. glutamicum* MA-4 increased its space-time-yield from 12.5 mM to 16.6 mM in 24 hours.
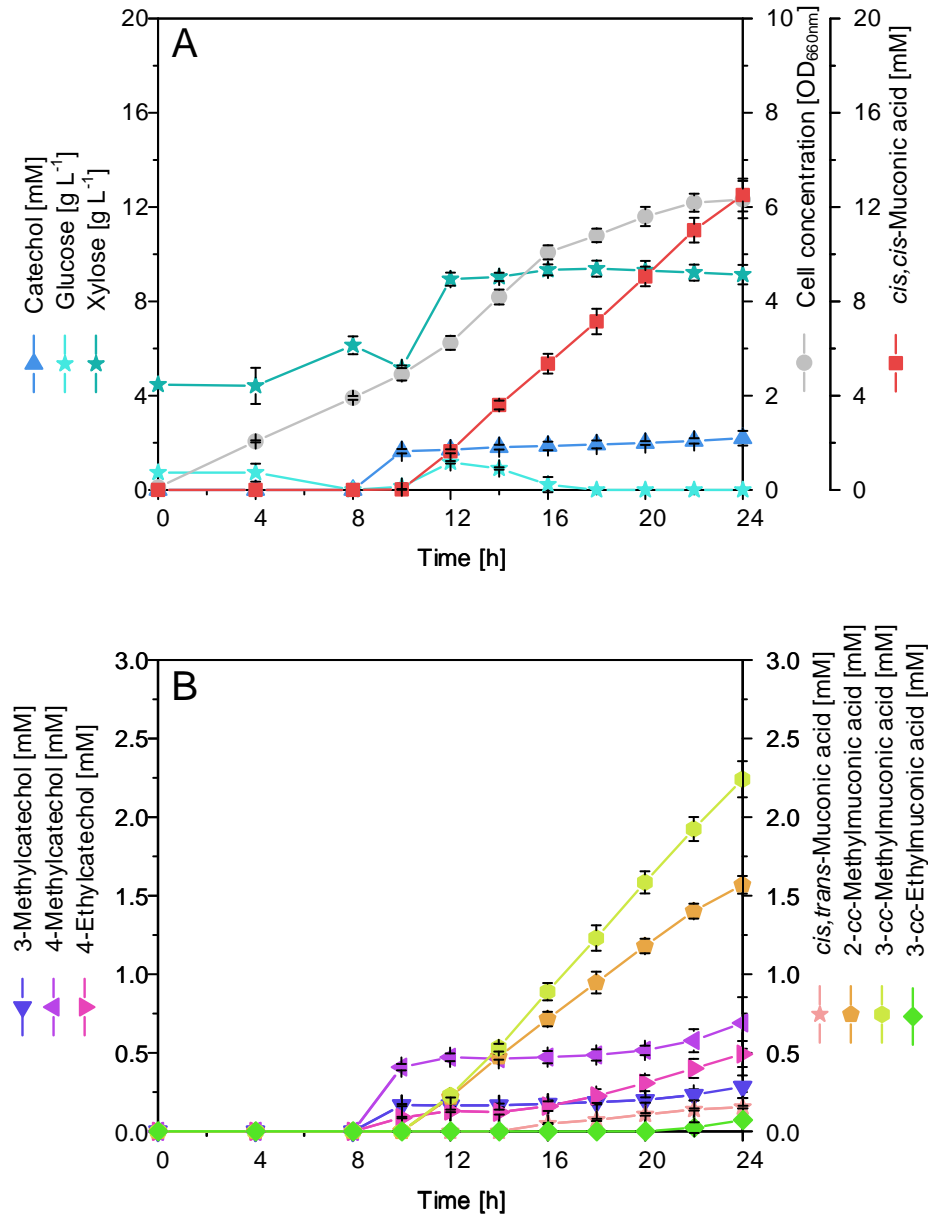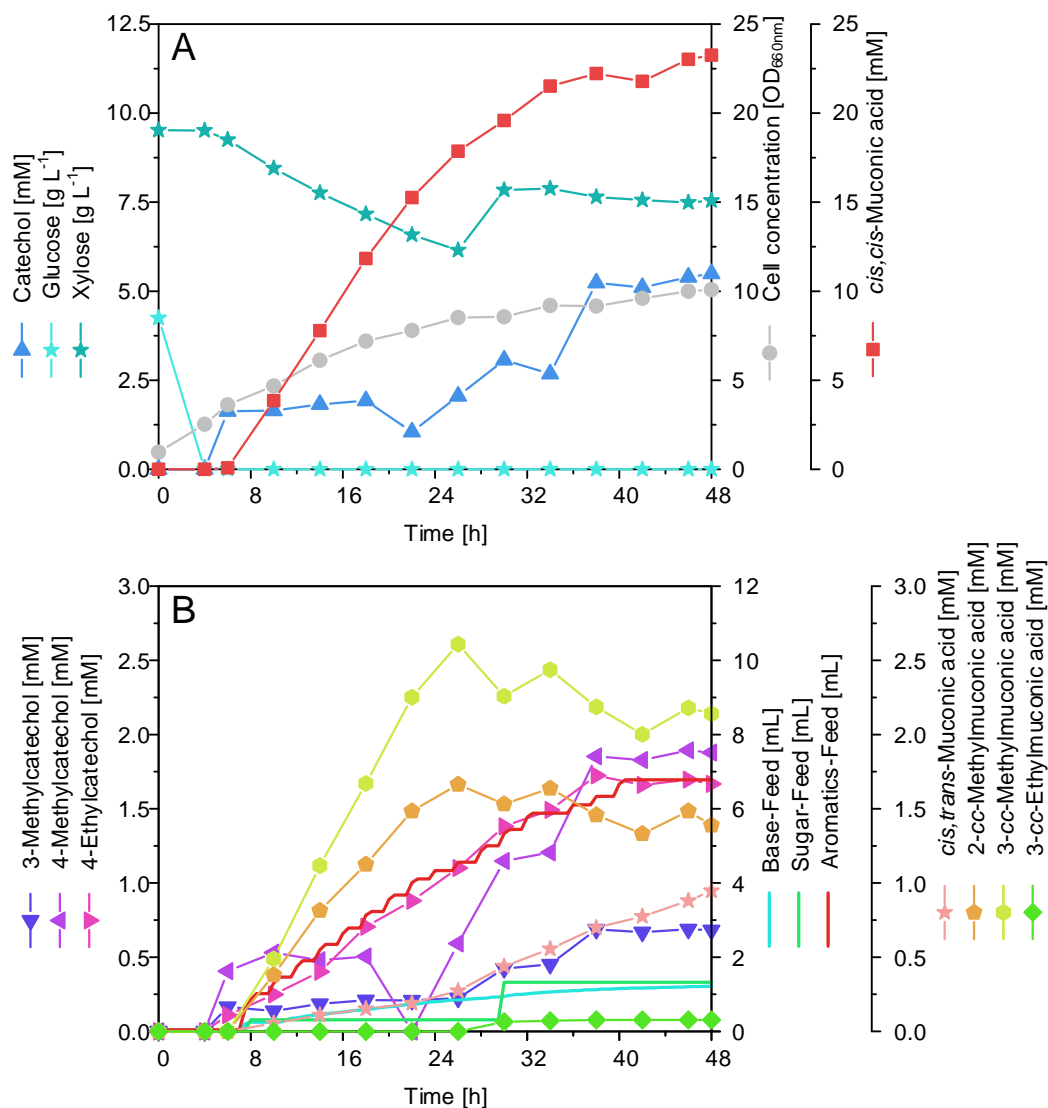
Figure 4.22: Fed-batch production of *cis,cis*-muconic acid from catechol-rich lignin hydrolysate using metabolically engineered *Corynebacterium glutamicum* MA-3 [16] at 30 °C in a bioreactor. Sugars, obtained from HTC of hemicellulose (200 °C, 50 g L^{-1}, 66 % loading, 10 minutes), followed by post-processing (distillation, vacuum concentration and treatment with activated charcoal), was repeatedly added as a co-substrate to support growth. Lignin hydrolysate was obtained from hydrothermal conversion of IndulinAT (395 °C, 30 g L^{-1}, 50 % loading, 1 hour), followed by vacuum concentration and was added pulse-wise every two hours. No phenol was detected. (n=1)

Table 4.4: Microbial production of *cis,cis*-muconic acid from lignin hydrolysates during fed-batch processes, using metabolically engineered microbes

Strain	Growth substrate	Raw material	Time [h]	Titer [g L^{-1}]	Yield [%]	Ref.
P. putida IDP/pTS110	Glucose	Alkali lignin hydrolysate	-	0.02	1	[151]
S. paucimobilis SME257/pTS084	Glucose	Alkali lignin hydrolysate	-	0.03	45	[151]
C. glutamicum MA-3	Hemicellulose hydrolysate	IndulinAT hydrolysate	48	0.9	96 ± 6	this work
Amycolatopsis sp. MA-2	Glucose	IndulinAT hydrolysate	30	1.3	101 ± 5	this work
C. glutamicum MA-3	Hemicellulose hydrolysate	IndulinAT hydrolysate	48	1.6	97	this work (appendix)
C. glutamicum MA-4	Hemicellulose hydrolysate	IndulinAT hydrolysate	24	1.8	94 ± 7	this work
C. glutamicum MA-2	Glucose	IndulinAT hydrolysate	27	1.8	100	[16]
P. putida MA-9	Glucose	IndulinAT hydrolysate	24	2.1	97 ± 4	this work
C. glutamicum MA-2	Glucose	IndulinAT hydrolysate	30	2.6	101 ± 5	this work
C. glutamicum MA-4	Hemicellulose hydrolysate	IndulinAT hydrolysate	48	3.3	97	this work
P. putida MA-9	Glucose	IndulinAT hydrolysate	54	13.0	100	[87]

Previous attempts to produce *cis,cis*-muconic acid from lignin hydrolysate have mainly used glucose as co-substrate [14, 16, 87, 164] (Table 4.4), which originated from (corn) starch and thus competes with feed and food industry [150]. One alternative concept solely used alkali lignin hydrolysate, however only achieved a rather low maximum titer of 0.03 g L^{-1} [151]. In contrast, the present result marks a new record of 4 g L^{-1} (28 mM) muconic acids, produced entirely in a renewable process, non-competitive to feed and food industry. It is therefore a promising starting point for a potential future application in a bio-based industrial setting.

5 Conclusion and Outlook

As most abundant carbon and aromatic deposit, lignocellulose is a promising renewable resource in the growing bio-based economy [92]. However, both the recalcitrance of lignin and the sensitivity of most microorganisms towards aromatic compounds, needs to be overcome [20]. So far, bioconversion of lignin-derived aromatic compounds [14, 16, 87, 88, 94, 96, 110, 149, 151, 164, 166, 172], had limited success.

In this work, a new record of 4 g L^{-1} muconic acids, produced entirely from renewables, non-competitive to feed and food, was achieved. Hydrothermal conversion of lignin and hemicellulose was tailored for selected microbial hosts. *Corynebacterium glutamicum* was engineered to metabolize hemicellulosic xylose and to accumulate *cis,cis*-muconic acid, an important chemical intermediate in bio-nylon production [87], from lignocellulosic aromatics (Figure 5.1).
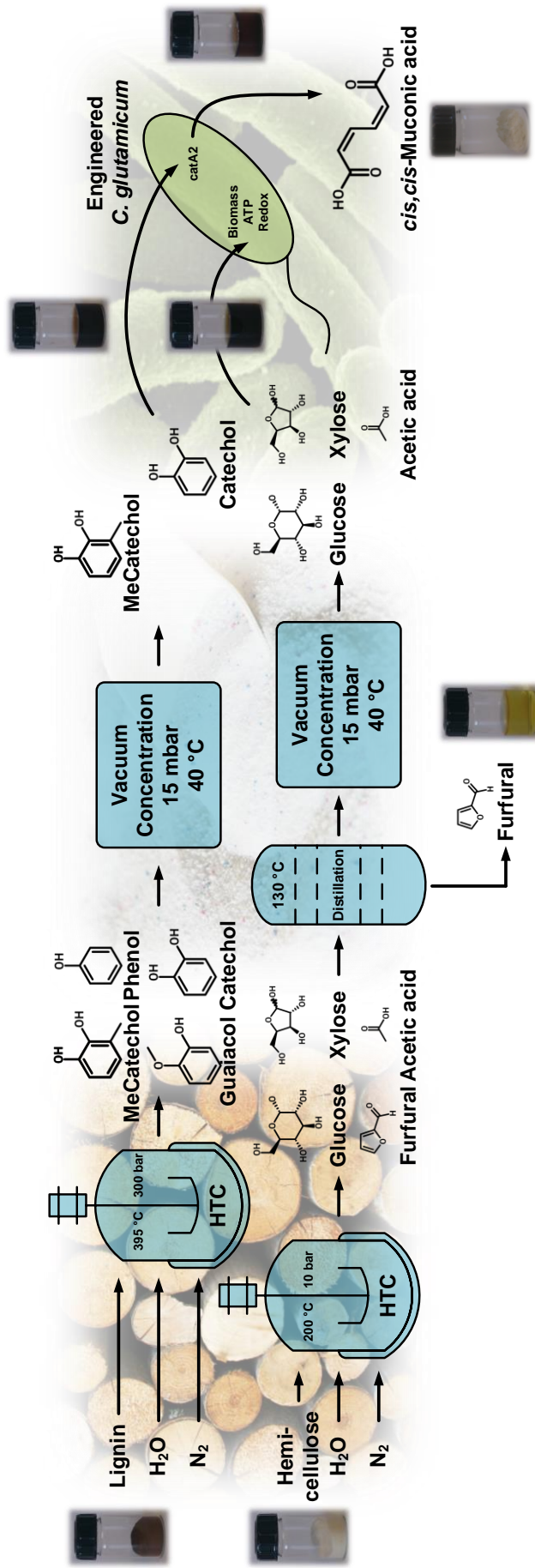
Figure 5.1: Process flow from powdered lignin and hemicellulose to cultivation supernatant containing *cis,cis*-muconic acid. Samples of every important step are shown.

It is a promising start for a potential future application in a bio-based industrial setting. Further efforts might even lead to additional improvement.

If a lignin-hydrolysate with higher purity could be generated, possibly significantly higher titers of *cis,cis*-muconic acid could be reached as bioconversion experiments with pure aromatic monomers have shown titers of up to 85 g L^{-1} [16]. Very recently, a 100 % pure guaiacol solution was acquired from lignin through Lewis acid La(OTf)$_3$ catalysis in methanol/water solvent [148]. Likely, also a pure catechol solution could be attained from such a pristine guaiacol extract through hydrolysis. Although, environmental considerations would need to be weighed [132]. In addition to modulating the aromatic spectrum, also the aromatic yield could be improved. Possible strategies include the use of additives, such as salts, and the use of different solvents [70, 145]. As the residual solvents and salts in the lignin hydrolysate could potentially hamper bioconversion performance [130], techniques for detoxification of the hydrolysates will need to be applied [74]. On the other hand, the development of a continuous-flow processing system for hydrothermal conversion could both increase the yield and support in scale-up [49].

Another aspect to be considered is bioconversion efficiency. Genetic optimization of the production host could further increase performance, e.g. by tailoring active transport of *cis,cis*-muconic acid [16], optimizing energy and redox metabolism [19] or genomic integration of the DNA [139]. Identifying the optimal process conditions could further contribute in enhancing efficiency of the superior microbial cell factory. A more advanced setup with automated feeding of lignin-hydrolysate, depending on the

dissolved oxygen signal, has been proposed previously [16]. Also optimization of the medium composition should be attempted, as it has proven effective in increasing the lipid-yield at least 2-fold when *Rhodococcus opacus* was grown on lignin-hydrolysate [96].

Lastly, modular lignocellulose biorefineries have been proposed that comprise the fractionation, depolymerization and bioconversion processes [43]. Such modular refineries could be flexibly modified to utilize different biomass sources, depolymerization strategies or a variety of microbial hosts to produce an array of chemical intermediates and products such as furfural, lipids, PHA, methylated and ethylated muconic acids, *cis,cis*-muconic acid, vanillin, vanillate and organic acids [19]. For example, the furfural that is separated from the hemicellulose hydrolysate by distillation could be chemically converted into tetrahydrofuran (THF) [165], which is used during the conversion of *cis,cis*-muconic acid via adipic acid into nylon-6.6 [87]. Moreover, also furfural has a wide variety of useful applications as a platform chemical, including the use as a solvent, the production of pharmaceuticals and the use as a fungicide [6, 93, 103]. Modular biorefinery concepts could progress the existing lignocellulose value-chains significantly [43] and, thus, might help lignin-valorization reach industrial relevance.

Appendix

6 Appendix

6.1 Abbreviations

5-HMF	5-Hydroxymethylfurfural
Ara	Arabinose
BSTFA	N,O-bis(trimethylsilyl)trifluoroacetamide
BTX	Benzene, Toluene and Xylene
*cc*MA	*cis,cis*-Muconic acid
Conc.	Concentration
ECN	Energy Research Centre of the Netherlands
EMP	Embden-Meyerhof-Parnas
EU	European Union
FH	Fraunhofer Institute CBP
g	Gravity constant
Gal	Galactose
GC-MS	Gas chromatography mass spectrometry
Glc	Glucose
GVL	γ-Valerolactone
HPLC	High performance liquid chromatography
HTC	Hydrothermal Conversion
Man	Mannose
MBDSTFA	N-methyl-N-tert-butyldimethylsilyl-trifluoroacetamide
OD	Optical Density
OS	Organosolv
PHA	Polyhydroxyalkanoate
PPP	Pentose phosphate pathway
TCA	Tricarboxylic Acid
THF	Tetrahydrofuran
US	United States of America
Xyl	Xylose

6.2   Cultivation of *P. putida* MA-9 on organosolv lignin hydrolysate
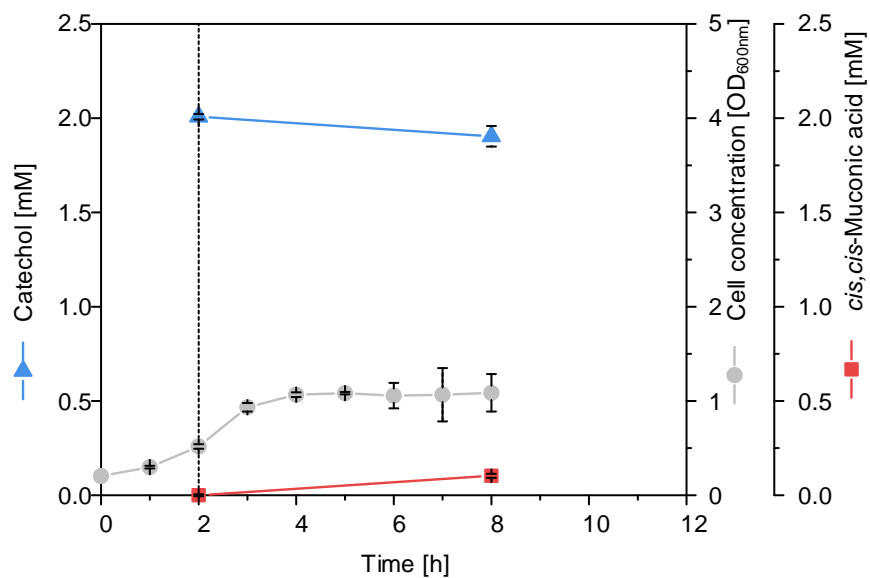
Figure 6.1: Production of *cis,cis*-muconic acid from a catechol-rich organosolv lignin hydrolysate using metabolically engineered strain *Pseudomonas putida* MA-9 at 30 °C. To the cultivation, glucose was added as a co-substrate to support growth. The lignin hydrolysate was obtained from hydrothermal conversion of organosolv lignin at 395 °C for 1 hour Details on the metabolic improvements of the used strain can be obtained from the literature [87]. (n=3)

6.3 Aromatic monomers obtained from multiple runs of hydrothermal conversion
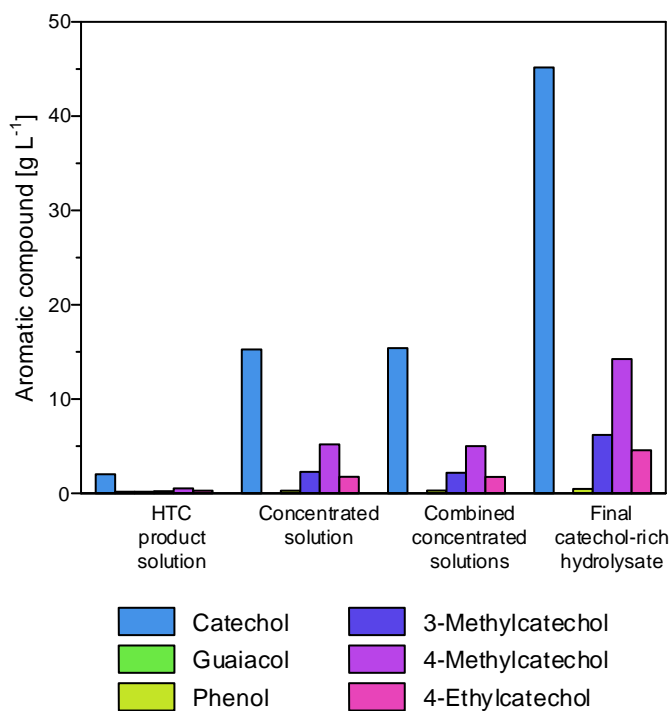
Figure 6.2: Concentration of aromatic monomers obtained from multiple runs of hydrothermal conversion (395 °C, 1 hour, IndulinAT) and subsequent vacuum concentration. The HTC product solution and its concentrated solution represent one exemplary run out of seven. The combination of the seven runs was represented by the combined concentrated solution, which was further concentrated to the final catechol-rich hydrolysate. (n=1)
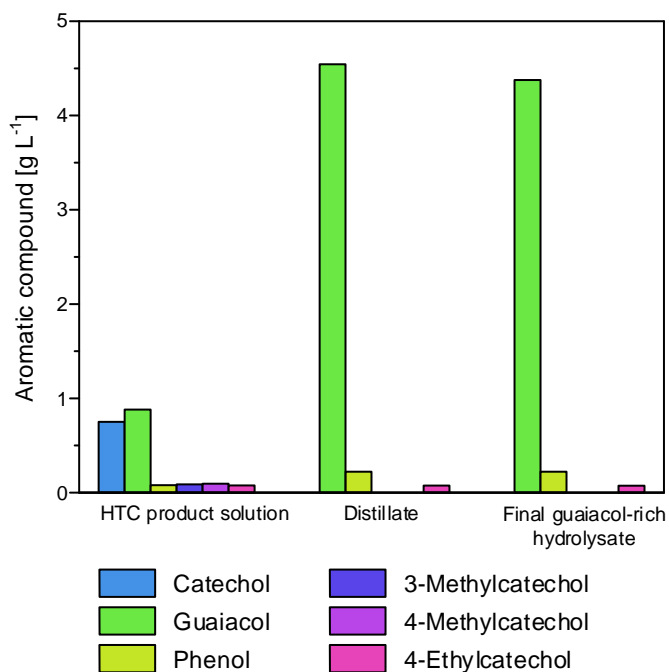
Figure 6.3: Concentration of aromatic monomers obtained from multiple runs of hydrothermal conversion (370 °C, 20 minutes, IndulinAT) and subsequent distillation. The HTC product solution and its distillate represent one exemplary run out of three. The combination of the three runs was represented by the final guaiacol-rich hydrolysate. (n=1)

6.4 Cultivation of *C. glutamicum* MA-3 on hemicellulose hydrolysate in a bioreactor with addition of lignin hydrolysate

Cis,cis-muconic acid was produced to a final titer of 11.2 mM (1.6 g L^{-1}), while 3-*cis,cis*-methylmuconic acid (1.9 mM), 2-*cis,cis*-methylmuconic acid (0.6 mM) and *cis,trans*-muconic acid (0.6 mM) accumulated as well. Using this improved setup, *C. glutamicum* MA-3 was able to produce double the amount of *cis,cis*-muconic acid in the same time as compared to cultivation in shake flasks. Additionally, the accumulation of catechol and (m)ethylcatechol was significantly reduced in comparison.
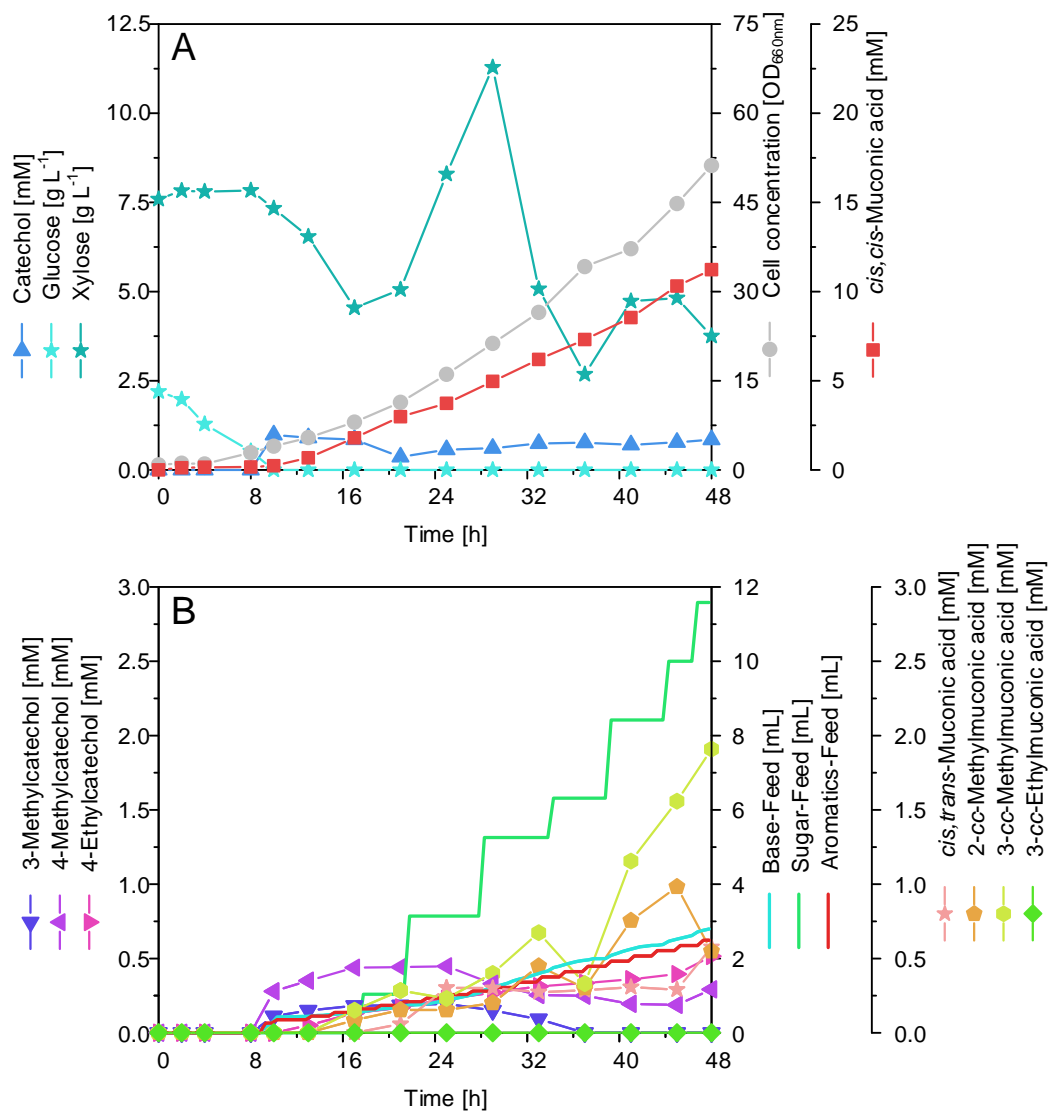
Figure 6.4: Fed-batch production of *cis,cis*-muconic acid from catechol-rich lignin hydrolysate using metabolically engineered *Corynebacterium glutamicum* MA-3 at 30 °C in a bioreactor. Xylose, obtained from HTC of hemicellulose (200 °C, 50 g L^{-1}, 66 % loading, 10 minutes) was repeatedly added as a co-substrate to support growth. Lignin hydrolysate was obtained from hydrothermal conversion of IndulinAT (395 °C, 30 g L^{-1}, 50 % loading, 1 hour), and was added pulse-wise every two hours. Details on the metabolic improvements of the used strain can be obtained from the literature [16]. No phenol was detected. (n=1)

6.5 Overview of results from hydrothermal conversion of lignin and subsequent bioconversion to muconic acids
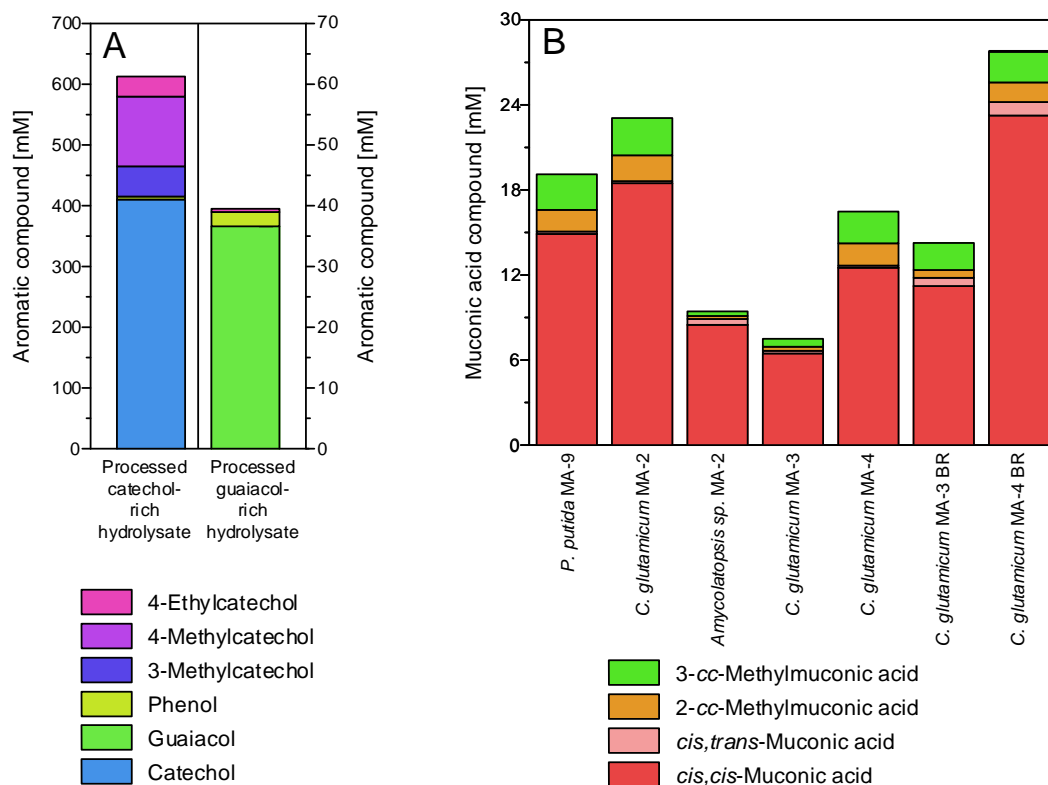
Figure 6.5: Overview of results from hydrothermal conversion of lignin (IndulinAT) at 395 °C, 1 hour and 370 °C, 20 minutes and subsequent processing (A) and bioconversion to muconic acids (B). (BR = Bioreactor)

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