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

The cover features a detailed process flow diagram. It starts with two parallel Hydrothermal Conversion (HTC) units. The top unit takes Lignin, H₂O, and N₂ as input and produces MeCatechol, Phenol, and Guaiacol Catechol. The bottom unit takes Hemicellulose, H₂O, and N₂ as input and produces Glucose, Xylose, and Furfural Acetic acid. The MeCatechol and Phenol products are then processed through a 'Vacuum Concentration' step (15 mbar, 40 °C) to yield MeCatechol and Catechol. The Glucose and Xylose products are processed through another 'Vacuum Concentration' step (15 mbar, 40 °C) to yield Glucose, Xylose, and Acetic acid. The MeCatechol and Catechol are then fed into a bioreactor containing 'Engineered *C. glutamicum*' which produces *cis,cis*-Muconic acid. The Acetic acid is also shown as a byproduct.

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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, CO₂-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 C₅ and C₆ 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,

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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 C₅ 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 Economic perspectives

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

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liters of biofuel should be produced each year [128], which would be CO₂-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 C₅ and C₆ sugar monomers and can constitute up to 30 % of plant dry weight [145].

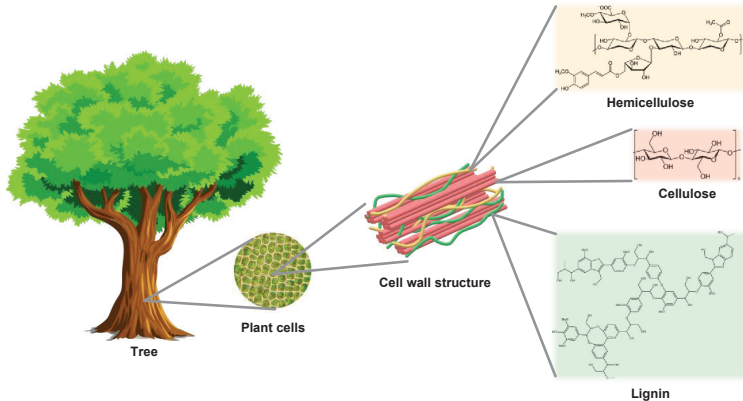


Figure 2.1: Composition of plant biomass. The wall of each individual plant cell is made of microfibrils in which cellulose, hemicellulose and lignin are intertwined. The lignin structure is an exemplary molecular structure.

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].

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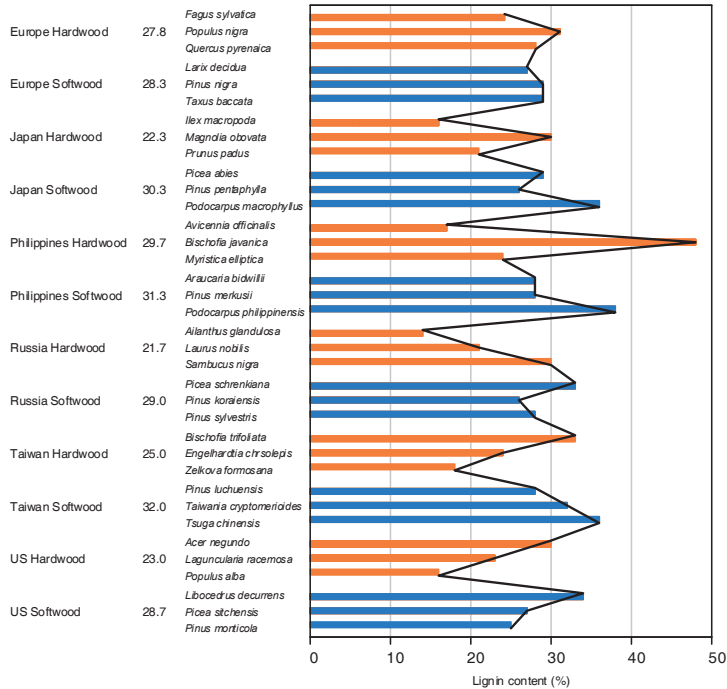


Figure 2.2: Lignin content [%] of soft- and hardwoods from different countries. The values next to the country are the average of the given species. The data are taken from literature, where more species can be found [2, 4, 50, 119].

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).

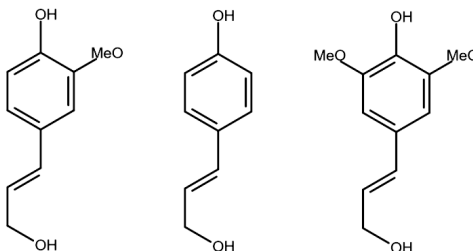


Figure 2.3: The three typical lignin monomers (monolignols) are coniferyl alcohol, *p*-coumaryl alcohol and sinapyl alcohol (from left to right).

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

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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 CO₂. 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 ₃ [%]	Glc [%]	Man [%]	Gal [%]	Ara [%]	Xyl [%]
Beech	Hardwood	23.00	73.98	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
<i>Eucalyptus globulus</i>	Hardwood	15.94	80.41	62.80	17.61	74.50	2.28	1.32	0.60	21.31
<i>Eucalyptus urograndis</i>	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