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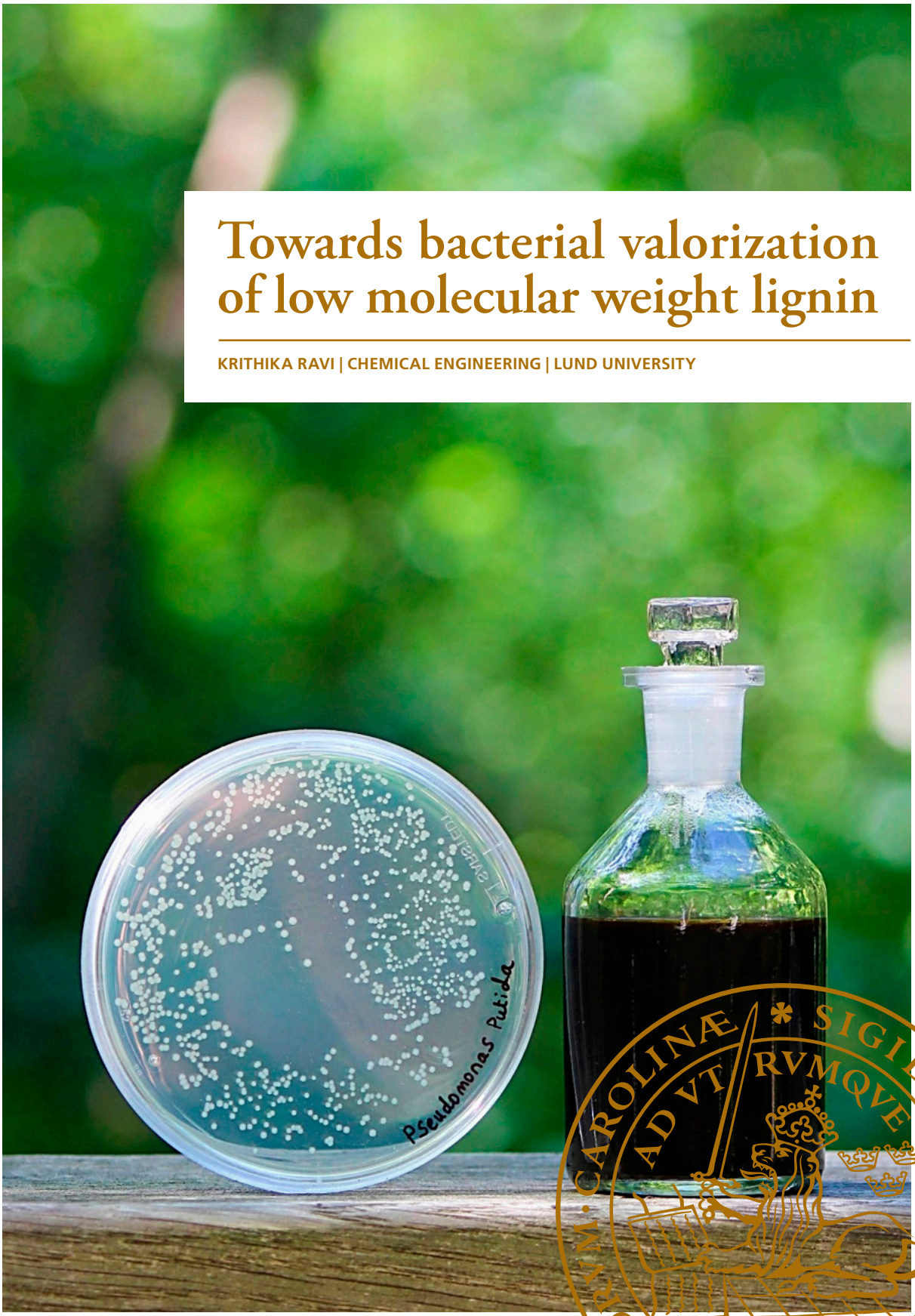

Towards bacterial valorization of low molecular weight lignin

DOCTORAL DISSERTATION

2019

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Title and subtitle
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Abstract

Lignin is one of the major constituents in lignocellulosic biomass and is the most abundant source of renewable aromatics on terrestrial ecosystems. The work carried out in this thesis concerns bacterial conversion of low molecular weight lignin. This thesis is divided into three major sections, with an initial emphasis on screening and characterization of selected bacterial species on lignin model compounds, followed by testing their performance on treated lignin substrates, and finally progressing towards strain improvement via metabolic engineering.

During screening for bacteria using samples from natural and man-made environments, *Pseudomonas* species were found dominant. Some of these isolates, and the well-known aromatic degrader – *Pseudomonas putida* KT2440 – were cultivated on lignin model compounds. *P. putida* and *Pseudomonas* sp. isolate 9.1 attained specific growth rates of about 0.21-0.27 h^{-1} and 0.12-0.30 h^{-1} respectively, on several compounds from the coniferyl, *p*-coumaryl and benzoyl branches of the funnelling pathways. Meanwhile, a contaminant was found growing on syringate plates, and was later identified to be a bacterium belonging to the *Microbacterium* genus. This Gram-positive bacterium, named RG1, was able to consume syringate and syringaldehyde besides other aromatic compounds from the coniferyl and *p*-coumaryl branches. Due to its interesting abilities to assimilate syringyl compounds, the genome of this strain was sequenced to identify genes involved in a putative syringyl pathway.

To assess the performance of selected bacteria on lignin substrates, cultivations were performed using alkaline- and oxidatively-treated Kraft lignin. *P. putida* and *P. fluorescens* consumed 4-HBA, vanillin, and vanillate in the complex lignin mixture that likely contained various toxic products. In addition, *Rhodococcus opacus* and *Sphingobium* sp. SYK-6 assimilated guaiacol and acetovanillone respectively, from the lignin mixture. Interestingly, *P. fluorescens* was able to break down the higher molecular weight lignin and produce several smaller molecules.

P. putida was selected as a host organism for genetic engineering aimed at expanding the range of substrates utilized. Heterologous expression of the cytochrome P450 and oxidoreductase genes from *R. rhodochrous* enabled *P. putida* to assimilate guaiacol – one of the major depolymerization products from softwood lignin – as the sole carbon source. Furthermore, the identification and deletion of an aldehyde reductase in a *P. putida* strain that converts ferulate to vanillin, increased the yield of vanillin by eliminating the formation of vanillyl alcohol as by-product.

Key words
Pseudomonas putida, Microbacterium sp., Guaiacol, Syringate, Kraft lignin, Bioconversion, Biorefineries

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"The greatest enemy of knowledge is not ignorance; it is the illusion of knowledge"

− Daniel J. Boorstin

Abstract

Lignin is one of the major constituents in lignocellulosic biomass and is the most abundant source of renewable aromatics on terrestrial ecosystems. The work carried out in this thesis concerns bacterial conversion of low molecular weight lignin. This thesis is divided into three major sections, with an initial emphasis on screening and characterization of selected bacterial species on lignin model compounds, followed by testing their performance on treated lignin substrates, and finally progressing towards strain improvement via metabolic engineering.

During screening for bacteria using samples from natural and man-made environments, *Pseudomonas* species were found dominant. Some of these isolates, and the well-known aromatic degrader – *Pseudomonas putida* KT2440 – were cultivated on lignin model compounds. *P. putida* and *Pseudomonas* sp. isolate 9.1 attained specific growth rates of about 0.21-0.27 h^{-1} and 0.12-0.30 h^{-1} respectively, on several compounds from the coniferyl, *p*-coumaryl and benzoyl branches of the funnelling pathways. Meanwhile, a contaminant was found growing on syringate plates, and was later identified to be a bacterium belonging to the *Microbacterium* genus. This Gram-positive bacterium, named RG1, was able to consume syringate and syringaldehyde besides other aromatic compounds from the coniferyl and *p*-coumaryl branches. Due to its interesting abilities to assimilate syringyl compounds, the genome of this strain was sequenced to identify genes involved in a putative syringyl pathway.

To assess the performance of selected bacteria on lignin substrates, cultivations were performed using alkaline- and oxidatively-treated Kraft lignin. *P. putida* and *P. fluorescens* consumed 4-HBA, vanillin, and vanillate in the complex lignin mixture that likely contained various toxic products. In addition, *Rhodococcus opacus* and *Sphingobium* sp. SYK-6 assimilated guaiacol and acetovanillone respectively, from the lignin mixture. Interestingly, *P. fluorescens* was able to break down the higher molecular weight lignin and produce several smaller molecules.

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Popular summary

The work carried out in this thesis is about the bioconversion of lignin. So, what is lignin?

Lignin is one of the three major components, which make up plants. Up to 30% of the dry matter in a tree can consist of lignin. Since the beginning of the 1900's, we have used the other two components (cellulose and hemicellulose) of wood to make paper and cardboard products. The leftover lignin is usually burnt to provide heat and power to the pulp mill. Cellulose and hemicellulose are polymers of sugars and can be hydrolyzed into sugars. In emerging new biorefineries, producing chemicals and biofuels by fermentation of sugars will generate huge amounts of lignin as by-product. For the sustainable consumption of renewable resources such as wood, all its components must be used efficiently. This situation calls for different strategies to utilize lignin.

Why is bioconversion of lignin a good idea?

Lignin is a complex macromolecule that is made up of several aromatic units linked by different bonds. In nature, some types of fungi can degrade lignin in order to access the cellulose and hemicellulose to feed upon. The lignin breakdown products are usually consumed by aromatic-degrading bacteria present in the same ecological niche. Some of these bacteria can metabolize a wide range of lignin compounds (monomers) and funnel them into a few central intermediates, like many small rivers forming a large river. This so-called biological funnel reduces the complexity of lignin. The intermediates are further processed to enter the central carbon metabolism of the microorganisms, where the cell gets energy for growth. Some of these bacteria could be easily modified by genetic engineering to generate products from lignin compounds. These features make the bioconversion of lignin a suitable approach for its efficient utilization.

In the first part of this thesis, a search for lignin-eating bacteria was conducted in several natural and man-made environments. A group of bacteria named '*Pseudomonas*' was found dominant in these environments. Some of these bacteria were grown in shake flasks using small lignin-related aromatic compounds as the source of nutrition. As expected, these microorganisms were able to utilize several aromatic compounds and grow on them.

In the second part of this thesis, growth of these bacteria was conducted using real lignin as substrates. As lignin is a macromolecule, it has to be cleaved into smaller molecules before the bacteria can ingest them. The breakdown of lignin by fungi is a very slow process in nature. However, there are several

thermochemical methods that can cleave lignin and generate a range of compounds that vary in size. A mixture produced by thermochemical methods was fed to bacteria, and some of these were able to convert most of the compounds into central intermediates and grow on them.

After the successful testing of bacteria on lignin, the next step was to modify bacteria in order to widen their substrate uptake and also to produce chemical compounds of interest. For this purpose, a popular bacterium (*Pseudomonas putida* KT2440) that is robust and can utilize a large range of lignin compounds was selected as a host. This bacterium was modified to consume one more of the major lignin compounds (guaiacol), which it normally does not consume. Later, an interesting new enzyme that catalyzes the reduction of vanillin was identified in one of the isolated bacteria. This was subsequently used to modify the host bacterium in order to produce high yields of vanillin – a food flavouring agent, from a lignin-related compound (ferulic acid).

The findings of the work carried out in this thesis contributes towards the utilization of lignin through bioconversion, which is an exciting new field. Further research will be required for the production of other high value compounds from lignin. The final goal will again be the efficient utilization of lignin, which is one of the most abundant sources of renewable aromatics on Earth.

ஆய்வுப்பணிச் சுருக்கம் (Research summary in Tamil)

உயிரியல் மாற்றத்தின் மூலம் லிக்னின் பயன்பாட்டை பற்றிய ஆராய்ச்சி இந்த ஆய்வறிக்கையில் மேற்கொள்ளப்பட்டுள்ளது.

தாவரங்கள் செல்லுலோஸ், ஹெமிசெல்லுலோஸ் மற்றும் லிக்னின் ஆகிய மூன்று முக்கிய கூறுகளைக் கொண்டுள்ளன. ஒரு மரத்தின் உலர்ந்த பொருளில் முப்பது விழுக்காடுகள் வரை லிக்னின் இருக்கக்கூடும். 1900களின் தொடக்கத்திலிருந்து காகிதம் மற்றும் அட்டை உற்பத்திகளைத் தயாரிக்க மரத்தின் மற்ற இரண்டு கூறுகள் (செல்லுலோஸ் மற்றும் ஹெமிசெல்லுலோஸ்) பயன்படுத்தப் படுகின்றன. எஞ்சியிருக்கும் லிக்னின் எரிக்கப்பட்டு கூழ் ஆலைக்கு வெப்ப ஆற்றலையும் மின்சாரத்தையும் வழங்கப் பயன்படுத்தப்படுகிறது. மரம் போன்ற புதுப்பிக்கத்தக்க வளங்களின் அனைத்துக் கூறுகளும் திறமையாகப் பயன்படுத்தப்பட வேண்டும். எனவே, லிக்னினின் மேம்பட்ட பயன்பாட்டிற்கு மாற்று வழிகள் பகுப்பாய்வு செய்யப்பட வேண்டும்.

உயிரியல் மாற்றத்தின் மூலம் லிக்னினைப் பயன்படுத்துவது ஒரு சாத்தியமான நுட்பமாகும்.

லிக்னின் என்பது ஒரு சிக்கலான பெருமூலக்கூறு ஆகும். இது வெவ்வேறு பிணைப்புகளால் இணைக்கப்பட்ட பல மூலக்கூறுகளால் ஆனது. சில வகையான பூஞ்சைகள் செல்லுலோஸ் மற்றும் ஹெமிசெல்லுலோஸை உண்பதற்காக லிக்னினின் கட்டமைப்பைச் சிதைக்கும் வல்லமை பெற்றவை. அவ்வாறு சிதைக்கப்பட்ட லிக்னினின் முறிவு மூலக்கூறுகள் அந்த பூஞ்சைகளின் சுற்றுச்சூழலில் இருக்கும் பாக்டீரியாக்களால் நுகரப்படுகின்றன. இதில் சில குறிப்பிட்ட பாக்டீரியாக்கள் பல்வேறு சிறிய லிக்னின் சேர்மங்களை வளர்சிதைமாற்றம் செய்து அவற்றை ஒரு சில மத்திய இடைநிலைகளாக மாற்றும். இந்த வளர்சிதைமாற்றம் லிக்னினின் சிக்கலான கட்டமைப்பை எளிமைப்படுத்துகிறது. இவ்வாறு உற்பத்தி செய்யப்பட்ட இடைநிலைகள் பாக்டீரியாக்களின் மத்திய கார்பன் வளர்சிதை மாற்றத்தில் பங்கேற்கின்றன. இதன் மூலம் பாக்டீரியாக்கள் வளர்ச்சிக்கான ஆற்றலைப் பெறுகின்றன. இந்த பாக்டீரியாக்களில் சிலவற்றை மரபணு பொறியியல் கொண்டு எளிதாக மேம்படுத்துவதன் மூலம் லிக்னின் சேர்மங்களிலிருந்து பல்வேறு தயாரிப்புகளை உருவாக்கலாம். இதன் மூலம் லிக்னினின் நேர்த்தியான பயன்பாடு சாத்தியமாகும்.

இந்த ஆய்வறிக்கையின் முதல் பகுதியில் லிக்னின் உண்ணும் பாக்டீரியாக்களைக் கண்டறிய பல இயற்கை சுற்றுச்சூழல்களில் ஆராய்ச்சி நடத்தப்பட்டது. இந்த சுற்றுச்சூழல்களில் 'சூடோமோனாஸ்' என்ற பாக்டீரியா குழு ஏராளமாகக் காணப்பட்டது. சிறிய லிக்னின் சேர்மங்களை ஊட்டச்சத்துக்கான ஆதாரமாகக் கொண்டு இவ்வகை பாக்டீரியாக்கள் ஆய்வகத்தில் வளர்க்கப்பட்டன. இந்த நுண்ணுயிரிகள் பல லிக்னின் சேர்மங்களை உண்டு அவற்றில் எதிர்பார்த்த வண்ணம் வளர்ந்தன.

இந்த ஆய்வறிக்கையின் இரண்டாம் பகுதியில் லிக்னினையே வினைவேதிமங்களாக பயன்படுத்தி பாக்டீரியாக்கள் வளர்க்கப்பட்டன. லிக்னின் ஒரு பெருமூலக்கூறு என்பதால் பாக்டீரியாக்கள் அவற்றை நுகர்வதற்கு முன் லிக்னினை சிறிய மூலக்கூறுகளாக உடைக்க வேண்டும். பூஞ்சைகள் இயற்கை சூழலில் லிக்னினைப் பிளக்க மிக அதிக நேரம் எடுத்துக்கொள்ளும். எனவே லிக்னைனை பிளக்க வேதியியல் முறைகள் பயன்படுத்தப்பட்டன. வேதியியல் முறைகளால் உடைக்கப்பட்ட சிறிய லிக்னின் மூலக்கூறுகளின் கலவை

பாக்டீரியாக்களுக்கு வழங்கப்பட்டது. இந்த பாக்டீரியாக்கள் பெரும்பாலான லிக்னின் கலவைகளை மத்திய இடைநிலைகளாக மாற்றி அந்த சேர்மங்களை வெற்றிகரமாக உட்கொண்டன.

லிக்னின் மூலக்கூறுகளைப் பயன்படுத்தி பாக்டீரியாக்களை வெற்றிகரமாகப் பரிசோதித்த பின்னர் ஆராய்ச்சியின் அடுத்த கட்டம் பாக்டீரியாவை மேலும் மேம்படுத்தி அதன் மூலம் பயன்தரும் இரசாயன சேர்மங்களை உற்பத்தி செய்தல். இந்த நோக்கத்துடன் வலுவான மற்றும் பெரிய அளவிலான லிக்னின் கலவைகளைப் பயன்படுத்தக்கூடிய பிரபலமான பாக்டீரியா (சூடோமோனாஸ் புடிடா KT2440) தேர்ந்தெடுக்கப்பட்டது. இந்த பாக்டீரியா இயற்கையில் உட்கொள்ளாத ஒரு குறிப்பிடத்தக்க லிக்னின் மூலக்கூற்றை (குயகால்) உட்கொள்ள மேம்படுத்தப்பட்டது. பின்னர் சுவாரஸ்யமான புதிய நொதி பாக்டீரியா ஒன்றில் அடையாளம் காணப்பட்டது. இந்த நொதியை மேற்குறிப்பிட்ட பிரபலமான பாக்டீரியாவில் செலுத்தி லிக்னின் மூலக்கூற்றிலிருந்து ஒரு விதமான நறுஞ்சுவைக் காரணி (வனிலின்) அதிக அளவில் உற்பத்தி செய்யப்பட்டது. இவ்வாறு லிக்னினின் பயன்பாடு மேம்படுத்தப்பட்டது.

இந்த ஆய்வறிக்கையில் மேற்கொள்ளப்பட்ட பணிகளின் கண்டுபிடிப்புகள் உயிரியல் மாற்றத்தின் மூலம் லிக்னினை மேன்மையாகப் பயன்படுத்த உதவும். இது ஒரு புதுமையான ஆராய்ச்சித் துறை ஆகும். லிக்னினிலிருந்து பிற உயர் மதிப்பு சேர்மங்களை உற்பத்தி செய்வதற்கு மேலும் பல புதிய ஆராய்ச்சிகள் இன்றியமையாதவை. பூமியில் அதிக அளவில் நிறைந்துள்ள ஆதாரங்களில் ஒன்றான லிக்னின் புதுப்பிக்கத்தக்க வளமாகும். எனவே, இயற்கை வளங்களின் நீடித்த நுகர்வுக்கு லிக்னின் திறம்படப் பயன்படுத்தப்பட வேண்டும்.

List of publications

This thesis is based on the following publications, referred to by their Roman numerals:

I. **Ravi K**, García-Hidalgo J, Gorwa-Grauslund MF, Lidén G (2017) Conversion of lignin model compounds by *Pseudomonas putida* KT2440 and isolates from compost. Appl Microbiol Biotechnol 101(12):5059-5070. doi:10.1007/s00253-017-8211-y

II. **Ravi K,** García-Hidalgo J, Nöbel M, Gorwa-Grauslund MF, Lidén G (2018) Biological conversion of aromatic monolignol compounds by a *Pseudomonas* isolate from sediments of the Baltic Sea. AMB Express 8(1):32. doi:10.1186/s13568-018-0563-x

III. García-Hidalgo J, Brink DP, **Ravi K,** Paul CJ, Lidén G, Gorwa-Grauslund MF (2019). Bacterial isolate genome annotation as a driver for improved microbial cell factories: *calA* from *Pseudomonas putida* encodes a vanillin reductase. Submitted.

IV. **Ravi K,** García-Hidalgo J, Brink DP, Skyvell M, Gorwa-Grauslund MF, Lidén G (2019) Physiological characterization and sequence analysis of a syringate-consuming Actinobacterium. Bioresource Technol 285:121327. doi:10.1016/j.biortech.2019.121327

V. **Ravi K,** Abdelaziz OY, Nöbel M, García-Hidalgo J, Gorwa-Grauslund MF, Hulteberg CP, Lidén G (2019) Bacterial conversion of depolymerized Kraft lignin. Biotechnol Biofuels 12(1):56. doi:10.1186/s13068-019-1397-8

VI. Abdelaziz OY, **Ravi K,** Mittermeier F, Meier S, Riisager A, Lidén G, Hulteberg CP (2019) Oxidative depolymerization of Kraft lignin for microbial conversion. ACS Sustain Chem Eng 7(13):11640-11652. doi:10.1021/acssuschemeng.9b01605

VII. García-Hidalgo J, **Ravi K,** Kuré L-L, Lidén G, Gorwa-Grauslund M (2019) Identification of the two-component guaiacol demethylase system from *Rhodococcus rhodochrous* and expression in *Pseudomonas putida* EM42 for guaiacol assimilation. AMB Express 9(1):34. doi: 10.1186/s13568-019-0759-8

Other related publications that are not a part of this thesis

VIII. Abdelaziz OY, Brink DP, Prothmann J, **Ravi K,** Sun M, García-Hidalgo J, Sandahl M, Hulteberg CP, Turner C, Lidén G, Gorwa-Grauslund MF (2016) Biological valorization of low molecular weight lignin. Biotechnol Adv 34(8):1318-1346. doi:10.1016/j.biotechadv.2016.10.001

IX. Brink DP, **Ravi K,** Lidén G, Gorwa-Grauslund MF (2019) Mapping the diversity of microbial lignin catabolism: experiences from the eLignin database. Appl Microbiol Biotechnol 103(10):3979-4002. doi: 10.1007/s00253-019-09692-4

X. Abdelaziz OY, **Ravi K,** Nöbel M, Tunå P, Turner C, Hulteberg CP (2019). Membrane filtration of alkali-depolymerised Kraft lignin for biological conversion. Bioresource Technology Reports 7:100250. doi: 10.1016/j.biteb.2019.100250

Author contributions

I. I participated in the design of the study and performed all cultivation experiments. I was involved in the discussion of results and preparation of manuscript.

II. I participated in the design of the study, the experimental work, and the discussion of results. I was involved in the preparation of manuscript.

III. I participated in the design of the study, strain characterization, and the discussion of results. I critically reviewed the manuscript.

IV. I designed the study and participated in the experimental work. I was involved in the discussion of results and preparation of manuscript.

V. I designed the study and coordinated the experimental work. I was involved in the discussion of results and wrote the manuscript.

VI. I designed and coordinated the bioconversion experiments and UHPLC analysis. I drafted the bioconversion part of the manuscript and critically reviewed the manuscript.

VII. I participated in the design of the study and performed cultivation experiments. I was involved in the discussion of results and critically reviewed the manuscript.

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Abbreviations and symbols

3MGA	3-*O*-methyl gallic acid
4-HBA	4-hydroxybenzoic acid
BCD	Base catalyzed depolymerization
BLAST	Basic Local Alignment Search Tool
CYP	Cytochrome P450 monooxygenase
DSMZ	Deutsche Sammlung von Mikroorganismen und Zellkulturen
FAD	Flavin adenine dinucleotide
GGE	Guaiacylglycerol-β-guaiacyl ether
GRAS	Generally regarded as safe
HV1	Host vector system safety level 1
MS	Mass spectrometry
MWD	Molecular weight distribution
NAD^+/NADH	Nicotinamide adenine dinucleotide
OD_{620}	Optical density at 620 nm
ODLB	Oxidatively depolymerized LignoBoost lignin
SEC	Size exclusion chromatography
SSF	Swedish Foundation for Strategic Research
TCA cycle	Tricarboxylic acid cycle
THF	Tetrahydrofolic acid
(U)HPLC	(Ultra) High performance liquid chromatography

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Chapter 1

Introduction

1.1 Biomass conversion in biorefineries

Lignocellulosic biomass is the most abundant source of renewable carbon in terrestrial ecosystems, and is a promising feedstock for the production of chemicals, materials, and fuels (DeMartini et al. 2013; Isikgor and Becer 2015). It is found in the cell wall of both woody (e.g. spruce, pine, birch, and poplar) and herbaceous plants (e.g. corn stover, switchgrass, wheat straw, and *Miscanthus*). It is mainly composed of cellulose (40-60%), hemicellulose (10-40%), and lignin (15-30%), linked together to form a macromolecular structure that is very recalcitrant to chemical and biological degradation (Himmel et al. 2007; Schutyser et al. 2018b). Cellulose is a long chain of glucose units linked by β-1,4 glycosidic bonds, while hemicellulose is a heteropolymer that consists of both hexose (e.g. glucose, mannose, and galactose) and pentose (e.g. xylose and arabinose) sugars, including also some sugar acids like glucuronic acid. Lignin is a rather complex aromatic macromolecule, with a three-dimensional structure composed of phenylpropanoid subunits, linked by various C-C and C-O bonds (Isikgor and Becer 2015).

Biorefining is the sustainable processing of biomass into a spectrum of marketable bio-based products (Bell et al. 2014), and over the years, several different biorefinery strategies for lignocellulosic biomass have been proposed (Bozell and Petersen 2010). Traditional biorefineries such as pulp/paper mills utilize this kind of raw material to produce a diverse range of paper and cardboard products (Ragnar et al. 2014). Newer biorefinery concepts aim to utilize lignocellulose and convert it into various other products such as bioenergy, biofuels, chemicals, and materials. A well-known example is the fermentative conversion of lignocellulosic carbohydrates into bio-ethanol (Hahn-Hägerdal et al. 2006; Limayem and Ricke 2012). So far, in both traditional and newer biorefineries, it is mostly the cellulose and hemicellulose fractions that are converted to products of various end use. Lignin is merely a by-product, which is burnt in most cases to produce heat and electricity to power the refinery (Tuck et

al. 2012). However, the efficient utilization of lignin, which is the most abundant source of renewable aromatics, is essential for the economic feasibility and environmental sustainability of biorefineries (Ragauskas et al. 2014).

1.2 Scope and outline of this thesis

This thesis is about lignin and the potential to use this feedstock for production of valuable chemicals by exploring microbial conversion pathways. The work has been conducted as part of a larger project funded by the Swedish Foundation for Strategic Research (SSF) aiming towards the biological valorization of lignin (Figure 1.1).

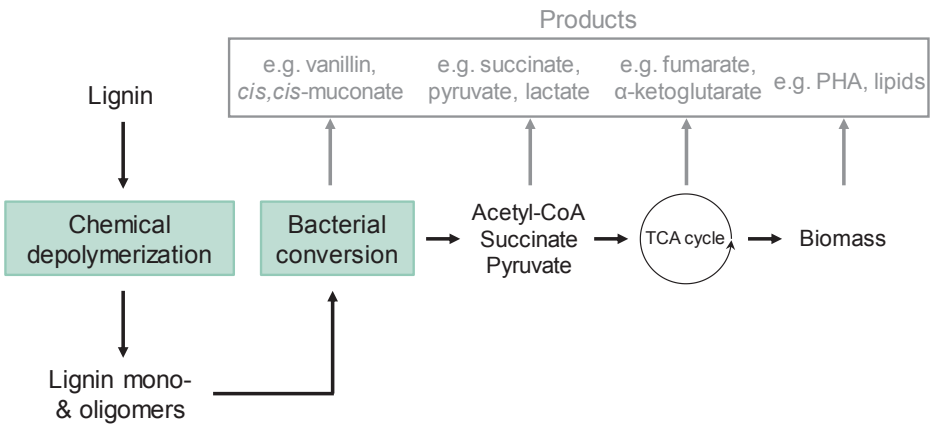


Figure 1.1. Strategy for the biological valorization of lignin.

The project has not directly aimed to produce a specific product or chemical from lignin, but rather to demonstrate a conceptual idea for production of chemicals using microbial hosts by shunting lignin mono- and possibly also short oligomers into central metabolites (acetyl-CoA, succinate, and pyruvate). These central metabolites can be used for cellular growth or be diverted into other compounds by metabolic engineering. Possible products from this process can derive from e.g. the aromatic funnelling pathways, the TCA cycle, or they can be intracellular storage polymers (Figure 1.1). The potential products have a large range of different applications.

The specific research questions in focus of this thesis work were:

- Are there bacteria with interesting lignin-degrading properties to be found from e.g. composts?
- What metabolic pathways for lignin conversion do these microbes contain?
- Will these microbes grow in a more "industrial" and complex lignin substrate?
- Can identified pathways be implemented in other hosts leading to more efficient cell factories for lignin conversion?

The work carried out for this thesis was mostly experimental. Shake flask experiments were performed for liquid cultivations, and solid agar plates were used for screening. Several analytical techniques such as spectrophotometry, (Ultra) High Performance Liquid Chromatography ((U)HPLC), Size Exclusion Chromatography (SEC), and enzyme activity assays were used to evaluate the experiments. Also, *in silico* sequence analysis were carried out to tentatively identify genes/enzymes responsible for certain reactions in the pathways for conversion of aromatics.

This thesis is divided into six chapters and supplemented with seven papers/manuscripts. Chapter 2 provides a general background on lignin chemistry, together with an overview of some lignin separation and treatment methods that are related to this work. In chapter 3, work done on physiological characterization of bacteria using aromatic model compounds is presented and discussed. This covers **papers I to IV**, out of which in papers I to III *Pseudomonas* spp. were characterized on several lignin model compounds; and in paper IV a novel isolate that grows on syringate was characterized and its whole genome sequence was used to map the putative syringate pathway. Chapter 4 summarizes work done on depolymerized lignin. This covers **papers V and VI** in which bioconversion/growth on depolymerized Kraft lignin from different pre-treatment methods was tested using selected bacterial species. The final topic addressed in the thesis is improvement of a bacterial host - *Pseudomonas putida* - by genetic engineering. Chapter 5 covers work from **papers VII and III**, in which *P. putida* was engineered for the uptake of guaiacol, and for improving the yield of vanillin from ferulic acid, respectively. Finally, chapter 6 provides an outlook and conclusion of this thesis.

Chapter 2

Lignin as a renewable feedstock

2.1 Lignin in nature

Lignin is a heterogeneous, water-insoluble, alkyl-aromatic macromolecule found in the cell wall of terrestrial plants. It is the most abundant source of renewable aromatics on Earth (Holladay et al. 2007). The yearly amount of natural lignin produced has been estimated to be between 5 and 36 x 10^8 tons (Gellerstedt and Henriksson 2008). In nature, lignin occurs strongly associated with cellulose and hemicellulose providing rigidity and structural stability to the biomass. Lignin waterproofs the plant cell wall enabling transport of solutes through the vascular tissues (Gellerstedt and Henriksson 2008). The fraction of lignin varies in different types of biomass (Figure 2.1), for example, woody plants such as softwoods and hardwoods contain up to 32% lignin based on dry matter, whereas straws and grasses contain only up to 20% (see review by Ragauskas et al. 2014). Lignin is almost absent in mosses and green algae (Vanholme et al. 2010). Variations also occur between different cell types, tissues, and life stages of a cell within a given plant.

Lignin protects the cell wall polysaccharides by its resistance to biological degradation. In nature, there are only few microorganisms, especially fungi, which can secrete extracellular enzymes to break down lignin (Martínez et al. 2005). Even so, the degradation rate of lignin is quite slow, and lignin contributes to a large fraction of humus in soil (Ayyachamy et al. 2013).

2.2 Lignin composition and structure

Lignin is more complex in structure and composition than cellulose and most hemicelluloses. In plants, it is synthesized via the phenylpropanoid pathway in which phenylalanine is first converted to cinnamic acid using phenylalanine ammonia-lyase and then hydroxylated to *p*-coumaric acid by cinnamate 4-hydroxylase. In some monocots (herbaceous plants), tyrosine may also be used

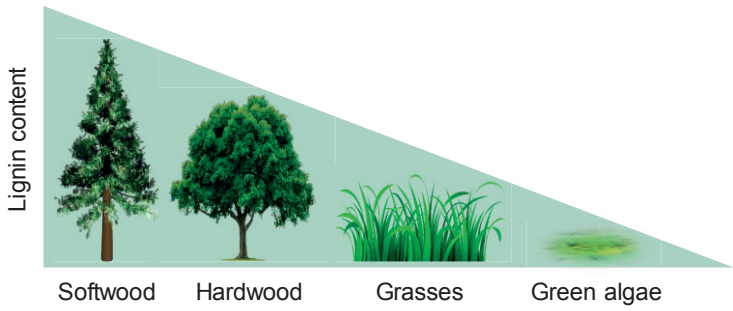


Figure 2.1. Lignin content in different types of biomass.

in which case is directly converted to *p*-coumaric acid by tyrosine ammonia-lyase (see reviews from Ayyachamy et al. 2013; Rinaldi et al. 2016). At *p*-coumaric acid, the enzymatic reactions diverge to finally form *p*-coumaryl alcohol, coniferyl alcohol, and sinapyl alcohol that are the three main building blocks of lignin (Figure 2.2). These building blocks differ in the number of methoxy groups on their aromatic ring and are often abbreviated as H (*p*-hydroxyphenyl), G (guaiacyl), and S (syringyl). The respective distribution of H, G, and S subunits in lignin varies between plant species (Table 2.1). Generally, softwood lignin, for example from pine and spruce, almost only contains G units, while hardwood lignin, such as from poplar and birch, contains both G and S subunits. Lignin from herbaceous plants contains all H, G, and S units; however, the H subunit is usually less than 5%. Apart from these three principal building blocks, to some extent also other compounds such as, *p*-coumaric acid, ferulic acid, *p*-hydroxybenzoic acid, dihydroconiferyl alcohol, tricin, monolignol acetates, acylated monolignols, and products from incomplete monolignol synthesis are incorporated into the lignin structure (Rinaldi et al. 2016).

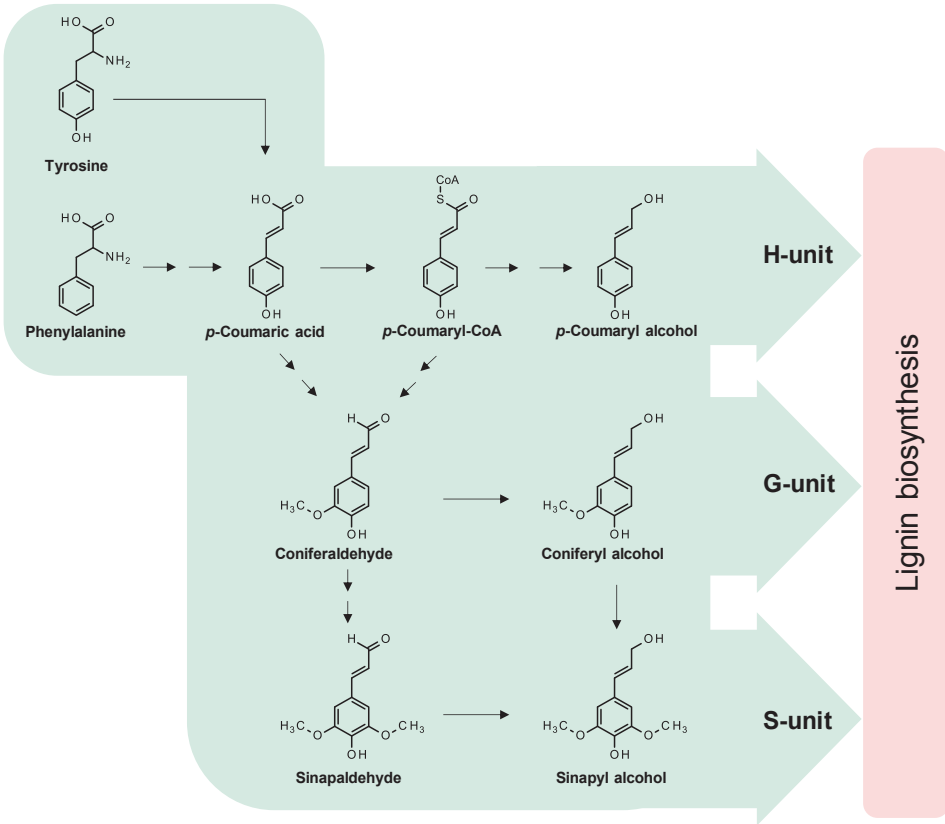


Figure 2.2. Biosynthesis of monolignols from phenylalanine/tyrosine via the phenylpropanoid pathway. Adapted from (Rinaldi et al. 2016).

Once the lignin monomers are synthesized in the cytosol, they are transported to the cell wall where lignification takes place with the help of laccases and peroxidases (Boerjan et al. 2003). These enzymes oxidise the phenol group of the monolignols to produce reactive phenoxy radicals. The process of lignin polymerization occurs through the combination of two radicals. As monolignols are conjugated systems, several inter-unit linkages can be formed, including carbon-carbon and ether bonds. The most abundant ether linkage is β-O-4, and other common ether bonds are α-O-4 and 4-O-5. These bonds are typically more easily cleaved than the C-C bonds such as 5-5, β-5, β-1, and β-β (Table 2.2). The amount of C-C bonds in native lignin depends on the ratio of monolignols present, since the methoxylated ortho-positions cannot take part in the formation of C-C linkages (Schutyser et al. 2018b). Hence, hardwood lignin that is to a large extent composed of S-units contains less C-C bonds than softwood lignin that is exclusively composed of G-units (Table 2.2). These differences significantly affect lignin depolymerization.

Table 2.1. Relative distribution of syringyl (S), guaiacyl (G), and *p*-hydroxyphenyl (H) lignin subunits in different types of biomass. (El Hage et al. 2009; Lapierre et al. 1995; Mansfield et al. 2012; Ziebell et al. 2010)

Source of biomass	%S	%G	%H
Spruce	Trace	98	2
Pine	-	99	1
Poplar	63	37	-
Birch	78	22	-
Oak	68	32	-
Corn	61	35	4
Miscanthus	44	52	4
Alfalfa	39	56	5
Arabidopsis	23	74	3

Table 2.2. Relative distribution of common linkages in lignin. Adapted from (Abdelaziz et al. 2016).

Linkages	Softwood (%)	Hardwood (%)
β-O-4	45-50	60
α-O-4	2-8	6-8
4-O-5	4-8	7-9
β-5	9-12	6
β-1	7-10	1-7
β-β	2-6	3-12
5-5	10-27	3-9

Apart from H, G, and S lignins, the recently discovered C-lignin (catechyl lignin) is present in the seed coat of vanilla orchids and various cacti species (Chen et al. 2012a; Chen et al. 2013). This catechyl lignin is a homopolymer of caffeyl alcohol and is almost exclusively linked by β-O-4 bonds to create benzodioxanes as the major unit in the polymer (Figure 2.3). This C-lignin represents a very interesting type of lignin, since it may, in principle, be depolymerized to a single product with high yields (Li et al. 2018).

Lignin is often referred in the literature as a "heteropolymer". However, even if the structure of native lignin is not fully known, it is highly questionable that it consists of repeating units and should therefore not be called a polymer. The molecular weight of native lignin is hard to assign as the macromolecule undergoes several structural changes during isolation. However, the closest numbers have been reported to be in the range of 8,300 to 78,400 g/mol (see review by Tolbert et al. 2014).

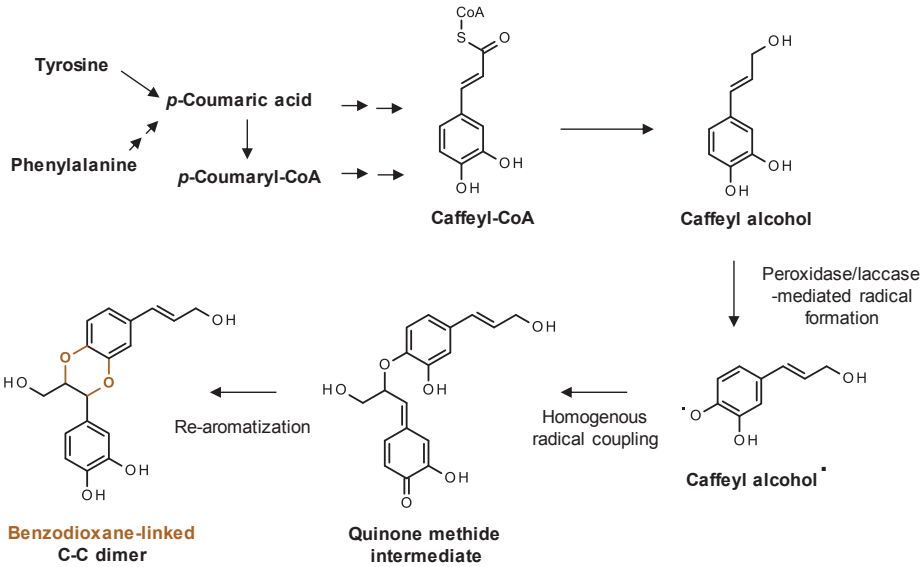


Figure 2.3. Coupling of caffeyl alcohol homodimers to form benzodioxane-linked C-C dimers. Caffeyl alcohol is synthesized from phenylalanine/tyrosine via the phenylpropanoid pathway. Adapted from (Berstis et al. 2016).

2.3 Technical lignin

When biomass is industrially treated, lignin is often (co-) produced. This lignin is here referred to as technical lignin. The structure of technical lignin is very different from the native lignin in terms of the proportion of C-O and C-C bonds. The relative abundance of linkages in technical lignin depends on the type and severity (temperature, concentration, and time) of treatment applied to isolate lignin (Constant et al. 2016). The composition of phenylpropanoid subunits again depends on the type of biomass used. Hence the term 'lignin' as such is broad and not very self-descriptive.

2.3.1 Kraft lignin

For more than a century, pulp mills have successfully separated lignin from the lignocellulosic biomass to produce paper and cardboard products from the holocellulosic fraction. The amount of pulp produced globally has been estimated to be around 400 million tons per year[1]. Kraft cooking is today the most common pulping method applied to solubilize lignin and it accounts for 95% of all pulps

[1] https://www.statista.com/topics/1701/paper-industry/

that are produced chemically (Ragnar et al. 2014). In a normal Kraft pulping process, wood chips are cooked with a solution of sodium hydroxide and sodium sulphide at a temperature ranging from 155 to 175 °C for several hours. Under such alkaline conditions several reactions take place, including the cleavage of lignin-carbohydrate linkages, lignin depolymerization by cleavage of α and β-aryl ether linkages, solubilization of resulting lignin fragments, and eventually lignin repolymerization (Gierer 1985). The resultant black liquor after cooking is rich in lignin and it is mostly incinerated not only to recover the cooking chemicals but also to generate the energy required for the pulp/paper mill. Kraft lignin can be precipitated from black liquor by acidification using CO_2, acetic acid, sulfuric acid, phosphoric acid, and hydrochloric acid (Alen et al. 1979; Loutfi et al. 1991). As Kraft lignin is highly condensed and comprises lower amount of β-O-4 bonds (compared to native lignin), its recovery for chemical uses is not very large. Additionally, the presence of sulfur in Kraft lignin in the form of thiol groups may complicate its valorization.

2.3.2 Sulfite lignin

Next to Kraft pulping, sulfite pulping is the most common chemical pulping process. The sulfite process is older than the Kraft pulping, but has gradually been replaced by the more efficient and versatile Kraft process. Sulfite cooking can be carried out in acidic, neutral, or alkaline conditions using either sulfurous acids or bisulfites (Calvo-Flores and Dobado 2010). In this process, ether bonds are cleaved hydrolytically, after which the reactive α-positions are sulfonated by sulfite ions (Lora 2008). The resultant lignin (lignosulfonate) in spent sulfite liquor is highly water soluble due to the presence of sulfonate groups and can be isolated by either extraction, ultrafiltration, or precipitation. Compared to Kraft lignin, lignosulfonates are higher in molecular weight and contain larger amounts of sulfur (4 to 8%) (Calvo-Flores 2015).

2.3.3 Other techniques to isolate lignin

Apart from the Kraft and sulfite processes, other commercial techniques to isolate lignin include soda pulping and organosolv processes, which both can produce sulfur-free lignin (Nadif et al. 2002). In addition, currently developing biorefineries may employ steam explosion, ammonia fiber explosion, CO_2 explosion, ionic liquid dissolution, hot water process, dilute acid process, alkaline oxidation process, and pyrolysis to separate lignin from biomass (Calvo-Flores 2015).

2.4 Current and potential uses of lignin

So far, the major use of lignin is as fuel and only a few percent of the industrially produced lignin is isolated for other purposes. Several million tons of Kraft lignin per year is used on-site to produce steam and electricity. In a mill where pulp is a major product, surplus energy will be generated and thus used for external purposes. Nevertheless, if a pulp mill is integrated to a paper manufacturing facility, this energy is used for drying paper and cardboard products.

There are a few established applications of lignin and the market is gradually expanding to other products. Lignosulfonates are currently used for applications such as concrete additives, binders/adhesives, and as dispersants in agroindustry (see reviews by Aro and Fatehi 2017; Plank 2004). The production of vanillin from softwood lignosulfonates via alkaline oxidative chemical process is a prominent niche (Pacek et al. 2013).

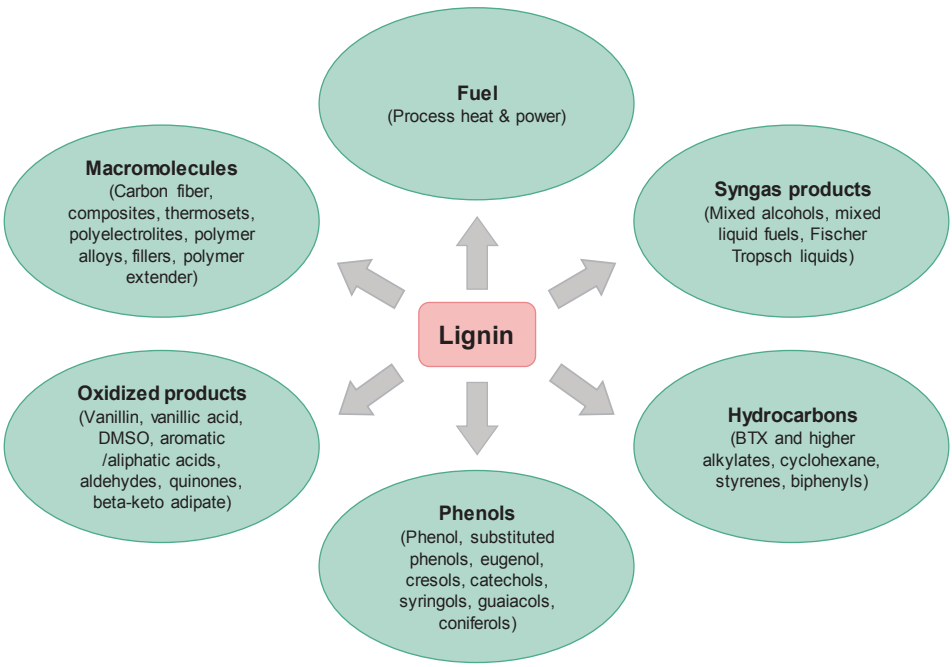


Figure 2.4. Potential products derived from the conversion of lignin. Adapted from (Holladay et al. 2007).

Several potential (new) uses of lignin were extensively analysed by researchers from NREL and PNNL, which resulted in the "Top-value added chemicals from biomass – volume II" (Holladay et al. 2007). In this report, the production of numerous products such as fuels, syngas, macromolecules, phenols, oxidised

products, and hydrocarbons are discussed (Figure 2.4). One of the hurdles for the valorization of lignin via production of high-value smaller molecules is the need of advanced technologies to selectively break bonds. In this following section, some of the depolymerization techniques to breakdown lignin are discussed.

2.5 Thermochemical lignin depolymerization

For the valorization of lignin through the proposed strategy (Figure 1.1), a central process step is lignin depolymerization intended to breakdown the lignin macromolecule into smaller fragments that are suitable for microbial uptake. This process of depolymerization is quite challenging especially with technical lignins, due to the higher relative abundance of C-C bonds (at the expense of C-O bonds) that are difficult to cleave. Even a small decrease in the relative amount of ether bonds can drastically reduce the monomers yield, as the theoretical yield of monomers is almost proportional to the square of the relative abundance of C-O bonds (Schutyser et al. 2018b). To avoid this loss in lignin reactivity, lignin depolymerization should preferably be performed directly on the native lignin in the lignocellulosic biomass. However, the traditional pulp and paper industries generate huge amounts of lignin through Kraft and sulfite pulping, which are of interest to valorize.

In the context of this thesis, the techniques applied (by project partners) to treat Kraft lignin were base-catalyzed and alkaline-oxidative lignin depolymerization. A brief summary of these two methods is presented in sections 2.5.1 and 2.5.2 respectively. Some other depolymerization techniques that are applicable to technical lignins are listed in section 2.5.3.

2.5.1 Base-catalyzed lignin depolymerization

Base-catalyzed lignin depolymerization (BCD) is usually performed at temperatures ranging from 200 to 330 °C in the presence of a solid (MgO, CaO, basic zeolites etc.) or soluble base (mostly NaOH) (Toledano et al. 2012). Water is used as a solvent in most cases, although aqueous organic solvents such as methanol, ethanol, and THF are often reported (Long et al. 2014). BCD of lignin is carried out at pressures ranging from 100-300 bars with a short residence time of around 2-15 minutes, preferably in a continuous flow reactor to avoid repolymerization due to condensation.

In principle, lignin is dissolved in alkaline solvent where the alkaline or alkaline metal ions polarize the ether bonds. At the given reaction conditions, bond cleavage occurs mainly at the weakest bonds such as β-O-4 and α-O-4, resulting

Table 2.3. Selected examples of base catalyzed (BCD) and alkaline oxidative lignin depolymerization. Adapted from (Schutyser et al. 2018b).

Depolymerization method	Lignin	Catalyst and conditions	Monomer yield	Reference
BCD	Alcell lignin	NaOH, ethanol/water (1:3 v/v), 240 °C, 30 min	4 wt%	(Thring 1994)
	Indulin AT	NaOH, water, continuous mode, 315 °C, 130 bar, 1.4 h^{-1} LHSV (Liquid hourly space velocity)	8 wt%	(Beauchet et al. 2012)
	Hardwood organosolv lignin	NaOH, water, continuous mode, 300 °C, 250 bar, 4.2. min	15 wt%	(Roberts et al. 2011)
	Softwood Kraft lignin	Na-X, ethanol/water (1:2 wt/wt), 250 °C, 1 bar N_2, 1 h	18 wt%	(Chaudhary and Dhepe 2017)
	Indulin AT	NaOH, water, continuous mode, 220 °C, 120-130 bar, 2 min	6.3 wt%	**Paper V**
Alkaline lignin oxidation	Spruce Kraft lignin	$CuSO_4$/$FeCl_3$, water+NaOH (3M), 170 °C, 5 bar O_2, 20 min	1.58 wt%	(Voitl and Rudolf von Rohr 2008)
	Hardwood Kraft lignin (LignoBoost, Innventia)	No catalyst, water+NaOH (2N), 120 °C, 3 bar O_2, 10-20 min	6 wt%	(Pinto et al. 2013)
	Softwood Kraft lignin (LignoBoost, Innventia)	No catalyst, water+NaOH (2N), 120 °C, 3 bar O_2, 40 min	5 wt%	(Rodrigues Pinto et al. 2011)
	Pine Kraft lignin (60 g/L)	$CuSO_4$, water+NaOH (2N), 120 °C, 3-4 bar O_2, 45-100 min	8.3 wt%	(Mathias and Rodrigues 1995)
	Softwood Kraft lignin (LignoBoost, Innventia)	No catalyst, water+NaOH (0.2M), 160 °C, 3 bar O_2, 30 min	3.2 wt%	**Paper VI**

in a product mixture that typically consists of phenolic mono-, di-, and oligomers together with some aliphatic degradation products such as methanol and formic/acetic acids (Beauchet et al. 2012). The reported monomer yields after BCD are usually around 10 wt%. At temperatures under 300 °C methoxyphenols are the major products, and these can be either substituted (acetophenone-derivatives and aromatic aldehydes) or unsubstituted (Lavoie et al. 2011). At higher temperatures, the selectivity usually shifts towards catechol and methyl/ethyl catechols (Katahira et al. 2016). Some selected examples of BCD along with their process parameters and yields are listed in table 2.3.

2.5.2 Alkaline-oxidative lignin depolymerization

Oxidation of lignin can either result in the release of side chains by cleavage of the C_α-C_β or C_4-C_α bonds with retained aromatic compounds or result in cleavage of the aromatic ring to yield aliphatic carboxylic acids. Here, the former type, with retained aromatic compounds, is primarily of interest. Alkaline-oxidative lignin depolymerization for the production of phenolic monomers is usually carried out in alkaline media (0.5-4 M NaOH or KOH in water) with oxygen (O_2) as an oxidant. A high initial pH is required to solubilize the lignin and to ionize the free (phenolic) hydroxyl groups that enables oxidation (Schutyser et al. 2018b). The reaction is initiated by the oxidation of phenolate ions into phenoxy radicals and further proceeds through a radical reaction mechanism. The most common pathways are reviewed by Ma et al. 2015, but the exact mechanism is not fully understood.

The reaction conditions of aerobic lignin oxidation, such as temperature (60-190 °C) and pressure (2-14 bar O_2), are usually milder than BCD (Table 2.3). Addition of a catalyst in the reaction mixture has been proven to accelerate the reaction and increase the yield of monomers (Schutyser et al. 2018a). Several homogeneous and heterogeneous catalysts that contain metals (Mn, Cu and Fe) have been frequently used; however, the reported monomer yields are usually below 10 wt%. Some studies have shown to yield a reasonable amount of phenolic monomers without the addition of any catalyst (Table 2.3).

Alkaline oxidation of lignin enables the selective production of aldehydes such as syringaldehyde, vanillin, and *p*-hydroxybenzaldehyde with a relative distribution that depends on the source of lignin. Apart from the aldehydes, aromatic acids such as *p*-hydroxybenzoic, vanillic and syringic acids, and acetophenone-derived monomers such as acetovanillone and acetosyringone are the most common products (Schutyser et al. 2018b).

2.5.3 Other lignin depolymerization techniques

Apart from the techniques mentioned above, some other methods reported to depolymerize technical lignin are reductive depolymerization, acid-catalyzed depolymerization, acidic/pH neutral oxidation, solvolytic depolymerization, pyrolysis, and gasification (see reviews by Abdelaziz et al. 2016; Rinaldi et al. 2016; Schutyser et al. 2018b). Reductive depolymerization employs redox catalysts and hydrogen to hydrocrack or disassemble lignin. Acid-catalyzed depolymerization employs Lewis acids or soluble/solid Brønsted acids in water/solvent mixture. Acidic lignin oxidation is usually performed either in

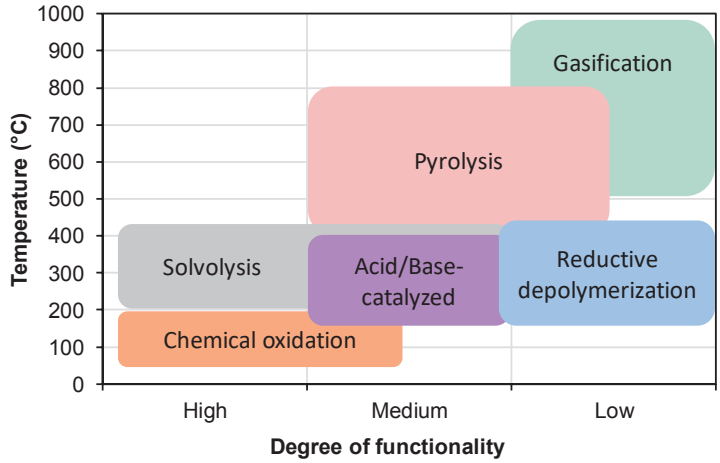


Figure 2.5. Summary of thermochemical processes for the depolymerization of technical lignins. Temperature applied for each process vs. the degree of functionality in the product mixture. Adapted from (Rößiger et al. 2018).

diluted strong acids such as H_2SO_4, HCl or in concentrated acetic acid under aerobic conditions. Oxidation can also be performed using (pH) neutral ionic liquids or solvents e.g. methanol. Solvolytic depolymerization involves a solvent such as water or organic solvent mixtures together with thermal action (225-450 °C). Pyrolysis involves the thermal and solventless decomposition of lignin at inert conditions. Finally, gasification is a process of converting lignin into a gas mixture (H_2, CO, CH_4, CO_2). Different techniques of depolymerization yield products with diverse functionality (Rößiger et al. 2018) (Figure 2.5).

Chapter 3

Bacterial conversion of lignin-related aromatics

For the efficient valorization of lignin using the proposed strategy in the current project, i.e. an initial chemical lignin depolymerization followed by microbial conversion of low molecular weight aromatic compounds (Figure 1.1), it is important to choose microorganism(s) that can funnel a wide range of lignin compounds into key intermediates and further incorporate them into the central carbon metabolism (TCA cycle) (Linger et al. 2014). This is essential as lignin is highly heterogeneous and the depolymerization of lignin results in a complex product mixture containing numerous aromatic compounds (Lavoie et al. 2011). In this context, investigating the growth of microorganisms on a variety of lignin-related compounds and assessing their innate metabolic capacities on such aromatics remain crucial.

In this thesis, physiological characterization of several bacterial species that can consume/convert lignin-related aromatic compounds was carried out. The starting point ("bench-mark" organism) was the well-known aromatic degrader *Pseudomonas putida* KT2440, and subsequently other isolated bacterial species from various natural or man-made environments were characterized. Selected microorganisms were tested for growth in shake flasks containing mineral media with lignin-related aromatic compounds as the only source of carbon and energy. Growth rates, uptake rates, and yields were calculated and compared between different organisms and carbon sources **(Papers I, II and IV)**. For two of the isolates, whole genome sequences were obtained (outsourced) and analyzed (in-house) to understand some of the aromatic metabolic pathways **(Papers III and IV)**.

This chapter starts with a brief overview of the microbial funnelling and fission pathways for aromatic compounds used in this study in section 3.1. Microbial screening techniques that were carried out for the isolation of bacterial species used are then briefly summarized in section 3.2. In sections 3.3 to 3.5, the results from the current work on characterization of aromatic metabolism of bacterial species are discussed. The readers are also directed to the eLignin Microbial

Database[2] that was created as a part of the SSF project. eLignin is an openly available database that indexes data from the lignin bibliome, such as microorganisms, aromatic substrates, and related metabolic pathways (Brink et al. 2019).

3.1 Bacterial aromatic catabolism - funnelling and fission pathways

3.1.1 Funnelling pathways

As already mentioned in chapter 2 (section 2.2), the three main building blocks of lignin are sinapyl (S unit), coniferyl (G unit), and *p*-coumaryl (H unit) alcohols. The catabolism of the aromatic building blocks follows pathways – called funnelling pathways (Harwood and Parales 1996; Linger et al. 2014) – according to the same grouping, i.e. there are sinapyl (S branch; two methoxy groups), coniferyl (G branch; one methoxy group), and *p*-coumaryl (H branch; no methoxy groups) pathways. Several organisms, such as a number of *Pseudomonas* and *Rhodococcus* species, have been reported to be able to consume compounds from the G and H branches, but much fewer organisms are known to be able to metabolize S-lignin compounds (see review by Xu et al. 2019). The building blocks of lignin as well as the bacterial catabolism of aromatic compounds are not limited to S, G, and H units, and some of the other routes that include lignin-related aromatics are, for example, benzoyl, caffeyl, resorcinol, and cresol pathways (Brink et al. 2019).

In the work of this thesis, the bacterial funnelling branches studied were sinapyl, coniferyl, *p*-coumaryl, and benzoyl pathways. These funnelling branches are usually linear with end points such as 3-*O*-methylgallate/gallate, protocatechuate, and catechol, after which the aromatic ring is cleaved (Figure 3.1). The compounds from the sinapyl branch are converted to 3-*O*-methylgallate/gallate, while the compounds from benzoyl and *p*-coumaryl branches are in most cases converted to catechol and protocatechuate, respectively (see reviews from Abdelaziz et al. 2016; Brink et al. 2019; Xu et al. 2019). The compounds from the coniferyl branch are converted to vanillate and can then be either (i) decarboxylated to guaiacol and eventually demethylated to catechol as reported in several *Bacillus* and *Streptomyces* species (Chow et al. 1999; Crawford and Olson 1978), or (ii) demethylated to protocatechuate as reported in several *Pseudomonas* species (Buswell and Ribbons 1988; Priefert et al. 1997).

[2] www.elignindatabase.com

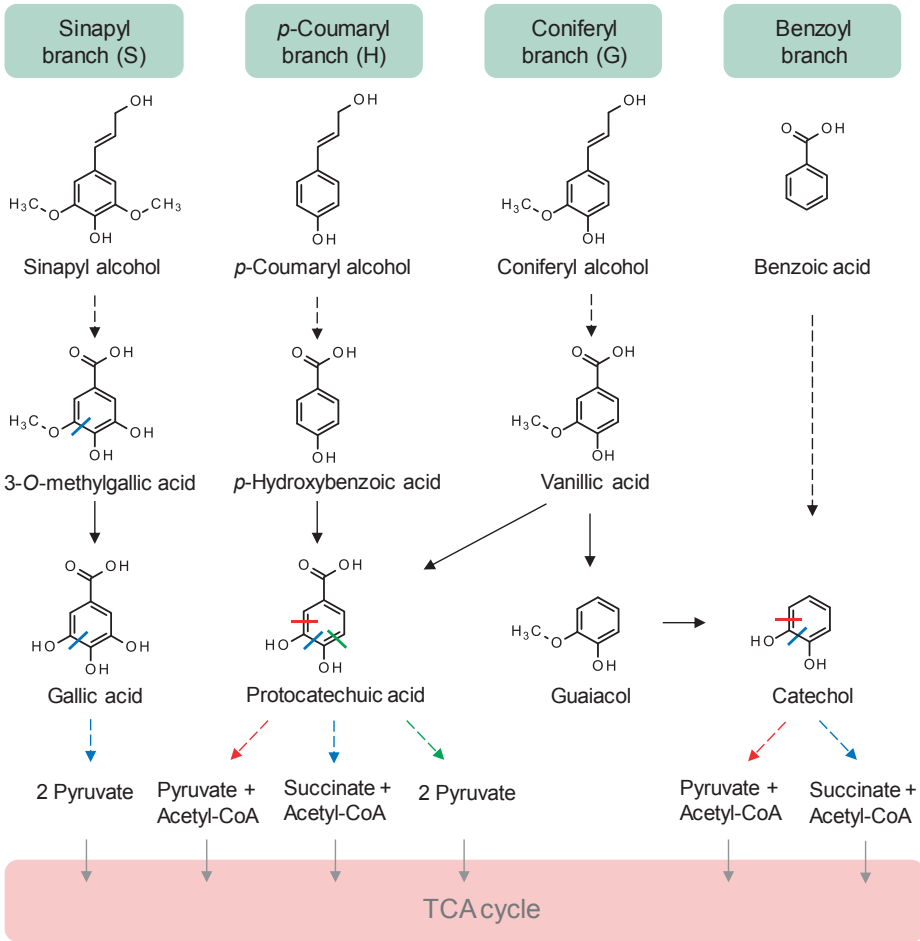


Figure 3.1 Simplified representation of funnelling and fission pathways for the bacterial metabolism of lignin model compounds. Blue, red, and green lines represent ortho, 2,3, and 4,5-meta cleavages, respectively. Dashed lines represent several reaction intermediates. Adapted and simplified from (Xu et al. 2019).

The ring cleavage pathways (also called fission pathways) start from the end points of the funnelling pathways, and the products of the fission pathways depend on the position of ring cleavage. Eventually, these products (acetyl-CoA, succinate, pyruvate and oxaloacetate) enter the TCA cycle to support growth of the microorganism (Figure 3.1). For the detailed reactions of fission pathways, see reviews by (Kamimura et al. 2017b; Xu et al. 2019).

3.1.2 Protocatechuate cleavage

Protocatechuate is usually a central intermediate in the catabolism of compounds from the *p*-coumaryl and coniferyl branches. It can be cleaved via one of the three cleavage pathways, yielding different end products.

i. Protocatechuate 2,3 meta-cleavage yields one molecule each of pyruvate and acetyl-CoA. This pathway is less common but has been reported in *Paenibacillus* sp. JJ-1b (formerly *Bacillus macerans*) (Kasai et al. 2009; Wolgel et al. 1993).

ii. Protocatechuate ortho-cleavage proceeds via β-ketoadipate and finally yields one molecule each of succinate and acetyl-CoA. This pathway (referred to as β-ketoadipate pathway) is more common and has been reported in several organisms including *Rhodococcus jostii* and *Pseudomonas putida* (Harwood and Parales 1996; Jiménez et al. 2002; Yamanashi et al. 2015).

iii. Protocatechuate 4,5 meta-cleavage yields two molecules of pyruvate. This type of cleavage has been reported in several organisms including *Sphingobium* sp. SYK-6, *Novosphingobium aromaticivorans* DSM 12444, and *Comamonas* sp. E6 (Kamimura et al. 2010; Masai et al. 2014; Perez et al. 2019).

3.1.3 Catechol cleavage

Catechol is an intermediate from the benzoyl branch but can also result from the coniferyl branch (via guaiacol) of the funnelling pathways. It can be cleaved via one of the two cleavage pathways, yielding different end products.

i. Catechol ortho-cleavage (also referred to as β-ketoadipate pathway), similar to protocatechuate ortho-cleavage, proceeds via β-ketoadipate and yields one molecule each of succinate and acetyl-CoA.

ii. Catechol meta-cleavage yields one molecule each of pyruvate and acetyl-CoA.

Both of these pathways have been reported in several organisms including *Pseudomonas* and *Rhodococcus* species (Fuchs et al. 2011; Harwood and Parales 1996; Veselý et al. 2007).

3.1.4 3-*O*-methylgallate/gallate cleavage

Aromatic compounds in the sinapyl branch are converted to either 3-*O*-methylgallate or gallate, and the aromatic ring is then cleaved to eventually produce 4-oxalomesaconate (OMA). This fission pathway yields one molecule each of pyruvate and oxaloacetate, and oxaloacetate is usually converted to pyruvate, finally yielding two molecules of pyruvate. This pathway has been reported in a few organisms such as *Sphingobium* sp. SYK-6, *Novosphingobium aromaticivorans*, and *P. putida* KT2440 (Cecil et al. 2018; Kasai et al. 2007; Nogales et al. 2011).

3.2 Bacterial screening from natural/man-made environments

Most of the microorganisms that can degrade lignin and lignin-related aromatic compounds in nature are fungi and bacteria (Crawford and Crawford 1980). Fungi are usually responsible for the breakdown of lignin macromolecule into smaller aromatics, thanks to the presence of extracellular enzymes such as laccases and peroxidases (see review by Martínez et al. 2005). The enzymatic breakdown of lignin, a slow process, is outside the scope of this thesis. The lignin breakdown products formed due to the action of fungi are partly consumed by fungi, but also consumed by the bacteria present in the same ecological niche (Brown and Chang 2014). The isolation of such bacterial species has been reported from several natural and man-made environments, with a majority being isolated from soil/forest environments, termite gut, and pulp/paper mill effluents (Table 3.1).

These bacteria mostly belong to the Actinobacteria and Proteobacteria phyla, which consist of Gram-positive and Gram-negative species respectively (Brink et al. 2019; Bugg et al. 2011; Tian et al. 2014). Among Proteobacteria, γ-Proteobacteria is the main class of aromatic-catabolizing bacteria. It mostly includes species of the genera *Pseudomonas*, *Acinetobacter*, *Enterobacter*, and *Serratia*. β-Proteobacteria also include a few aromatic-degrading genera e.g. *Comamonas*, *Cupriavidus*, and *Burkholderia*. Actinobacteria includes several different genera, out of which the most dominant species belong to the *Rhodococcus* and *Streptomyces* genera (Bugg et al. 2011). These organisms have been reported to metabolize several lignin-related aromatics such as 4-HBA, vanillic acid, ferulic acid, benzoic acid, and phenol (Tian et al. 2014). Some of these bacterial species have also been reported to excrete extracellular enzymes and degrade lignin to some extent (de Gonzalo et al. 2016). The other phyla that comprises fewer lignin-degrading bacteria are Firmicutes that includes *Bacillus*,

Table 3.1. Selected bacterial species that were isolated from various natural or man-made environments.

Isolates	Isolated from	Selected on	Reference
Rhodococcus erythropolis	Woodland soil	Wheat straw lignocellulose	(Taylor et al. 2012)
Microbacterium oxydans	Woodland soil	Wheat straw lignocellulose	(Taylor et al. 2012)
Oceanimonas doudoroffii	Allantoin-rich seawater	Lignin derivatives	(Numata and Morisaki 2015)
Bacillus licheniformis	Termite gut	Lignin	(Zhou et al. 2017)
Comamonas sp. B-9	Eroded bamboo slips	Kraft lignin	(Chen et al. 2012b)
Acetoanaerobium sp. WJDL-Y2	Sludge of a pulp and paper mill	Kraft lignin	(Duan et al. 2016)
Trabulsiella sp.	Termite gut	Guaiacylglycerol-β-guaiacyl ether (GGE)	(Suman et al. 2016)
Bacillus sp. LD003	Soil from beneath rotted logs	Kraft lignin	(Bandounas et al. 2011)
Gordonia sp.	Puerto Rican forest soil	Alkali lignin	(Woo et al. 2017)
Bacillus pumilus C6	Rainforest soil	Kraft lignin and GGE	(Huang et al. 2013)
Rhizobium sp. strain YS-1r	Decaying biomass	Alkali lignin	(Jackson et al. 2017)
Serratia sp. JHT01	Soil samples	Kraft lignin	(Tian et al. 2016)
Burkholderia sp. H1	Rotten wood samples	Alkali lignin	(Yang et al. 2017)
Streptomyces sp. F-6 and F-7	Forest soil samples from China	Alkali lignin	(Yang et al. 2012)
Pseudomonas sp. strain NGC7	Soil from Japan	Syringate	(Shinoda et al. 2019)
Pseudomonas sp. isolate B	Vegetable compost	Softwood stream	**Paper I**
Pseudomonas sp. isolate C	Vegetable compost	Softwood stream	**Paper I**
Pseudomonas sp. isolate Sigma	Vegetable compost	Kraft lignin	**Paper I**
Pseudomonas sp. isolate 9.1	Sediments of the Baltic Sea	Ferulate	**Paper II**
Microbacterium sp. isolate RG1	Contaminated syringate plate	Syringate	**Paper IV**

and Bacteroidetes, which contains *Sphingobacterium* (Brink et al. 2019; Tian et al. 2014).

From an applied perspective, a number of bacterial strains such as *Pseudomonas putida* KT2440, *Sphingobium* sp. SYK-6, *Rhodococcus opacus* PD630, *Rhodococcus jostii* RHA1, *Cupriavidus necator* JMP134, *Acinetobacter baylyi* ADP1, *Amycolatopsis* sp. 75iv2, and *Streptomyces viridosporus* T7A have been well characterized for their aromatic-degrading capabilities (Ahmad et al. 2011; Barbe et al. 2004; Brown et al. 2011; Jiménez et al. 2002; Masai et al. 2007; Pérez-Pantoja et al. 2008; Zhao et al. 2016). However, there may well be other interesting bacteria, which can metabolize lignin-related compounds yet to be discovered and explored. Recently, there have been several studies made intended towards the screening and identification of novel bacterial species from natural/man-made environments that can degrade lignin or related compounds (Table 3.1).

Related to this thesis work, two screenings were performed using vegetal compost and sediments from the Baltic Sea. In the first study, a growth-based screening was performed using samples from vegetable compost. This was regarded an interesting environment since the extended action of soil microbial communities has resulted in a wide range of aromatic compounds obtained from lignin breakdown. The bacteria present in such environment were selected using either lignin-enriched streams from Kraft pulping or Kraft lignin from Sigma as a sole carbon source (**Paper I**). The isolates found were identified using 16S rRNA (ribosomal RNA) sequencing. Out of the four identified bacteria, three belonged to the *Pseudomonas* genus, and these were characterized using several aromatic compounds (section 3.3.2).

Subsequently, a second screening was performed using samples from sediments of the Baltic Sea, nearby a pulp production plant in Kramfors (northern Sweden). This is an environment where lignin-rich residues have accumulated for many years, and microorganisms present could be expected to have metabolic capacities related to lignin. The isolation of bacterial species from those sediments was carried out using either ferulate, guaiacol, or a softwood waste stream rich in lignin, as a sole carbon source **(Paper II)**. Among the isolated organisms, the fastest growing strain tentatively identified as *Pseudomonas* sp. isolate 9.1 was selected for further characterization using lignin model compounds as carbon sources (Section 3.3.3).

Apart from *Pseudomonas* spp., other γ-Proteobacteria found were *Klebsiella*, *Lelliottia*, and *Acinetobacter*. Three species that belonged to Firmicutes (*Bacillus*), and Actinobacteria (*Rhodococcus*) were also found. As mentioned above, these phyla/classes of bacteria have previously been reported in lignin-

rich environments (Brink et al. 2019); hence, the results were in line with expectations. Interestingly, during growth on plates and in small-scale liquid cultivations it was found that the *Pseudomonas* spp. were rather fast growing and had excellent ability to grow on several aromatics. Hence, it was decided to further characterize these pseudomonads.

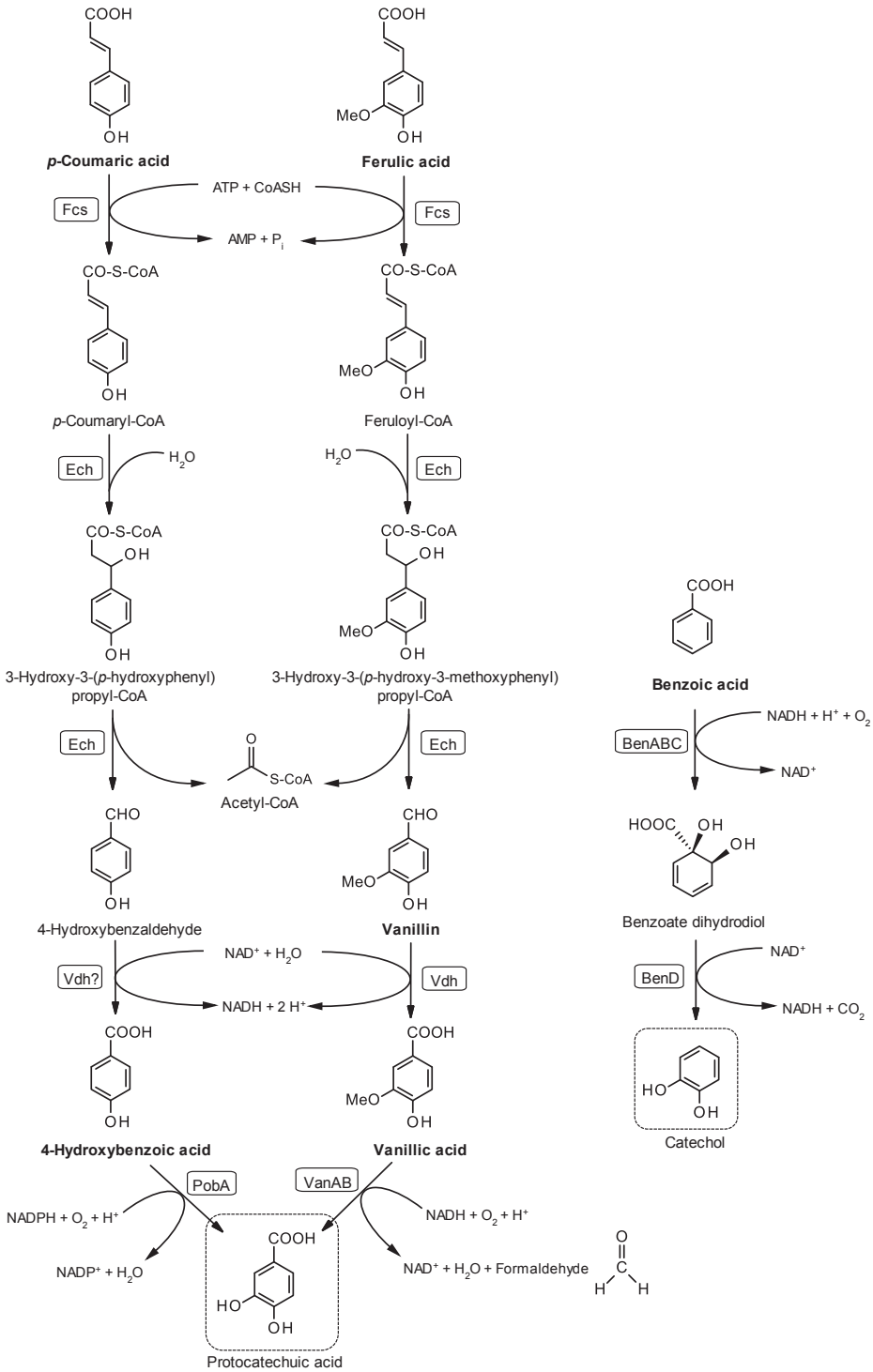
3.3 Physiological characterization of *Pseudomonas* spp.

Pseudomonas spp. are ubiquitous in lignin-rich environments, and in the present work several *Pseudomonas* isolates/strains were selected for further investigation of their metabolism of lignin-related aromatics. This is relevant, as the evaluation of aromatic metabolism – both in terms of the presence of catabolic pathways as well as the innate capacities of such pathways – is crucial for the design of efficient lignin conversion strategies. Starting with a well-known *Pseudomonas putida* strain (KT2440), the aromatic metabolizing capabilities of some *Pseudomonas* isolates are discussed in this section.

3.3.1 *Pseudomonas putida* KT2440

A natural starting point for examining aromatic metabolism in *Pseudomonas* spp. is the strain *P. putida* KT2440. This Gram-negative soil bacterium has been extensively studied in the past decades for its ability to degrade several aromatic compounds (Jiménez et al. 2002; Kim et al. 2006), and this strain is well-known for its fully annotated genome sequence (Belda et al. 2016; Nelson et al. 2002). Recently, it has gained even more attention due to the established CRISPR/Cas9 technology for advanced genome editing (Aparicio et al. 2018; Sun et al. 2018). Due to these characteristics, its use in industrial biotechnology for the production of fine chemicals is promising (Wackett 2003). The strain has often been designated as GRAS (Generally Regarded as Safe). However, it should more correctly be designated as HV1 certified, which still indicates that it is safe to work with in a ML1 (Microbiology Laboratory Class 1) environment (Kampers et al. 2019).

Figure 3.2. (Next page) Main upper degradation pathways of ferulic acid, *p*-coumaric acid, and benzoic acid in *Pseudomonas putida* KT2440. The six model compounds used in this study are indicated in bold; names of the enzymes involved in each step of the pathway are shown in boxes. *Fcs* feruloyl-CoA synthase, *Ech* enoyl-CoA hydratase/lyase, *Vdh* vanillin dehydrogenase, *PobA* 4-hydroxybenzoate hydroxylase, *VanAB* vanillate O-demethylase complex, *BenABC* benzoate dioxygenase complex, *BenD* 1,6-dihydroxycyclohexa-2,4-diene-1-carboxylate dehydrogenase. Metabolic nodes that can undergo ring cleavage are shown in dashed boxes.


COOH
OH
p-Coumaric acid
MeO
Ferulic acid
ATP + CoASH
AMP + Pi
Fcs
CO-S-CoA
p-Coumaryl-CoA
Feruloyl-CoA
H2O
Ech
3-Hydroxy-3-(p-hydroxyphenyl) propyl-CoA
3-Hydroxy-3-(p-hydroxy-3-methoxyphenyl) propyl-CoA
O
S-CoA
Acetyl-CoA
CHO
4-Hydroxybenzaldehyde
Vanillin
NAD+ + H2O
NADH + 2 H+
Vdh?
Vdh
4-Hydroxybenzoic acid
Vanillic acid
PobA
VanAB
NADPH + O2 + H+
NADP+ + H2O
NADH + O2 + H+
NAD+ + H2O + Formaldehyde
Protocatechuic acid
Benzoic acid
NADH + H+ + O2
NAD+
BenABC
HOOC
Benzoate dihydrodiol
BenD
NADH + CO2
Catechol

In **paper I**, *P. putida* KT2440 was investigated for its ability to metabolize lignin-related aromatic compounds as sole carbon sources. The compounds were chosen from the benzoyl (benzoate), *p*-coumaryl (*p*-coumarate, 4-hydroxybenzoate), and coniferyl (vanillin, vanillate, ferulate) branches of the funnelling pathways (Figure 3.2). KT2440 was able to catabolize all six compounds tested, which implies the presence of functional G and H funnelling branches together with their respective ring fission pathways. The specific growth rates on the compounds with one methoxy group were lower than the tested compounds with no methoxy groups (Figure 3.3A). This might be due to the presence of phenylmethyl ether linkages, which are relatively difficult to break and might be a bottleneck for the catabolism of these compounds (Okamura-Abe et al. 2016).

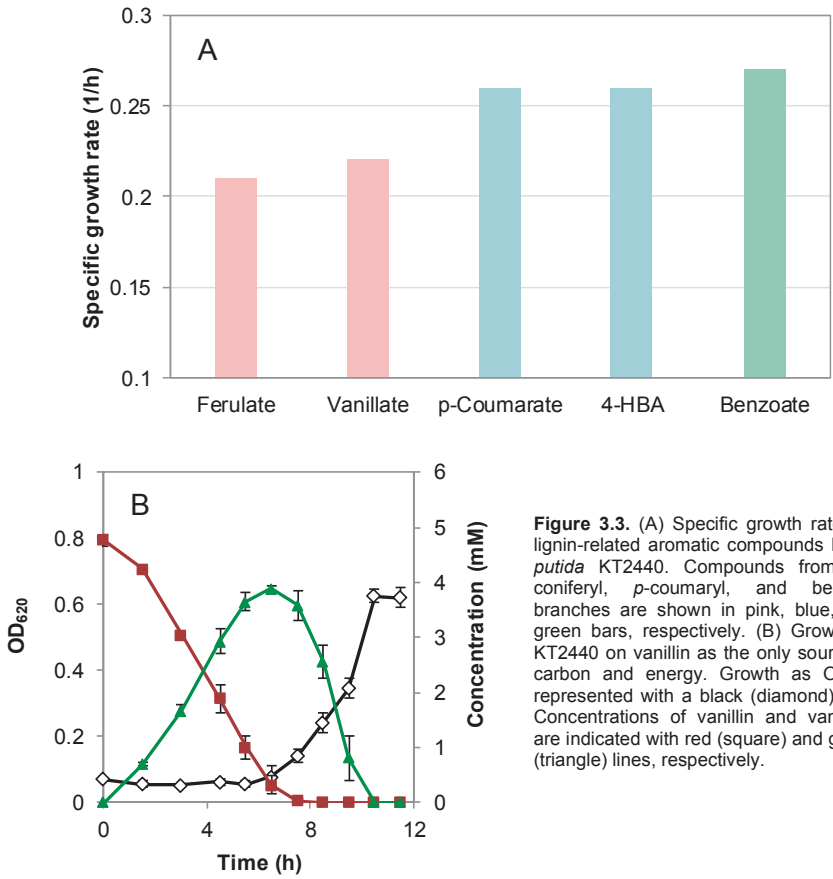


Figure 3.3. (A) Specific growth rates of lignin-related aromatic compounds by *P. putida* KT2440. Compounds from the coniferyl, *p*-coumaryl, and benzoyl branches are shown in pink, blue, and green bars, respectively. (B) Growth of KT2440 on vanillin as the only source of carbon and energy. Growth as OD is represented with a black (diamond) line. Concentrations of vanillin and vanillate are indicated with red (square) and green (triangle) lines, respectively.

KT2440 rapidly oxidized the aromatic aldehyde vanillin and converted it to its first downstream product - vanillate (Figures 3.2 and 3.3B). As the specific consumption rate of vanillate (i.e. oxidized vanillin) (3.8 mmol/g_{CDW}/h) was lower than the uptake/conversion rate of vanillin (4.9 mmol/g_{CDW}/h), a net

accumulation of vanillate was observed in the medium. The excreted vanillate was eventually consumed by KT2440 (Figure 3.3B). The rapid conversion of a rather toxic aldehyde, vanillin, seems to be an efficient action of Vdh (vanillin dehydrogenase), but previous studies in *P. putida* KT2440 showed that Vdh is not the only enzyme involved because there were major changes in expression of nearly 662 proteins in response to vanillin (Simon et al. 2014).

A substrate based on depolymerized lignin, will consist of a *mixture* of several aromatic compounds (as well as other compounds). For this reason, the growth of KT2440 on a mixture of model compounds was also studied. Three compounds were mixed at a time in the following way: (i) all three compounds from the coniferyl branch (ii) one compound each from coniferyl, *p*-coumaryl, and benzoyl branches (iii) two compounds from coniferyl branch and one from *p*-coumaryl branch.

In the first case when three compounds (vanillin, vanillate, ferulate) from coniferyl branch were mixed, vanillin was initially detoxified to vanillate, and vanillate was subsequently co-consumed with ferulate. When compounds from different branches were mixed, KT2440 preferred benzoate, followed by *p*-coumarate, and ferulate was the least preferred substrate. There was an overlap in the consumption of benzoate and *p*-coumarate, but benzoate and ferulate were consumed sequentially (Figure 3.4). In the third case, vanillin was detoxified to vanillate but there was partly a co-consumption of 4-HBA. The accumulated vanillate was eventually consumed within 8 hours. Overall, the consumption pattern of compounds in the mixture was in agreement with the experiments using individual model compounds, and this further confirmed the preference of substrates by KT2440, with the compounds from coniferyl branch being the least preferred of the two.

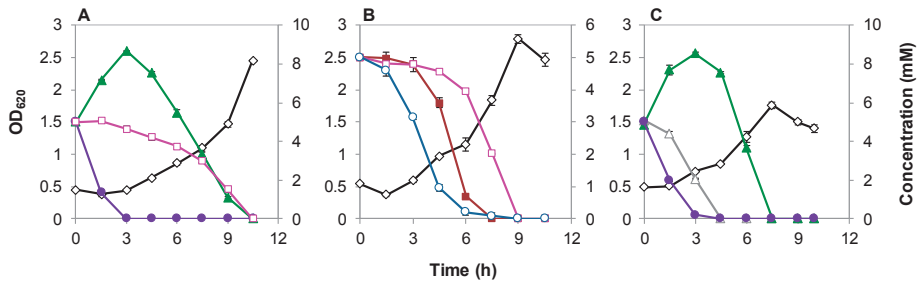


Figure 3.4. Growth of *P. putida* KT2440 on the mixture of (A) vanillin, vanillate, ferulate (B) benzoate, *p*-coumarate, ferulate (C) vanillin, vanillate, 4-hydroxybenzoate. OD is indicated with a black diamond line. The concentration of model compounds is shown in violet closed circle (vanillin), pink open square (ferulate), green closed triangle (vanillate), blue open circle (benzoate), red closed square (*p*-coumarate), and grey open triangle (4-HBA).

Interestingly, no growth was observed when guaiacol was provided as a sole carbon source although *P. putida* KT2440 was able to catabolize methoxylated compounds such as vanillate and ferulate. Guaiacol is usually demethylated to catechol and consumed via the catechol branch of the β-ketoadipate pathway (Figure 3.1) (Barton et al. 2018; Dardas et al. 1985). As KT2440 is known to possess the catechol fission pathway (Jiménez et al. 2002), the lack of growth on guaiacol implies the absence of cytochrome P450 monooxygenases that are involved in the conversion of guaiacol to catechol.

No growth of *P. putida* KT2440 was observed on compounds with two methoxy groups, such as syringate and syringaldehyde. These compounds are usually catabolized to gallate (via 3-*O*-methylgallate), where the aromatic ring is cleaved to form pyruvate and oxaloacetate (Figure 3.1). KT2440 has been shown to possess enzymes (GalA, -B, -C, -D) for the conversion of gallate (Nogales et al. 2011), but the lack of growth on syringyl compounds might be due to the absence of enzymes (syringate *O*-demethylase, 3MGA *O*-demethylase) responsible of the demethylation of such compounds. In a recent study by Masai and co-workers, KT2440 was shown to co-consume syringate in the presence of vanillate (Sonoki et al. 2018), which implies the action of VanAB (vanillate *O*-demethylase complex) on syringate; however, the introduction of exogenous *vanAB* under control of a synthetic promoter did not result in the assimilation of syringate as a sole carbon source (Sonoki et al. 2018). Further efforts are required to understand the mechanism of syringate assimilation in order to construct a *Pseudomonas* strain that can grow on syringyl compounds.

3.3.2 *Pseudomonas* isolates from vegetal compost

Three other *Pseudomonas* strains were isolated from mature vegetal compost, by selection on lignin-containing softwood stream (isolates B and C) and Kraft lignin from Sigma-Aldrich (isolate Sigma). The growth characteristics of these isolates were also assessed on the mixture of lignin model compounds similar to what was done for *P. putida* KT2440 (**Paper I**). Growth on the mixture of vanillin, vanillate, and 4-HBA revealed that the isolates C and Sigma were much slower growing (growth completed after 26 h) than KT2440 (7.5 h). Nevertheless, the consumption of model compounds by isolate B was similar to that of KT2240, with isolate B in fact reaching a higher OD (2.15) than KT2440 (1.76). In the mixture of compounds from different funnelling branches, isolate B had the same preferences as KT2440 (cf. figure 5 in **paper I**), i.e. benzoate was consumed first, followed by *p*-coumarate, and ferulate was the least preferred of the tested substrates. Similar to KT2440, isolate B detoxified vanillin to vanillate and the net accumulated vanillate was eventually consumed. Another observation was that isolate B had a stationary phase after the depletion of available carbon sources. This is in contrast to KT2440, which had no apparent stationary phase

and the biomass concentration declined as soon as the available carbon sources were depleted.

This study showed the presence of similar metabolic pathways in the *Pseudomonas* isolates, but also showed clear differences between isolates in terms of their growth. While isolate C and Sigma had longer lag phases, the stability of isolate B in the stationary phase could be of importance for industrial applications. Further investigations on the genome/transcriptome level are required to identify the mechanistic reasons for these differences.

3.3.3 *Pseudomonas* sp. isolate 9.1

Additional bacterial species were isolated from sediments of the Baltic Sea, by selection on ferulate, guaiacol, and lignin-containing softwood stream (**Paper II**). Based on small-scale cultivations in M9 medium containing lignin model compounds (benzoate, *p*-coumarate, and ferulate), the fastest growing isolate was found to be a *Pseudomonas* sp. - here designated isolate 9.1. This isolate was more thoroughly characterized on lignin model compounds, and its metabolic capacity was assessed.

The model compounds were selected from the benzoyl (benzoate), *p*-coumaryl (*p*-coumarate), coniferyl (vanillin, ferulate, and guaiacol), and syringyl (syringate) branches of the upper funnelling pathways and isolate 9.1 was able to grow on four out of six tested compounds – in agreement with KT2440 (Figures 2&3 in **Paper II**). The highest specific growth and uptakes rates (Table 4 in **Paper II**) were obtained for benzoate, followed by *p*-coumarate, and the least preferred substrates were from the coniferyl branch. This trend was similar to the preferences observed in KT2440.

In contrast to KT2440, however, there was an excretion of pathway intermediates for isolate 9.1 in some cases. During growth of isolate 9.1 on *p*-coumarate, small amounts (< 1 mM) of 4-HBA were excreted as an intermediate (Figure 3.5). From cultivations on 4-HBA as the sole carbon source, it was found that the assimilation rate of 4-HBA (2.9 mmol/g_{CDW}/h) was similar to the uptake rate of *p*-coumarate (2.9 mmol/g_{CDW}/h). Possibly, the rate was in fact slightly lower, which could explain the excretion of 4-HBA. Catechol and *cis,cis*-muconate were excreted during growth on benzoate (Figure 3.5) and although the excreted catechol was eventually consumed, *cis,cis*-muconate remained unconsumed. Cultivations were also attempted on 5 mM *cis,cis*-muconate as the sole carbon source, but neither consumption, nor conversion of muconate to other products, were observed. This might be due to the absence of transporters for the uptake of *cis,cis*-muconate.

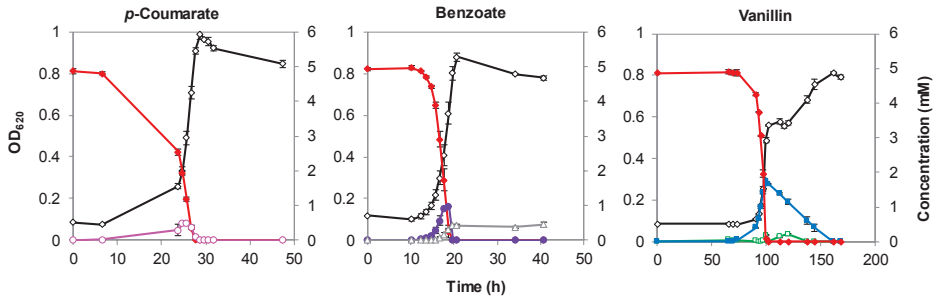


Figure 3.5. Growth of *Pseudomonas* sp. isolate 9.1 on lignin model compounds as the only source of carbon and energy. The OD and model compounds concentration (mM) are shown in black open diamond and red closed diamond, respectively. The excreted intermediate compounds, if any, are also shown (Read from right axis). Pink open circle, 4-hydroxybenzoate; Violet closed circle, catechol; Grey open triangle, *cis,cis*-muconate; Blue closed square, vanillyl alcohol; Green open square, vanillate.

A notable difference was that unlike *P. putida* KT2440 that oxidized vanillin to vanillate, isolate 9.1 excreted vanillyl alcohol during growth on vanillin (Figure 3.5). Owing to the higher uptake rate of vanillin (3.2 mmol/g_{CDW}/h) compared to the assimilation rate of vanillate (2 mmol/g_{CDW}/h), one might have expected the excretion of vanillate as for KT2440. The excretion of vanillyl alcohol may indicate an inefficient aldehyde dehydrogenase for the oxidation of vanillin to vanillate leading to the reductive channelling of vanillin to vanillyl alcohol. Other studies have also reported the excretion of vanillyl alcohol when the downstream genes related to catabolism of vanillin were deleted in *Pseudomonas putida* (Graf and Altenbuchner 2014).

Prior to growth on model compounds, isolate 9.1 had longer lag phases compared to KT2440; however, after the depletion of available carbon sources, isolate 9.1 maintained its biomass with a noticeable stationary phase – in contrast to KT2440.

No growth was observed on guaiacol or syringate, which indicates the absence of enzymes responsible for the demethylation of such compounds. However, *Pseudomonas* sp. strain NGC7 recently isolated from a soil sample in Japan has been shown able to assimilate syringate and syringaldehyde as sole carbon sources (Shinoda et al. 2019). This strain was also further engineered for the production of *cis,cis*-muconate from syringate-rich birch lignin and lignin-derived compounds (Shinoda et al. 2019).

In conclusion, isolate 9.1 was able to consume the aromatic compounds from the benzoyl, *p*-coumaryl, and coniferyl branches of the funnelling pathways, which shows the presence of aromatic catabolic pathways similar to *P. putida* KT2440. However, the excretion and re-consumption of downstream intermediates revealed rate-limited bottlenecks in some funnelling reactions. The excretion of

vanillyl alcohol during growth on vanillin, which is an uncommon trait for *Pseudomonas*, called for the identification and characterization of oxidoreductase(s) that are involved in this reaction. For this purpose, isolate 9.1 was subjected to whole genome sequencing, and the identified enzymes together with its characteristic activities are discussed below (Section 3.4).

3.4 Oxidoreductases in *Pseudomonas* spp.

Whole genome sequencing may enable identification of genes encoding novel enzymes, and by further characterization of these enzymes their metabolic actions can be better understood. These enzymes may then be used to upgrade more suitable host strain(s) than the isolates in which the enzymes were found.

In the present work, the genome of *Pseudomonas* sp. isolate 9.1 was sequenced to identify the oxidoreductase(s) responsible for the observed reduction of vanillin to vanillyl alcohol **(Paper III)**. At the same time, a gene encoding an enzyme with similar activity in the host strain *P. putida* KT2440 was found and the enzyme activity was confirmed. This was later used to eliminate the formation of vanillyl alcohol as by-product during the production of vanillin from ferulic acid in an engineered *P. putida* strain (discussed in chapter 5).

A starting point for the identification of a vanillin reductase in the sequenced genome of isolate 9.1 was Adh6p, an NADPH-dependent alcohol dehydrogenase from *Saccharomyces cerevisiae* that has been reported to reduce vanillin to vanillyl alcohol (Hansen et al. 2009). The protein sequence of Adh6p was used in a BLASTp[3] search to identify candidate enzymes in both isolate 9.1 and *P. putida* KT2440. This translated in a few hits at the gene level; FEZ21_09870 from isolate 9.1 and two putative genes, *calA* and PP_3839, from KT2440. These genes were cloned and expressed in *Escherichia coli*, and the whole cell assays revealed that the open reading frames FEZ21_09870 and *calA* encode aldehyde reductases that can convert vanillin to vanillyl alcohol (Figure 3.6). Similarly, when coniferaldehyde was provided as a substrate, the production of coniferyl alcohol was observed, which showed extended substrate specificities of the enzymes FEZ21_09870 and CalA. In addition, *in vitro* enzyme assays (details can be found in **Paper III**) revealed that these oxidoreductase enzymes, FEZ21_09870 and CalA, were also capable of performing the reverse reactions (i.e. alcohol to aldehyde), and it further demonstrated that these enzymes were mainly NADPH-dependent, with a minor affinity also to NADH.

[3] The Basic Local Alignment Search Tool (BLAST) finds regions of local similarity between nucleotide or protein sequences.

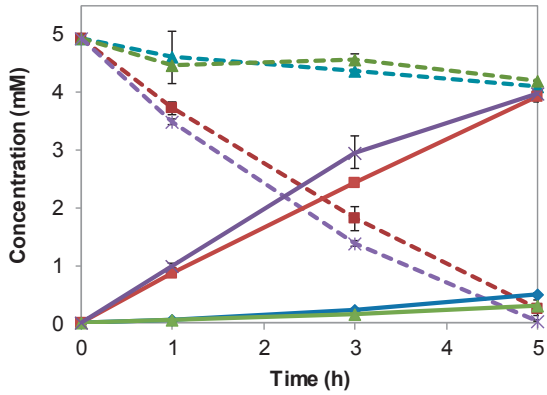


Figure 3.6. Whole-cell bioconversion of vanillin to vanillyl alcohol with *E. coli* BL21(DE3) cells expressing the three candidate genes FEZ21_09870 (red squares), PP_3839 (green triangles), and *calA* (purple crosses), and the negative control pNIC28-Bsa4KpnI (blue diamonds). Dashed and solid lines represent the concentrations of vanillin and vanillyl alcohol, respectively.

Surprisingly, PP_3839 gene product was found to be involved in the conversion of coniferyl alcohol to coniferaldehyde, which was the function previously proposed for the enzyme CalA (Belda et al. 2016). This was further confirmed by the deletion of *calA* and PP_3839 in *P. putida* KT2440, which resulted in the delayed (around 10 h) assimilation of coniferyl alcohol in the latter compared to the wild-type and Δ*calA* strains. The (delayed) growth of *P. putida* KT2440ΔPP_3839 on coniferyl alcohol might be due to the action of other promiscuous alcohol oxidases. In addition, PP_3839 gene product was found to be strictly NADH-dependent, and this enzyme was highly specific to coniferyl alcohol (i.e. no activity on vanillyl alcohol).

In conclusion, the enzymes encoded by FEZ21_09870 and *calA* were found to be responsible for the conversion of vanillin to vanillyl alcohol in *Pseudomonas* sp. isolate 9.1 and *P. putida* KT2440, respectively. These findings were used to engineer a *P. putida* strain for higher yield of vanillin from ferulic acid, by eliminating the formation of vanillyl alcohol (discussed in chapter 5). This study showed the potential of new isolates to further improve the host strains by resolving unknown metabolic traits.

3.5 *Microbacterium* sp. RG1 – a syringate consuming bacterium

3.5.1 Physiology on lignin model compounds

During the handling and incubation of plates in the laboratory, a contaminant bacterium was found growing on an M9 syringate plate. This is interesting as hardwood lignin is made of up to 75% syringyl units, and the bioconversion of syringate is of considerable interest for the biological valorization of lignin. So

far, only a few bacterial species have been reported to consume syringyl compounds, which include *Sphingobium* sp. SYK-6 (Masai et al. 2007), *Novosphingobium aromaticivorans* DSM 12444 (Cecil et al. 2018), *Pandoraea norimbergensis* LD001 (Bandounas et al. 2011), *Serratia* sp. JHT01, *P. chlororaphis* PT02 (Tian et al. 2016), and *Oceanimonas doudoroffii* JCM21046T (Numata and Morisaki 2015). Among these, *Sphingobium* sp. SYK-6 is the only strain that has been characterized in detail with regard to the enzymes involved in the catabolism of syringyl compounds. Due to the limited reports concerning the utilization of syringate, it was decided to further investigate the contaminant bacterium for its ability to catabolize syringate and syringaldehyde, as well as other lignin-related aromatic compounds as the sole source of carbon and energy **(Paper IV)**.

First, this contaminant was purified, and by 16S rRNA sequencing it was identified as belonging to the *Microbacterium* genus and the strain was designated sp. RG1. For its characterization, the compounds were selected from the syringyl (syringaldehyde, syringate, 3MGA), coniferyl (ferulate, vanillin, vanillate, and guaiacol), *p*-coumaryl (*p*-coumarate and 4-HBA), and benzoyl (benzoate) branches of the upper funnelling pathways and some of the key results are discussed below.

- Out of the ten compounds tested, *Microbacterium* sp. isolate RG1 was able to grow on eight of them, which include syringaldehyde, syringate, 3-*O*-methylgallate, and vanillin (Figure 3.7). However, the specific growth rates of isolate RG1 (Table 3 in **Paper IV**) were approximately 10 times lower than for *Pseudomonas putida* KT2440 **(Paper I)** in cases where both organisms showed growth.
- During growth on syringate, 3MGA – the first downstream product of syringate – was excreted as an intermediate, which was eventually consumed. This proves that syringate was in fact assimilated via 3MGA, similar to *Sphingobium* sp. SYK-6 (Masai et al. 2004). The excretion of 3MGA by RG1 might be due to its lower consumption rate (0.3 $mmol/g_{CDW}/h$) compared to the uptake of syringate (0.5 $mmol/g_{CDW}/h$).
- Among aromatic aldehydes, syringaldehyde was consumed faster (< 75 h) than vanillin (200 h) (Figure 3.7). The specific uptake rate of syringaldehyde was also five times higher than that of vanillin (Table 3 in **Paper IV**). The minimum concentration of vanillin and syringaldehyde reported to inhibit the growth of *Escherichia coli* on xylose was 1.5 and 2.5 g/L respectively, which indicates that vanillin is more toxic than syringaldehyde (Zaldivar et al. 1999).

- No growth was observed on guaiacol or benzoate. As guaiacol and benzoate are usually assimilated via the catechol branch of the β-ketoadipate pathway, the lack of growth on these compounds might be due to the absence of enzymes/genes related to this pathway.

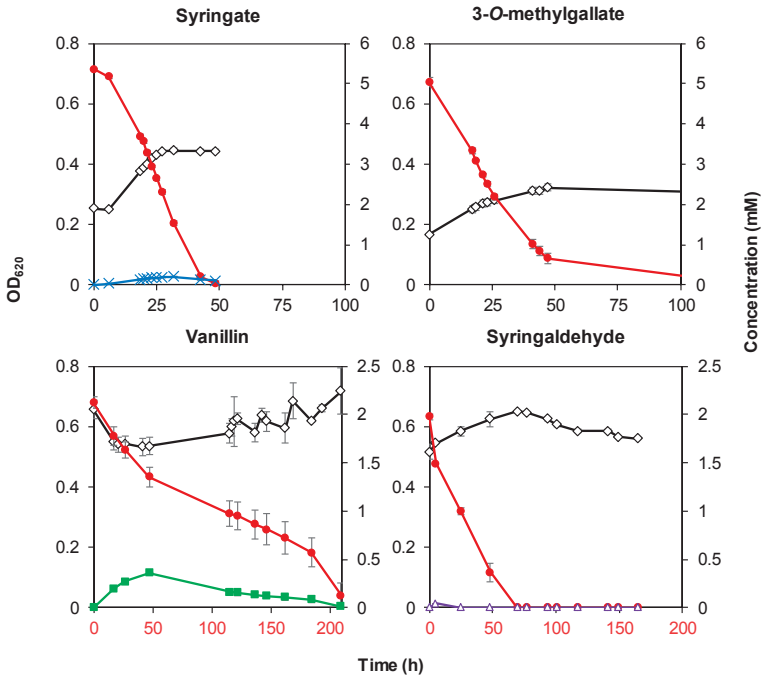


Figure 3.7. Growth of *Microbacterium* sp. isolate RG1 on selected lignin model compounds (2-5 mM) as the only source of carbon and energy in M9 mineral medium. OD_{620} (left axis) and model compounds concentration in mM (right axis) are shown as black diamonds and red circles respectively. Note the highlighted difference in time scale (X-axis). Concentration of excreted intermediates, such as 3MGA (blue line with cross markers), syringate (purple triangles), and vanillyl alcohol (green squares) are also shown.

In conclusion, the assimilation of compounds by RG1 confirmed the presence of fully functional syringyl, coniferyl, and *p*-coumaryl funnelling pathways together with their respective fission pathways. The specific growth rates and biomass yields of isolate RG1 were in general much lower than for the *Pseudomonas* strains; however, RG1 stood out with its ability to assimilate syringyl compounds such as syringaldehyde, and syringate.

3.5.2 *In silico* analysis of syringyl genes

Due to the interesting capabilities of isolate RG1 to assimilate syringyl compounds, whole genome sequence of this organism was obtained to further predict the genes/enzymes involved in the syringyl pathway **(paper IV)**. This is

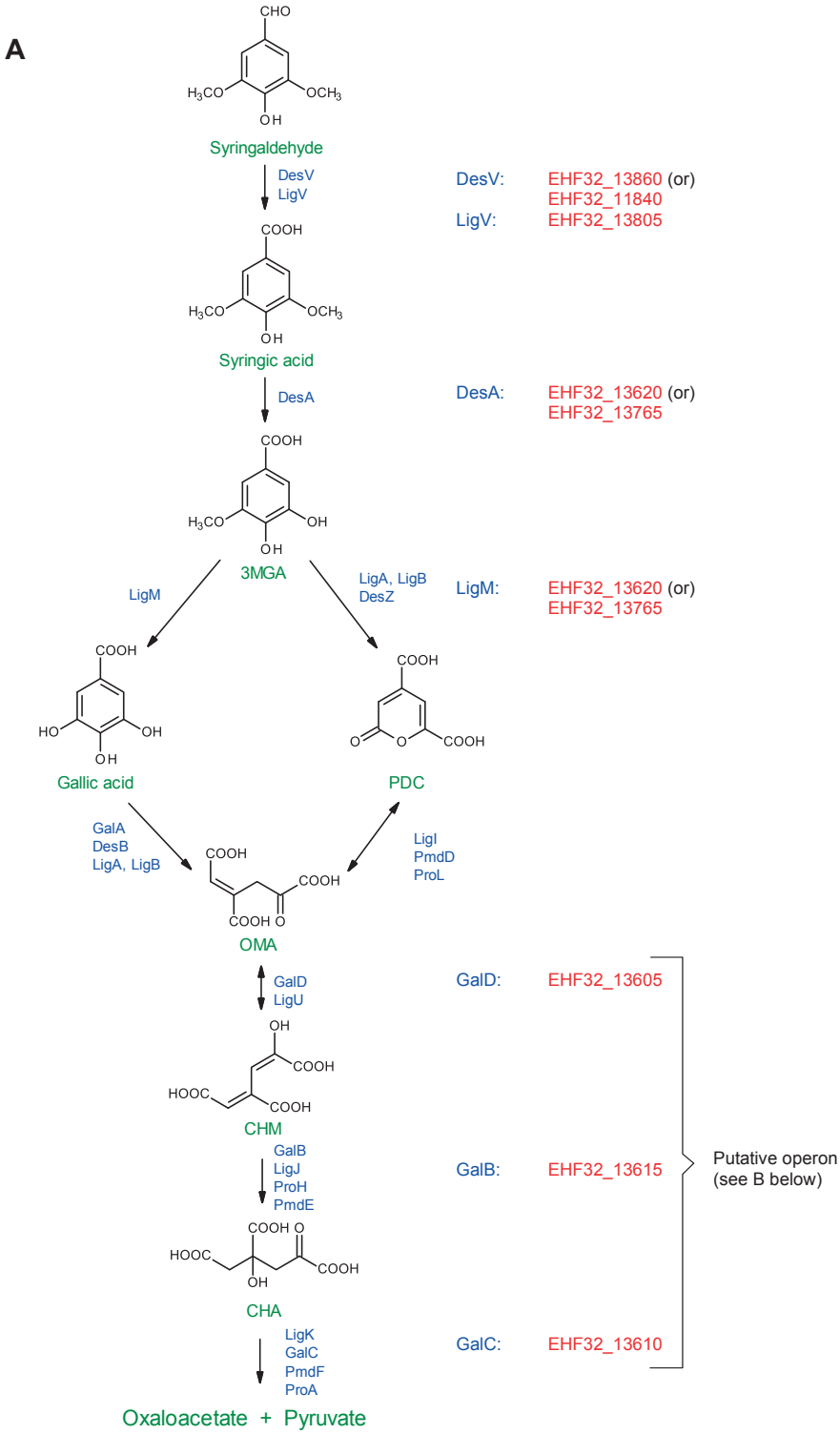
the first reported Gram-positive bacterium that presents this pathway, and the suggested genes might be of interest to express in other host(s) to broaden their range of substrate utilization.

To find the relevant genes, previously known enzymes related to the catabolism of syringyl compounds reported in literature were collected from Uniprot and blasted[4] against the genome of RG1. At the time of completion of this work, genes involved in the assimilation of sinapate were reported in *Novosphingobium aromaticivorans* DSM 12444 (Cecil et al. 2018); hence, these were not included in the blast of the current study. The blasted proteins included the enzymes responsible for the conversion of syringaldehyde → syringate → 3MGA/gallate that are characterized in *Sphingobium* sp. SYK-6 (Kamimura et al. 2017a; Masai et al. 2007), together with the enzymes responsible for gallate ring opening and reactions downstream, reported in *P. straminea* NGJ1, *P. putida* KT2440, and *Comamonas* sp. E6 (Kamimura et al. 2010; Maruyama et al. 2001; Nogales et al. 2011). The resultant enzymes in RG1 with query coverage over 85% were used to construct a putative syringyl pathway (Figure 3.8A).

The results of sequence analysis in isolate RG1 revealed the presence of enzymes similar to (i) aldehyde/vanillin dehydrogenase (DesV and LigV) for the conversion of syringaldehyde, (ii) syringate *O*-demethylase (DesA) for the conversion of syringate to 3MGA, and (iii) vanillate/3MGA *O*-demethylase (LigM) for the conversion of 3MGA to gallate. No similar enzymes related to the ring opening of gallate were found in RG1. However, a putative operon with genes encoding the enzymes (GalB/C/D) responsible for the conversion of 4-oxalomesaconate (first downstream product of gallate ring opening) to oxaloacetate and pyruvate were found in the genome of RG1 (Figure 3.8B). Also found in this putative operon is a gene annotated as catechol 2,3-dioxygenase, an enzyme responsible for the meta-cleavage of catechol. Possibly, this gene encodes the enzyme responsible for the ring-cleavage of gallate.

Further cloning and characterization of the suggested genes/enzymes are required to confirm their activities. However, this is the first study reporting the physiology together with the predicted genes/enzymes related to the catabolism of syringyl-compounds in a Gram-positive bacterium. These findings expand the pool of resources available to amend the host organism(s) for the efficient valorization of hardwood lignin that contains up to 75% syringyl units (Gellerstedt and Henriksson 2008).

[4] The Basic Local Alignment Search Tool (BLAST) finds regions of local similarity between sequences. The program compares nucleotide or protein sequences to sequence databases and calculates the statistical significance.


A
Syringaldehyde
DesV
LigV
Syringic acid
DesA
3MGA
LigM
LigA, LigB
DesZ
Gallic acid
PDC
GalA
DesB
LigA, LigB
LigI
PmdD
ProL
OMA
GalD
LigU
CHM
GalB
LigJ
ProH
PmdE
CHA
LigK
GalC
PmdF
ProA
Oxaloacetate + Pyruvate
DesV: EHF32_13860 (or) EHF32_11840
LigV: EHF32_13805
DesA: EHF32_13620 (or) EHF32_13765
LigM: EHF32_13620 (or) EHF32_13765
GalD: EHF32_13605
GalB: EHF32_13615
GalC: EHF32_13610
Putative operon (see B below)

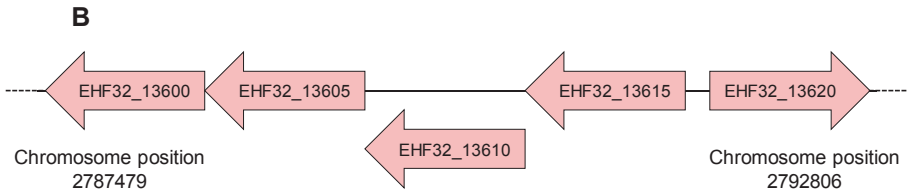


Figure 3.8. A) Catabolic pathway for the conversion of syringaldehyde via syringate and 3MGA. The reported proteins from other organisms are shown in blue. Proteins in *Microbacterium* sp. isolate RG1 that are homologous to the reported enzymes are shown in red. B) Schematic map of the operon/gene cluster that contains five adjacent genes that putatively encode five of the enzymes of this pathway. Abbreviations: 3MGA, 3-*O*-methylgallate; PDC, 2-pyrone-4, 6-dicarboxylate; OMA, 4-oxalomesaconate; CHM, 4-carboxy-2-hydroxy-cis,cis-muconate; CHA, 4-carboxy-4-hydroxy-2-oxoadipate. Enzymes: DesV, NAD-dependent aldehyde dehydrogenase; LigV, Vanillin dehydrogenase; DesA, Syringate *O*-demethylase; LigM, Vanillate/3-*O*-methylgallate *O*-demethylase; LigA, Protocatechuate 4,5-dioxygenase alpha chain; LigB, Protocatechuate 4,5-dioxygenase beta chain; DesZ, 3-*O*-methylgallate 3,4-dioxygenase; GalA, DesB, Gallate dioxygenase; LigI, PmdD, 2-pyrone-4,6-dicarboxylate hydrolase; ProL, 2-pyrone-4,6-dicarboxylate lactonase; GalD, LigU, 4-oxalomesaconate tautomerase; GalB, LigJ, ProH, PmdE, 4-oxalomesaconate hydratase; LigK, 4-hydroxy-4-methyl-2-oxoadipate aldolase; GalC, PmdF, ProA, 4-carboxy-4-hydroxy-2-oxoadipate aldolase.

Chapter 4

Bioconversion of Kraft lignin

In the previous chapter, growth of bacteria on pure aromatic compounds was discussed. However, in a commercial process it is likely desirable for cost reasons to use a crude, or only partly purified, lignin stream instead of purified aromatics. This stream will contain, in addition to known aromatics, several unknown compounds together with salts and acids that might be toxic to bacteria. As pointed out before (Section 2.2), the composition of a lignin stream depends on the plant origin of lignin, the method of recovery, and the technique of depolymerization (where applicable); hence, it is important to evaluate the performance of the intended bacterial strains on the particular substrate of interest.

Studies have previously been carried out to evaluate the capability of a number of microorganisms to grow on, or convert, lignin substrates. Alkaline pre-treated liquor from corn stover was used to evaluate 14 bacterial strains, out of which *Acinetobacter* ADP1, *Amycolatopsis* sp., and *Pseudomonas putida* were reported to depolymerize and catabolize 30% of the initial lignin present (Salvachúa et al. 2015). In another study, a substrate from base-catalyzed depolymerization of corn stover was tested for microbial growth using seven aromatic-catabolizing bacteria and two yeasts, and the reported results showed that 15% lignin was converted by *Pseudomonas putida* KT2440, *Corynebacterium glutamicum*, and *Rhodotorula mucilaginosa* (Rodriguez et al. 2017). Oxygen-treated Kraft lignin was used as a substrate to accumulate lipids in *Rhodococcus opacus* DSM 1069, and the maximum reported titre was 0.07 g/L lipids mainly composed of palmitic and stearic acids (Wei et al. 2015).

Softwood Kraft lignin, generated at the pulp factories, is by far the largest industrial lignin stream in Scandinavia (Gellerstedt 2015), and the valorization of such feedstock has become widely interesting (Shi et al. 2017; Wei et al. 2015). As the molecular weight of Kraft lignin is rather high, it has to be depolymerized to generate smaller aromatic compounds –preferably monomers – that the bacteria can ingest if a biological valorization is to be used (Abdelaziz et al. 2016).

In this thesis, Kraft lignin treated using two different techniques (supplied by collaboration partners) were used as substrates for bacterial growth **(Papers V and VI)**. The performance of different bacterial strains on the alkaline- and oxidatively-treated Kraft lignin are summarized and discussed in sections 4.1 and 4.2, respectively.

4.1 Bacterial conversion of alkaline-treated Kraft lignin

As discussed in chapter 2 (section 2.5), there are several different methods for the depolymerization of lignin. Depending on the severity of treatment, the resultant product might contain a mixture of different aromatic compounds, organic acids, and unknown cleavage products. The product from these treatments should be biocompatible without the presence of metal catalysts or solvents that are toxic for microbial growth.

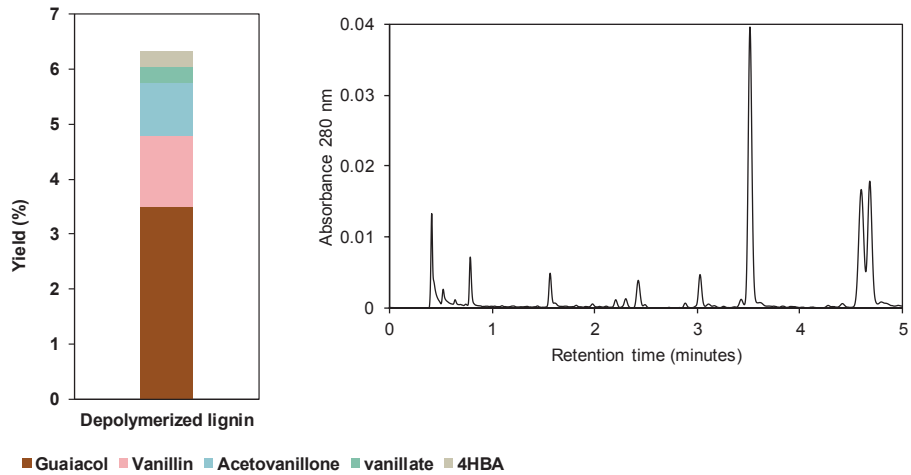


Figure 4.1. (Left) Yield (wt%) of identified aromatic monomers in the mixture of alkaline-treated Kraft lignin at 220 °C. (Right) UHPLC chromatogram of alkaline-treated Kraft lignin showing peaks at retention time 1.6 (4-HBA), 2.4 (vanillate), 3.5 (vanillin), 4.6 (guaiacol), and 4.7 (acetovanillone).

One substrate examined in the present work was alkaline treated Kraft lignin from a continuous process **(Paper V)**. Indulin AT, i.e. a commercial softwood Kraft lignin, was subjected to alkaline (NaOH) treatment in a continuous flow reactor at temperatures around 200 °C and 1 to 2 min residence time. It should be noted that this kind of treatment mainly targets the cleavage of the ether bonds such as α-O-4 and β-O-4, and as Kraft lignin is highly condensed with fewer of these bonds compared to native lignin, the yield of monomeric aromatic compounds is

expected to be limited to not more than 10% (Schutyser et al. 2018b). The product streams obtained in the present work consisted of monomers/dimers in this yield range. The main identified monomers were guaiacol, vanillin, acetovanillone, vanillate, and 4-HBA, which together accounted for slightly more than 6 wt% (Figure 4.1).

4.1.1 Assessing growth and monomer conversion

Plate screening

Plate screening is a typical first method for the qualitative assessment of growth on a substrate. Although results can be somewhat ambiguous (due to e.g. lack of appropriate nutrients, growth on storage compounds, or cryptic growth), this is a simple method to quickly get a first impression of growth of microbes on various substrates. Lack of growth may indicate either lack of ability of the microbe to metabolize the substrate, or toxicity of the medium due to inhibiting compounds. In the present work, nine bacteria[5] were screened for growth on agar plates using alkaline-treated lignin as the sole source of carbon and energy. Of the tested bacteria, *Rhodococcus opacus* and *Pseudomonas fluorescens* showed growth on the largest number of plates containing alkaline treated Kraft lignin at different temperatures and residence times (cf. Table 1 in **Paper V**).

Shake flask cultivations

The next step in characterizing growth is the assessment of liquid cultures. Cultivations in shake flasks can be performed at relatively small volumes (i.e. requiring only small quantities of substrates), and it allows both identification and quantification of excreted products as well as substrate consumed (using e.g. UHPLC/MS).

The two bacterial species, *R. opacus* and *P. fluorescens*, which showed growth on plates, and *Pseudomonas putida* EM42 (Martinez-Garcia et al. 2014) - a genome-edited version of the well-known aromatic degrader *P. putida* (strain KT2440) were chosen for liquid cultivations in shake flasks. The objective was primarily to investigate the consumption of aromatic monomers present in the treated-lignin mixture, but possible changes in the molecular weight distribution of the dissolved lignin were also of interest.

The sole carbon source used for cultivations was 1 g/L Kraft lignin (treated at 220 °C with 2 min residence time). All three organisms were able to grow on the given

[5] *P. putida* KT2440, *P. putida* EM42, *P. fluorescens* (DSM 50090), *R. erythropolis* (DSM 43066), *R. opacus* (DSM 1069), isolates 9.1, B, C (*Pseudomonas* spp.), and isolate 19 (*Rhodococcus* sp.).

substrate and reached an OD of 0.1, which is almost three times higher than the growth of these species on untreated lignin. This is in line with the higher availability of readily consumable monomers in the treated lignin, compared to the untreated Kraft lignin. All organisms were able to consume vanillin, vanillate, and 4-HBA. In addition, *R. opacus* also consumed guaiacol and an unknown peak at 0.8 min. This made it the "best" of three organisms in the sense that it could metabolize most of the readily available monomers in the complex lignin mixture. None of the tested species consumed acetovanillone.

Changes in the molecular weight distribution of the (larger) lignin fragments during liquid cultivation are due to the action of various lignolytic enzymes (see review by e.g. de Gonzalo et al. 2016). The secretion of extracellular enzymes by bacteria for the breakdown of higher molecular weight lignin is a very slow process. Higher density of cells might be required to observe this process within a few days/weeks. For this purpose, shake flask cultivations with lignin were performed together with glucose, where the initial growth on glucose helped the cells to achieve higher densities.

Size exclusion chromatography (SEC) is a simple technique to observe the changes in molecular weight distribution (MWD) of lignin samples (Baumberger et al. 2007). However, the absolute molecular weight of lignin measured using SEC can be inaccurate due to the unavailability of exact standards that represent the structure of lignin. Nevertheless, this technique is useful to compare the MWDs of different lignin samples (e.g. control and treated lignin). SEC chromatograms of *P. fluorescens* cultivations showed a one-third decrease in height of the peak (2-7 kDa) that corresponds to the larger molecular weight fraction and a substantial shift in molecular weight distribution towards lower average weights, compared to the control (Figure 4.2).

Interestingly, there was also a significant change in colour of the samples from *P. fluorescens* cultures (Figure 4.2). The results from UHPLC analysis showed the appearance of new peaks at 0.5 min (Figure 4.2), which could represent the degradation products formed that were not consumed by *P. fluorescens*. The inability of *P. fluorescens* to consume the breakdown products subsequent to lignin breakdown was previously reported in a study where alkaline-treated corn stover lignin was used for bacterial growth (Salvachúa et al. 2015). Further assays to determine enzyme activities were not possible due to the interference of lignin background. However, *in silico* analysis on the genome of *P. fluorescens* revealed the presence of DyP-type peroxidases (Table S1 in **Paper V**), which might be responsible for the breakdown of lignin.

As opposed to the case with *P. fluorescens*, SEC chromatograms of *R. opacus* and *P. putida* EM42 did not show any major changes in the lignin MWD.

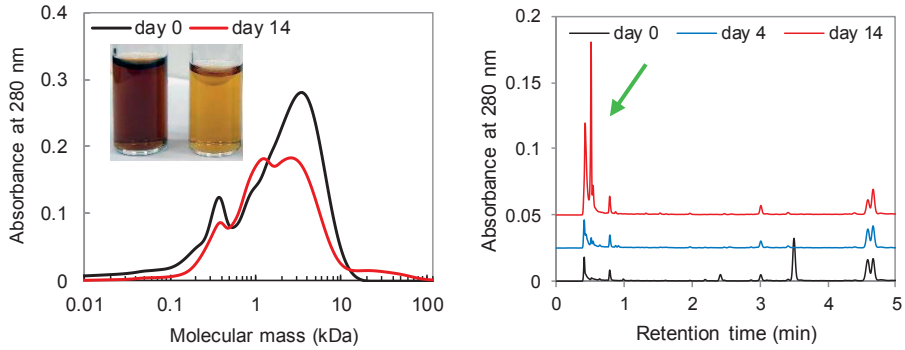


Figure 4.2. (Left) SEC chromatograms of 1 g/L depolymerized lignin on days 0 and 14 after high-cell density bacterial conversion by *P. fluorescens*. Note the change in media colour on day 14. (Right) UHPLC chromatograms of 1 g/L depolymerized lignin on days 0, 4, and 14 after high-cell density bacterial conversion by *P. fluorescens*. The occurrence of new large peaks at retention times 0.4–0.5 min for *P. fluorescens* on day 14 are shown with a green arrow.

However, in the previously mentioned study on alkaline pretreated liquor from corn stover (Salvachúa et al. 2015), *P. putida* KT2440 was shown to degrade the higher molecular weight lignin under nitrogen-limiting conditions.

In conclusion, the tested strains were able to assimilate the expected aromatic monomers in the pre-treated lignin mixture, i.e. the presence of a rather complex background did not prevent their conversion. *R. opacus* was able to consume most of the available aromatic monomers, including guaiacol - the most abundant of these. An additional observation was that *P. fluorescens* was able to decrease the average molecular weight of lignin, and also form some small molecules. More studies will be required to explore the characteristics of secreted enzymes and their specific actions on lignin. A further comment is that this study only intends to indicate possibilities. The concentration of depolymerized lignin used – 1 g/L – is far below what will be required for conceivable commercial processes. Higher concentrations were, however, not possible to test in the present case due to the precipitation of larger lignin molecules at neutral pH suitable for bacterial growth. To achieve higher feed concentrations for bacterial conversion other approaches will be required.

4.1.2 Cultivations on model compound mixtures

Depolymerized lignin will not only contain a number of inhibitors but also a mixture of different "edible" aromatics. In order to obtain more detailed data on the preference for substrates and possible excretion of intermediates, cultivations were performed with a synthetic mixture of a few key monomers (guaiacol, vanillin, and 4-HBA) found in alkaline-treated Kraft lignin. Vanillin and 4-HBA

are from the coniferyl and *p*-coumaryl branches, respectively (Figure 3.1), whereas guaiacol is usually metabolized via the benzoyl branch.

The following characteristics were noticed during these cultivations.

- The consumption of monomers by *R. opacus* was almost sequential starting with 4-HBA, followed by guaiacol, and vanillin was detoxified to vanillyl alcohol and eventually consumed at the end of cultivation (Figure 4.3). This highlighted the preferred substrates/pathways by *R. opacus*.
- The excretion of protocatechualdehyde by *R. opacus* (Figure 4.3) suggested a possible action of guaiacol demethylase on vanillin.
- As expected, both *Pseudomonas* strains consumed vanillin via oxidation to vanillate. The consumption/detoxification of vanillin was in parallel to the assimilation of 4-HBA (cf. **paper V**, Figure 7).

R. opacus was the only organism tested that could metabolize guaiacol. This is of significant interest since guaiacol was one of the major depolymerization products found in alkaline treated Kraft lignin. In a separate growth experiment it was found that *R. opacus* was able to grow on guaiacol with a specific growth rate of 0.2 h^{-1}, which is in fact higher than the growth rate of this strain on vanillate and 4-HBA (0.13 h^{-1}) (Kosa and Ragauskas 2012). Also, the biomass yield of *R. opacus* on guaiacol was higher than the yield on other methoxylated compounds such as vanillate by *P. putida* KT2440 **(Paper I)**. This underlines the effectiveness of guaiacol assimilation by *R. opacus*. However, other hosts, such as *P. putida*, may be preferable to *R. opacus* due to other metabolic traits and/or suitability for industrial bioprocessing. Expanding the substrate range of such hosts by introducing heterologous pathways will clearly be beneficial. The expression of the guaiacol demethylase genes from one of the *Rhodococcus* strains in *P. putida* will be discussed later in this thesis (section 5.1 or **Paper VII**).

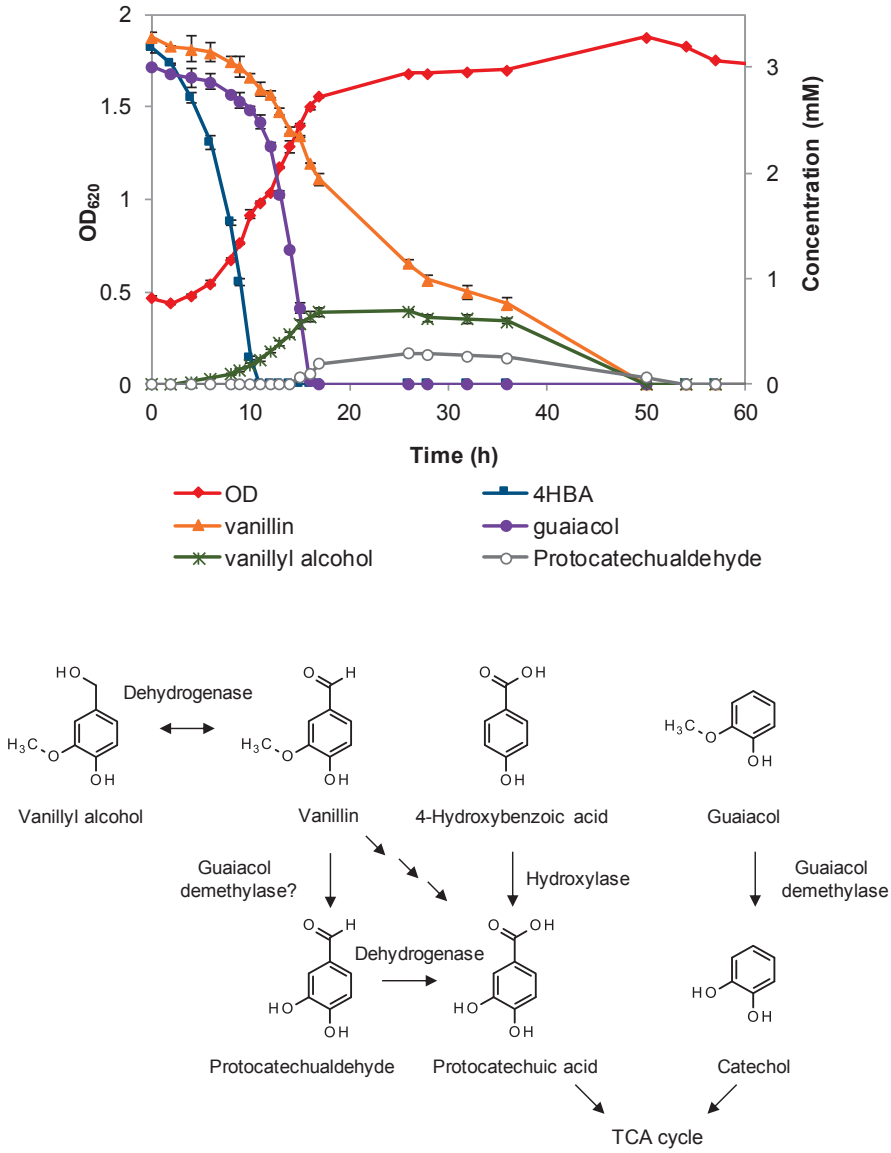


Figure 4.3. (Top) Growth (OD) and consumption of model compounds by *R. opacus* in M9 medium. 3 mM each of guaiacol, 4-HBA, and vanillin were provided as the only source of carbon. (Bottom) Proposed biochemical reactions for the assimilation of guaiacol, 4-HBA, and vanillin by *R. opacus* DSM 1069.

4.2 Bacterial conversion of oxygen-treated Kraft lignin

An alternative to alkaline depolymerization is oxidative or alkaline-oxidative treatment of Kraft lignin (Pinto et al. 2013; Rodrigues Pinto et al. 2011; Schutyser et al. 2018a). As discussed in chapter 2 (Section 2.5.2), the advantages of this approach compared to alkaline treatment are the possibility to use milder operating conditions, both lower temperature and the reduced concentration of base (NaOH). This decreases the energy needs in the process, and as the reaction mixture is less corrosive, capital costs for the reactor will be lower. In addition, the alkaline-oxidative conversion of lignin to phenolics is more selective compared to the base-catalyzed depolymerization (Schutyser et al. 2018b). The oxidation of lignin might result in a higher solubility of resultant product mixture at neutral pH, and the reduced concentration of alkali will generate less salts upon neutralization, which makes this approach even more suitable for bioconversion.

In the current work **(Paper VI)**, a softwood-based LignoBoost Kraft lignin (Zhu et al. 2014) was subjected to alkaline oxidative treatment in a batch reactor at various combinations of temperature (120-200 °C) and O_2 partial pressure (3-15 bar). The obtained oxidatively treated LignoBoost (ODLB) product streams were used as substrates for bacterial growth and the subsequent consumption of aromatic monomers were investigated (Section 4.2.2).

4.2.1 Analysis of monomers

Within the context of this thesis, the generation of aromatic monomers from Kraft lignin for the subsequent conversion by bacterial species was the primary aim. The main aromatic monomers identified in ODLB were guaiacol, vanillin, vanillate, and acetovanillone. These aromatics are similar to the ones found in alkaline-treated lignin (section 4.1), as both feedstocks used were softwood-based lignin emerging from a Kraft pulping process; however, the distribution of aromatics and their yields differed from the alkaline-treated lignin case. The total yield of aromatic monomers increased with increasing O_2 partial pressure for the reactions at 120 °C. At 160 °C, the maximum yield (3.2 wt%) of aromatic monomers was obtained for the reaction at 3 bar, after which the monomers yield decreased with increasing pressure (Figure 4.4). This suggests oxidation of aromatics under increased severity (especially at increased O_2 partial pressure) into other products, predominantly non-aromatic carboxylic acids via ring opening (Fernandes et al. 2019). The maximum yield of aromatics obtained through oxidative treatment (3.2 wt%) is only half of the yield generated by alkaline depolymerization (6.3 wt%). This might be due, mainly, to the differences in the depolymerization methods applied and may slightly be due to the differences in Kraft lignin. Nevertheless, the highest yield of aromatic

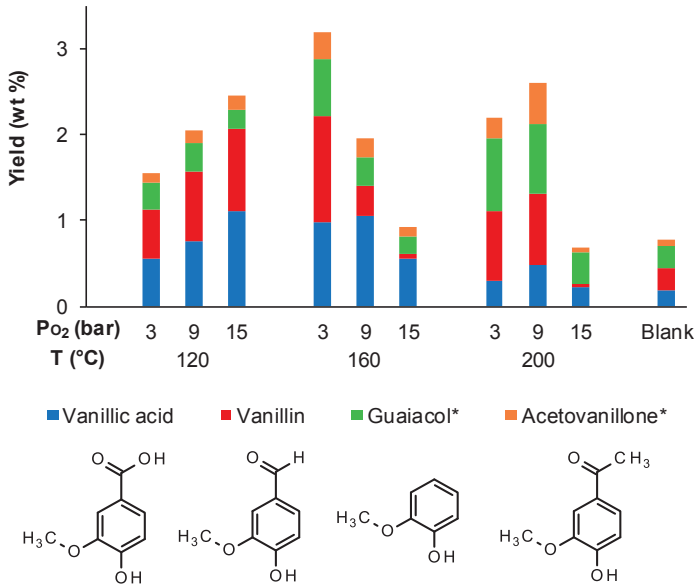


Figure 4.4. Impact of reaction temperature and oxygen pressure on the yield of monophenols from the alkaline oxidative treatment of Kraft lignin. Reaction conditions: 20 g/L LignoBoost lignin, 0.2MNaOHaqueous solution, 30 min. (*: Including coeluting compound, quantified with guaiacol and acetovanillone standards, respectively). The ratio of NaOH to lignin used was 0.4, compared to the equal weight ratio for alkaline treatment.

compounds obtained in this study (at 160 °C and 3 bar) was four times higher than the untreated lignin (Figure 4.4).

The major non-aromatic carboxylic acids identified in ODLB were formic and acetic acids, together with some minor amounts of succinic acid (cf. figure 2b, **paper VI**). The amount of acids increased with increasing O_2 partial pressure at the expense of aromatic monomers, especially for reaction temperatures starting from 160 °C. The highest yield of organic acids (13.5 wt%) was found in the lignin stream that was treated at the highest severity (200 °C and 15 bar) in which the subsequent yield of aromatic monomers was found to be the lowest – even lower than the untreated (blank) lignin. This shows the oxidation of not only generated but also previously available aromatics into ring-opened products. A high ratio of organic acids to mono-aromatic compounds at severe conditions (high temperature and oxygen pressure) was likewise reported by Lyu et al. (2018) in alkaline oxidative cracking of biorefining lignin from corn stover. Furthermore, Schutyser et al. (2018a) reported that the higher loading of NaOH and higher temperatures promoted the formation of aromatics, while higher oxygen pressure and longer reaction times led to the degradation of aromatics to formic, acetic, and malonic acids.

The highest amount of aromatic monomers was found at 160 °C and 3 bar with a rather low content of oxidized products (acids). These conditions were considered favourable and were reproduced in larger quantities to evaluate the performance of different bacterial species.

4.2.2 Cultivations using ODLB

As mentioned above, the depolymerized mixture obtained using a specific type of lignin and/or treatment method is always unique, irrespective of the similar aromatic monomers. The obtained ODLB streams in the current study consist of organic acids and other unknown products that might be different from the mixture generated through alkaline treatment in **paper V**. In addition, the solubility of ODLB streams at neutral pH was much higher than the alkaline-treated lignin mixtures, which allowed us to use higher feed concentrations - up to 10 g/L for some cultivations. The three organisms (*R. opacus* DSM 1069, *P. putida* KT2440, and *P. fluorescens*) used in the previous study were selected for cultivation experiments together with *Sphingobium* sp. SYK-6. This bacterium has been reported to assimilate several lignin-related aromatics such as vanillate and syringate (Masai et al. 1999), but it has been less studied on complex lignin feedstocks.

High cell density (OD ≈ 4) shake flask cultivations were performed with 2 g/L ODLB using the four abovementioned bacteria. To assess the growth and tolerance at higher substrate concentrations, *Sphingobium* sp. SYK-6 and *P. putida* KT2440 were also cultivated using 10 g/L ODLB (initial OD < 0.01). Clear growth was observed for both strains and the maximum OD reached was around 0.7. Consumption of aromatic monomers was in agreement with the high cell density cultivation of these species, and the key findings (cf. figure 6, **paper VI**) are as follows:

- Except for *R. opacus*, the tested bacteria were able to convert vanillin to vanillate and subsequently consume the excreted and available vanillate. In agreement with our previous results, *R. opacus* instead detoxified vanillin to vanillyl alcohol and eventually consumed the excreted vanillyl alcohol together with vanillate in 21 h.

- *R. opacus* was the only bacteria tested that could assimilate guaiacol. *Sphingobium* sp. SYK-6 could metabolize aromatic compounds with two methoxy groups (e.g. syringate) (Masai et al. 2004), but surprisingly the organism was unable to consume the mono-methoxylated compound guaiacol. SYK-6 holds genes encoding *β*-etherases that can cleave the *β*-aryl ether linkages in aromatic dimers to generate guaiacol (Masai et al. 2014), yet the uptake of guaiacol has not been reported.

- *R. opacus* was able to metabolize an as of yet unknown compound that co-elutes with acetovanillone in 20 h, leaving the acetovanillone in culture medium rather unconsumed. *Sphingobium* sp. SYK-6 assimilated acetovanillone together with the co-eluting compound in around 100 h. The bioconversion of some aromatic compounds, i.e. 4-vinylguaiacol and ferulic acid, to acetovanillone has been described in a few organisms (Mabinya et al. 2010; Shanker et al. 2007). However, to the best of our knowledge, no bacterial strains have been reported to assimilate acetovanillone as the sole source of carbon and energy.

Analysis of molecular weight distribution

SEC analysis revealed that there were no major modifications of the higher molecular weight lignin by any of the organisms used. Even the slightest peak shifts observed were towards the polymerization side, rather than depolymerization. This is in contrast to the breakdown of larger lignin molecules by *P. fluorescens* during cultivations using alkaline-treated Kraft lignin **(Paper V)**. This might be due to the differences in source of lignin used or treatment method applied. Some of the bacterial strains used in this study have been reported to secrete extracellular lignin-modifying enzymes (He et al. 2017; Salvachúa et al. 2015). However, the action of such enzymes in the depolymerization or polymerization of lignin depends on several factors, including the origin of lignin, pre-treatment method, reaction time/temperature, substrate loading, source of enzyme, and presence of mediators (Brzonova et al. 2017; Ghoul and Chebil 2012).

In conclusion, *R. opacus* DSM 1069, *P. putida* KT2440, and *P. fluorescens* were able to assimilate all the expected aromatic monomers present in the ODLB stream. Although *Sphingobium* sp. SYK-6 was able to consume acetovanillone, it was not capable of assimilating guaiacol. SYK-6 may therefore be more useful for the valorization of hardwood-based lignin that contains syringyl-units. The solubility of oxygen-treated lignin enabled the use of higher substrate concentrations (10X) compared to the alkaline-treated lignin. As *P. putida* KT2440 was able to grow and assimilate the monomers from this mixture without being inhibited at such concentrations, this approach can be useful for the future valorization of Kraft lignin in large scale. However, from a commercial standpoint, the yield of aromatic monomers from neither the alkaline-treatment nor the oxygen-treatment was sufficient yet. To obtain a reasonable yield of around 30-40 wt% from Kraft lignin that is highly condensed, other approaches might be required.

Chapter 5

Conversion of aromatic compounds using engineered *Pseudomonas putida*

As discussed in chapter 3, the bacterium *Pseudomonas putida* KT2440 has a large metabolic ability to convert a wide range of aromatic compounds, including vanillin, vanillate, benzoate, 4-HBA, and *p*-coumarate (sections 3.2 and 3.3). The usefulness of this was early recognized and a genetically modified version of *P. putida* was in fact the first patented engineered microorganism (Kevles 1994). Its high specific growth rate and short lag phases on a number of lignin-related compounds, and capacity to detoxify aldehydes such as vanillin, were observed throughout the course of this thesis work. These characteristics were not only limited to the performance on model compounds but also applicable to complex lignin substrates. Its robustness, the fully annotated genome sequence (Belda et al. 2016), and available molecular biology tools such as CRISPR/Cas9 (Aparicio et al. 2018) makes *P. putida* a logical host organism for applications in lignin biotechnology (see review by Nikel and de Lorenzo 2018).

However, for the efficient valorization of lignin using *P. putida*, this organism should be further improved by metabolic engineering enabling the use of a wider substrate range and/or the channelling of carbon into desired products. Some examples of work towards this aim by genetically modifying *P. putida* for the production using lignin-related substrates are listed in Table 5.1. One common target compound is *cis,cis*-muconate which is a direct precursor for adipic acid and can be used also for production of terephthalic acid (Beckham et al. 2016). It is a natural intermediate in the catechol fission pathway of *P. putida* (Figure 3.1). The production of *cis,cis*-muconate using model compounds as substrates (with co-substrates for growth) at yields approaching 100% (cf Table 5.1) as well as the use of "real" treated lignin as substrates is a breakthrough in the biological valorization of lignin (see e.g. recent review by Becker and Wittmann 2019).

Table 5.1. Some examples of genetically modified *P. putida* KT2440 for the production of value-added compounds using lignin-related substrates. Adapted from (Becker and Wittmann 2019).

Strain	Substrate	Product	Yield (mol/mol)	Reference
P. putida KT2440-JD1	Benzoate, Glucose (co-substrate)	*cis,cis*-muconate	100 %	(van Duuren et al. 2012)
P. putida KT2440-CJ238	*p*-Coumarate, Glucose (co-substrate)	*cis,cis*-muconate	95 %	(Johnson et al. 2017)
P. putida KT2440-MA9	HTC[a] Lignin (Indulin AT)	*cis,cis*-muconate	13[b]	(Kohlstedt et al. 2018)
P. putida KT2440-CJ516	Guaiacol, Glucose (co-substrate)	*cis,cis*-muconate	100%	(Tumen-Velasquez et al. 2018)
P. putida KT2440-CJ122	*p*-Coumarate	Lactate Pyruvate	76 % 77 %	(Johnson and Beckham 2015)
P. putida KT2440-AG2162	*p*-Coumarate	*mcl*-PHA	50[c]	(Salvachua et al. 2019)
P. putida KT2440-AG2162	Lignin stream from corn stover	*mcl*-PHA	17.7[c]	(Salvachua et al. 2019)
P. putida KT2440-CJ251	4-HBA, Glucose (co-substrate)	2-Pyrone-4,6-dicarboxylic acid	80.7 %	(Johnson et al. 2019)
P. putida KT2440-CJ263	4-HBA, Glucose (co-substrate)	β-ketoadipic acid	107.8 %	(Johnson et al. 2019)
P. putida KT2440-GN442ΔcalA	Ferulate	Vanillin	82 %	(Graf and Altenbuchner 2014) **Paper III**

[a] base catalysis and hydrothermal conversion
[b] titer – g/L
[c] g per g CDW

In this thesis, *P. putida* modified for the use of guaiacol as the only source of carbon and energy was studied **(Paper VII)**. An additional metabolic engineering work, resulting from the characterization and genome sequencing of a *Pseudomonas* isolate, was devised to increase the yield of vanillin from ferulic acid by removal of by-product formation **(Paper III).** Results of these metabolic engineering works are discussed in sections 5.1 and 5.2, respectively.

5.1 Assimilation of guaiacol

Similar to some other studies (Table S3 in **Paper V**), we also found guaiacol (2-methoxyphenol) as one of the major depolymerization products from softwood-based lignin using our methods of depolymerization **(Paper V and VI)**. The prevalence of guaiacol in depolymerized lignin makes its conversion a crucial step for the efficient utilization of lignin via bioconversion (Beckham et al. 2016). While *P. putida* KT2440 is able to demethylate and metabolize other methoxy-compounds such as vanillate, it is not capable of utilizing guaiacol as carbon source. As this strain comprises genes for the uptake of catechol, which is the demethylation product of guaiacol, the expression of genes for the demethylation of guaiacol in *P. putida* should –in theory– result in the assimilation of this compound.

Several bacterial species, both Gram positive and negative, have been reported to utilize guaiacol as a carbon source. The enzymes responsible for the aerobic demethylation of guaiacol to catechol have been identified as cytochrome P450 monooxygenases (CYP) in some bacteria, namely *Rhodococcus rhodochrous* 116 (Eltis et al. 1993), *Amycolatopsis* sp. ATCC 39116 (Sutherland 1986), and *Moraxella* sp. GU2 (Dardas et al. 1985). These enzymes require one or two redox partner protein(s) for the transfer of electrons from a cofactor such as NAD(P)H to the heme group of CYP (Figure 5.1).

In a recent study, the guaiacol demethylation system of *Amycolatopsis* sp. ATCC 39116 has been described, in which only one redox partner protein (GcoB) was shown to be involved in the transfer of electrons from NADH to the CYP monooxygenase (GcoA) (Mallinson et al. 2018). This system was successfully expressed in *P. putida* yielding a strain that could assimilate guaiacol. However, the transfer of similar genes from *Rhodococcus jostii* RHA1 and *R. pyridinivorans* AK37 to *P. putida* did not result in the growth on guaiacol (Tumen-Velasquez et al. 2018).

Due to the poor results reported with the above mentioned rhodococci, the present work in this thesis was aimed at investigating the guaiacol demethylation system of another reported bacterium, namely *R. rhodochrous* (Eltis et al. 1993; Karlson et al. 1993), with the intention to subsequently express this activity in the host strain *P. putida* EM42[6]. The original authors of the *R. rhodochrous* work reported a partial amino acid sequence of the enzyme CYP presumably responsible for guaiacol demethylation in *R. rhodochrous* (Eltis et al. 1993). However, no

[6] The strain EM42 that is a genome-reduced version of the parental strain KT2440 was constructed with the aim to achieve higher NAD(P)H and ATP availability, and increased genetic stability for heterologous expression, by eliminating the undesired traits (e.g. flagellar machinery, four prophages, three components of DNA restriction-modification systems, two transposons, etc.) (Martinez-Garcia et. al. 2014).

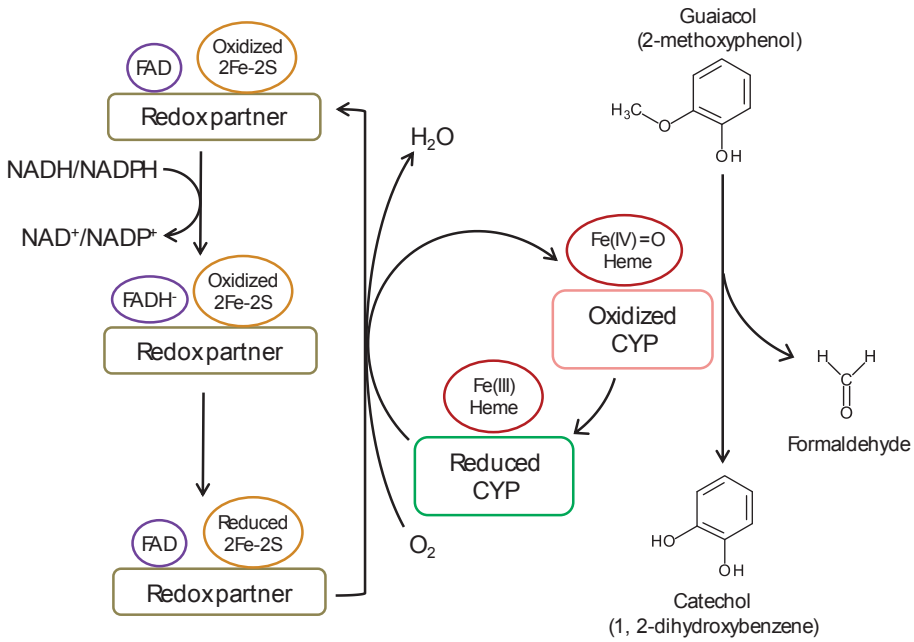


Figure 5.1. The reaction carried out by the guaiacol *O*-demethylation system of *Rhodococcus rhodochrous* and the electron transport involved between the two components.

identification or characterization of the essential redox partner proteins involved in this reaction were described. In the current work, the reported partial amino acid sequence was used to identify the operon in *R. rhodochrous* J3 that contained the coding sequence of the protein. This operon consisted of the CYP gene together with a gene encoding an oxidoreductase that might be a potential redox partner (ferredoxin/ferredoxin reductase) for the CYP. Some ferredoxin elements (second redox partner) were also identified in *R. rhodochrous* J3 as well as in *Amycolatopsis* ATCC 39116, and the combination of all three components (CYP, ferredoxin reductase, and ferredoxin) were heterologously expressed in *P. putida* EM42 giving rise to four different engineered strains (cf. **paper VII**, table 1) (More exact details regarding the identification of ferredoxin partners and the subsequent construction of CYP-encoding plasmids are found in **Paper VII**).

All the constructed strains (GI to GIV) were able to assimilate guaiacol as the sole carbon source, whereas the control strains were not (G0 and GIV are shown in figure 5.2). There were, furthermore, no major differences in the uptake of guaiacol between the engineered strains, irrespective of the presence or absence of the ferredoxin element. This suggests that only the oxidoreductase gene in addition to the CYP gene is needed for the demethylation of guaiacol, which is in agreement with the recently described system for *Amycolatopsis* sp. ATCC 39116

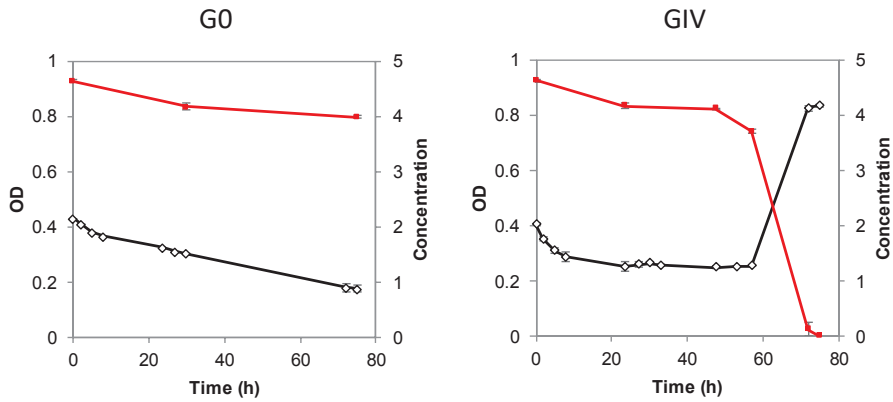


Figure 5.2. Cultivation of *P. putida* EM42 strains G0 and GIV using 5 mM guaiacol as the only source of carbon and energy in M9 mineral medium. Growth is represented as OD_{620} in black and guaiacol concentration (mM) is shown in red. The strain G0 consists of cytochrome P450 from *R. rhodochrous* J3, whereas the strain GIV consists of cytochrome P450 together with ferredoxin reductase from *R. rhodochrous* J3.

(Mallinson et al. 2018). These results also confirmed the role of oxidoreductase as an essential redox partner for CYP in the native *R. rhodochrous* J3 strain (Figure 5.1).

In conclusion, expression of the guaiacol demethylation system from *R. rhodochrous* resulted in the assimilation of guaiacol by *P. putida* EM42.

5.2 Improved yield of vanillin

Vanillin is one of the most widely used aromatic flavouring agents in food, fragrances, and pharmaceuticals. The global demand of vanillin (~ 20,000 tons in 2018) is almost fully (> 85%) met by synthetic production from petroleum-based sources (Havkin-Frenkel 2019). However, the Norwegian company Borregaard also produces vanillin from the alkaline-oxidative depolymerization of lignosulfonates. The yield of vanillin from this process is around 7 wt% of dry lignin (Bjørsvik and Minisci 1999; Pacek et al. 2013).

There is an increasing demand for "natural" or "natural flavour" vanillin in the society (Ciriminna et al. 2019). According to FDA, only vanillin that comes from vanilla beans can be labelled "natural"[7], while vanillin produced by natural processes (such as fermentation) can be marketed with a "natural flavour" label

[7] The European regulations allow vanillin produced from natural sources and by natural processes to be labelled as "natural" vanillin.

(Havkin-Frenkel 2019). Currently, the Belgian company Solvay produces vanillin by fermenting ferulic acid that is extracted from rice bran oil[8].

There have been several reports concerning the bioconversion of ferulic acid to vanillin using various bacterial species; however, in most cases, the yields were low and/or the biotransformation reactions were slow (Achterholt et al. 2000; Di Gioia et al. 2011; Fleige et al. 2013). Interestingly, a study by Graf and Altenbuchner (2014) reported a high-yield production of vanillin from ferulic acid using an engineered *P. putida* strain. In this strain (GN442), several genes in the aromatic funnelling pathways were deleted in order to stop the metabolism at vanillin. However, an undesired formation of the by-product vanillyl alcohol was observed (Graf and Altenbuchner 2014). This is likely the same phenotype that we observed in one of our isolates, *Pseudomonas* sp. isolate 9.1 (section 3.3.3).

The results reported in this work (section 3.4) showed that the enzymes FEZ21_09870 and CalA were responsible for the reduction of vanillin to vanillyl alcohol in isolate 9.1 and *P. putida* KT2440, respectively **(Paper III)**. Consequently, the deletion of the gene *calA* in *P. putida* GN442, i.e. the strain engineered for vanillin production from ferulate, resulted in the almost complete removal of vanillyl alcohol formation - from 17% in GN442 down to 1% in GN442ΔcalA in 18 h (Figure 5.3). Within a short conversion time of only 5 hours, the yields of vanillin were 76 and 82% in GN442 and GN442ΔcalA, respectively. However, after 18 h, due to the reduction of vanillin to vanillyl alcohol in GN442 the yield of vanillin decreased to 69%, while the yield was retained as 82% in GN442ΔcalA (Figure 5.3). This shows that there is almost no background activity of aldehyde reductase that acts on vanillin in GN442 once *calA* was deleted.

[8] https://www.solvay.com/en/article/natural-vanillin-ensures-resource-efficiency

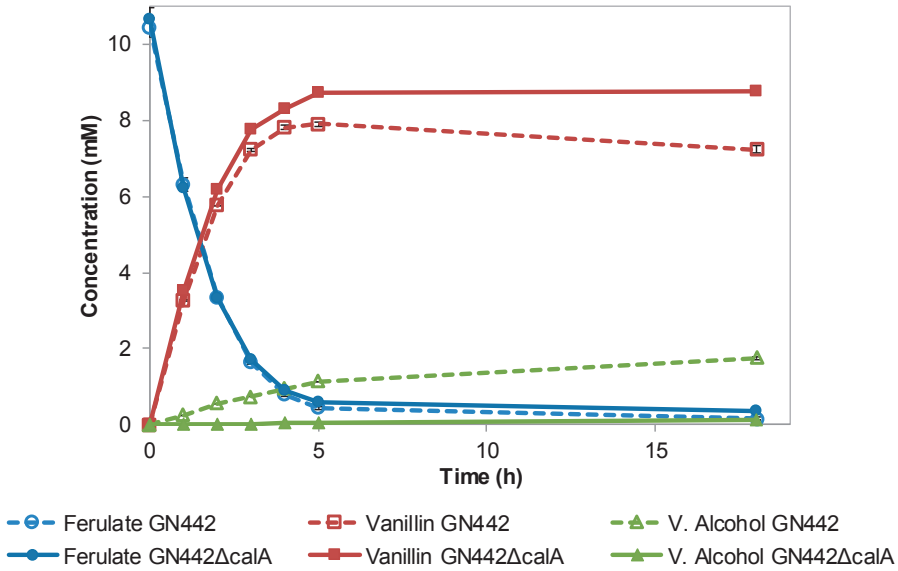


Figure 5.3. Bioconversion of ferulic acid into vanillin with the engineered *P. putida* strains GN442 and GN442ΔcalA. Deletion of *calA* in the GN442 strain (engineered for the production of vanillin from ferulic acid) completely abolished the formation of vanillyl alcohol as by-product.

In conclusion, an aldehyde reductase that is responsible for the formation of vanillyl alcohol was identified in *P. putida* KT2440 with the help of isolate 9.1 that had a different turnover of vanillin compared to KT2440. This was subsequently used to increase the yield of vanillin from ferulic acid in a *P. putida* strain, by eliminating the formation of vanillyl alcohol. This study demonstrates that the characterization of novel isolates does not only provide information about the diversity of metabolic phenotypes in nature, but also how this information can be applied to address unresolved traits of model strains leading to improved host strains for diverse biotechnological applications.

Chapter 6

Conclusions and outlook

In this thesis, possibilities of lignin bioconversion using bacteria were explored. Lignin bioconversion holds complex challenges, many of which are related to the variability of lignin composition and structure, and its decomposition into smaller fractions. In this work, however, the focus was the biological conversion of already obtained low molecular weight aromatic compounds. Initially, screening was performed on samples from natural and man-made environments to identify bacteria that could metabolize lignin, and/or related aromatic compounds. Some of the isolates found, together with previously reported aromatic-catabolizing bacteria, were physiologically characterized on several aromatic compounds from different funnelling pathways, followed by evaluating their performance on treated-lignin substrates. A final step in this thesis was to improve a chosen host organism (*Pseudomonas putida*) via metabolic engineering for i) a wider substrate uptake range and ii) increased product yield – exemplified by vanillin production from ferulic acid. The main findings of this work are summarized below.

Part I: Screening for bacteria and growth characterization on lignin-related aromatics

In the screening work made from compost and sea sediment using several lignin-related carbon sources, *Pseudomonas* spp. were frequently isolated. This underlines the importance of these bacteria in lignin-rich environments. The strain *P. putida* KT2440 was found able to assimilate most of the compounds from the benzoyl, *p*-coumaryl, and coniferyl branches of the funnelling pathways, with a preference in this order. Demethylation of compounds from the coniferyl branch, i.e. demethylation of vanillate, seems to be a rate-limited bottleneck. In contrast, the upstream conversion (and presumable detoxification) of vanillin to vanillate by KT2440 was very efficient, with several enzymes acting on vanillin. However, KT2440 was not able to consume guaiacol due to the absence of a suitable cytochrome P450 system.

Microbacterium sp. isolate RG1 was able to assimilate syringyl compounds, i.e. di-methoxylated aromatic compounds, such as syringate and syringaldehyde. The

genome of this isolate was also sequenced to construct a putative syringyl pathway and suggest the genes/enzymes involved in their metabolic reactions. This is the first reported Gram-positive bacterium to consume syringyl compounds, and the proposed genes will expand the pool of available resources to upgrade host strain(s).

In general, a fair number of microorganisms can degrade compounds from the H and G funnelling branches, but fewer organisms can metabolize S-lignin compounds. As demethylation often requires a cofactor such as THF, NADH or FAD, the assimilation of methoxylated aromatics, especially di-methoxylated syringyl-compounds, might be limited by the rate of cofactor regeneration.

Part II: Performance of bacteria on treated-lignin substrates

Rhodococcus opacus was the only microorganism tested that could metabolize guaiacol from the mixture of both alkaline-treated and oxidatively-treated Kraft lignin. As guaiacol is one of the major aromatic monomers expected after the depolymerization of softwood-based Kraft lignin, its efficient assimilation by *R. opacus* is a crucial factor. *P. fluorescens* was able to degrade the larger molecular weight lignin into smaller molecules. Further investigations will be required to identify the secreted extracellular enzymes and their mode of action on lignin. These bacterial enzymes might be interesting for modifying lignin towards various end applications. *P. putida* KT2440 and *Sphingobium* sp. SYK-6 were able to grow and assimilate several of the aromatic monomers present in the mixture of 10 g/L oxidatively-treated lignin, without being inhibited in such complex background.

The low solubility of alkaline-treated Kraft lignin at neutral pH prevented the supply of higher substrate concentrations for bacterial conversion. Despite the better solubility of oxygen-treated lignin, the yield of aromatic monomers for the direct uptake by bacterial species was less than 5%, which is likely too low for commercial applications. In the future, effective depolymerization methods should be developed for the higher yield of aromatic monomers from Kraft lignin for the subsequent uptake by bacterial strains. Nevertheless, this study demonstrated the possibilities of utilizing treated Kraft lignin in bioconversion processes.

Part III: Metabolic engineering of P. putida

Heterologous expression of cytochrome P450 and oxidoreductase genes from *R. rhodochrous* resulted in a *P. putida* strain that could assimilate guaiacol. In the future, potential host strains such as *P. putida* should be further upgraded for an even wider range of substrate uptake; this includes hardwood-derived lignin compounds such as syringate and syringaldehyde that may be utilized via

demethylation using engineered cytochrome P450 systems – as already demonstrated with syringol (Machovina et al. 2019). Eventually, the host strains shall also be engineered for channelling the carbon towards selected end products.

An interesting conversion activity was found in *Pseudomonas* sp. isolate 9.1, which was able to convert vanillin to vanillyl alcohol – a conversion typical for yeast detoxification but not *P. putida*. The isolate was genome-sequenced, and the enzyme responsible for the conversion of vanillin to its alcohol was identified. This was later used to construct a *P. putida* strain for the increased yield of vanillin from ferulic acid, by eliminating the formation of vanillyl alcohol. This shows how physiological characterization of isolates can be highly useful and give a better understanding of the biochemical reactions and associated enzymes/cofactors, which in turn enables genetic improvements of host strain(s).

Together with the ongoing research, the findings of the work carried out in this thesis contribute towards the valorization of lignin through bioconversion - a still largely unexplored field. The efficient utilization of lignin will lead to the sustainable consumption of renewable resources such as lignocellulosic biomass, which is crucial for the long-term success of the emerging bio-based economy.

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I am Krithika from Chennai, India and have been living in Europe for the past five years. While pursuing my master's degree in Indian Institute of Technology, I moved to Germany to complete my master thesis, and eventually found a PhD position in Sweden. During these years, I have learnt a lot of science, and improved my competence. I like to work in a challenging environment where I can learn something day-to-day. I also enjoy teaching and being around young students who are curious and determined to learn.

I like to travel around the world and experience different cultures. I love to do adventurous stuff, like jumping from a plane for example, of course with a parachute. I enjoy cooking Indian food, but also like to eat almost everything. Like every other Indian, I am serious about Cricket and enjoy cheering up for the Indian team. Finally, I love to talk and talk and talk…

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