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Lignin degradation and oxygen dependence

Ligninnedbrytning och syreberoende

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Abstract

This work concerns itself with the biological degradation of the lignin polymer. The chemical composition of the polymer makes it recalcitrant toward hydrolysis and other common chemical reactions occurring under biological conditions. The rumen of animals is considered as a place of high turnover rates for plant matter where lignin is an integral constituent. This makes it a promising place to look for microorganisms adapted to degrade the lignin fraction of the plant matter. In this work, steps were taken to create a metagenomic library of the microorganisms present in the rumen of sheep. Bioinformatical searches were performed to investigate the possible existence of enzyme systems explaining the widespread presence of the genera *Clostridium* and *Desulfovibrio* in environments rich in lignin. While several species of *Clostridium* contained an *O*-demethylase system, the presence of *Desulfovibrio* could not be explained.

Contents

Words and terms

- Technical lignins - A collective term for different lignin qualities. Depending on the chemistry of the pulping process, where the lignin is separated from the polysaccharide-fraction of the wood, different types of lignins are produced.
  - Kraft lignin is produced by the sulfate cooking process where phenolic hydroxyl groups and sulfur-containing groups are introduced into the polymer. The size of the water-soluble polymer ranges between 1500 and 5000 g/mole.
  - Lignosulfonate is a water-soluble polymer with a molecular weight ranging between 1000 and 50000 g/mole. It is produced by sulfite cooking and contains phenolic hydroxyl groups, carboxylic groups and sulfur-containing groups.
  - Alkali lignin is a water-soluble polymer devoid of sulfur with a size ranging from 1000 to 3000 g/mole.
  - Klason lignin is the remaining lignin fraction which is not soluble in concentrated acid.
- Avicel - A water-insoluble crystalline cellulose polymer.
- Fenton chemistry - Reactions between ferrous iron (Fe^{2+}) and hydrogen peroxide to create hydroxyl radicals which acts as non-selective oxidants.

1 Introduction

As the struggle continues to move away from an economy dependent on fossil carbon sources for energy and material, more and more biomass is targeted as a potential feedstock. Residues and leftovers from wood and agricultural industries constitutes a carbon source that does not compete for area with food sources, an issue often voiced as an intrinsic problem of growing biomass exclusively for bioenergy or -material. To consider the residues and leftovers as a realistic alternative, an efficient utilisation of the carbon is necessary. This means that as much as possible of the carbon locked up as cellulose, hemicellulose and lignin have to be released, reconstituted and extracted from the process. The saccharification of cellulose and, to some extent, hemicellulose is well under way to be expanded from the laboratory into larger pilot plants where the liberated sugar units are forming new compounds.

One of the major bottlenecks in the processing of biomass is the presence of lignin which inhibits an efficient transformation of the polysaccharides. There are a lot of chemical and physical processes aimed at unlocking the cellulose and hemicellulose from the lignin, many of which are destructive, energy intense or both. Together with the realisation that lignin can be considered as a possible renewable feedstock for aromatic chemicals, there are incentives to further investigate processes for separating the wood fractions from each other. Within a biological framework, this is done in various environments such as gastrointestinal systems, forest floors and lake sediments. It is the microbes residing in each environment that are responsible for the carbon cycling, which have to involve a more or less complete degradation of both the lignin and the polysaccharides. Ruminant animals are relying heavily on their gut microbes, mainly residing in the rumen, for the degradation of the vegetal feed. The microbes have to have the collected capacity to degrade the different carbon chains making up the cell walls of the plants and release compounds considered nutritious for the animals. As the rumen is devoid of oxygen it is a promising environment to find microbes and enzymes well adapted to plant matter degradation in the absence of oxygen. The ability for both catabolic and metabolic processes without the need of oxygen is considered an important aspect within the biorefinery concept, the production of fuel, energy and value-added chemicals from renewable biomass.

2 Research question

This work focuses on the question if and how lignin can be degraded in a biorefinery concept. This will be investigated by answering the questions

- By which mechanism is degradation most feasible?
- Which type of organism possess this mechanism?
- Where is it most probable to find these species?

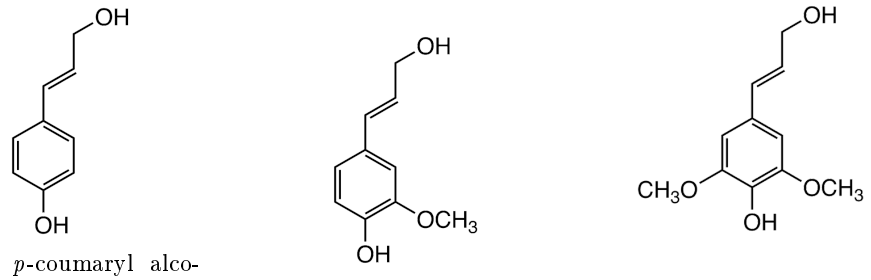
3 Background

3.1 A necessary shift

With mounting evidence of anthropogenic causes of the increase in mean temperature (IPCC, 2014), actions are needed to offset the increase. As the atmospheric concentration of CO_2 and global temperature are highly correlated, focus should be towards reducing future emissions thereby allowing the sequestration to catch up. There is a continuous flow of carbon to and from the atmosphere, oceans and terrestrial ecosystems which over large time scales varies within relatively strict limits. These limits are the result of a feedback system which inhibits that rapid changes drives the carbon concentration in any of the reservoirs beyond its capacity. However, since the industrial revolution, the inactive carbon reservoir represented by the different types of fossil fuels have been tapped leading to changes beyond the control of the feedback mechanism (Falkowski et al., 2000).

Since the global economy is tightly bound to the usage of petrochemical commodities for both energy, transport and material, it would be hard to make the necessary shift from fossil carbon without a severe economic impact on a global scale. But to idly sit by will also result in tremendous losses, both economic and humanitarian, as a majority of the worlds largest cities are situated dangerously close to the current sea level (Hallegatte et al., 2013). With the temperature projections offered in the latest report by IPCC, even modest increase will affect a large part of the worlds population (Stocker et al., 2013; Hallegatte et al., 2013). One way of combating the rising CO_2-levels, and all that it entails, while maintaining much of the petro-based infrastructure is to derive the commodities from biomass instead of fossil carbon. A first step in this direction was the production of bioethanol from biomass rich in starch and sugars and biodiesel through transesterification of vegetable oils. However, the production of biomass for the first generation of biofuels competes with the production of food crops in the face of an increasing population. To be able to properly feed a growing population while at the same time decreasing the usage of fossil carbon, the second generation of biofuels derives the biomass needed from non-competing sources like forestry and agricultural residues. This demands other processes as these types of biomass contains cellulose, hemicellulose and lignin rather than starch and simple sugars (Stöcker, 2008).

The second generation biofuels are only one of the products that can be derived from the biorefinery, the biomass processing- and production plant. The overall goal of the production plant is to reconstitute the biomass into a range of marketable products, equivalent to the oil refinery of today. Ideally these products would replace the petro-based products, thereby maintaining much of industrial infrastructure already in place. This diversification of end-products is recognized as a way of making the biorefinery economically viable as high-value materials and chemicals covers the costs of the low-value biofuels (Bozell & Petersen, 2010). However, before the end-products can be obtained, a key issue is the fragmentation of the raw biomass and how to find processes, chemical, physiochemical and biological, that produces the



(a) *p*-coumaryl alcohol **(b)** Coniferyl alcohol **(c)** Sinapyl alcohol

Figure 1: The three phenylpropanoids making up lignin. Figures from Ulrich et al. (2008).

right building blocks for further processing.

3.2 Lignin - the glue of the secondary cell wall

The cell wall of plants confers a mechanical stability which enables the cell to withstand variations in osmotic pressure without breaking. All cells have a primary cell wall containing cellulose organized into microfibrils embedded in a gel-like matrix made up of hemicellulose and pectins. In addition to the primary wall, some cell types develops a secondary cell wall with a different composition. In this secondary layer the concentration of cellulose is higher, replacing the water-containing matrix. In the xylem, the cells governing the water transport in the plant, the secondary cell wall is reinforced by the incorporation of lignin, between 15 and 50% depending on the species (Bowsher et al., 2008).

The amorphous lignin polymer is made up of the three phenylpropanoid subunits *p*-coumaryl, coniferyl and sinapyl alcohol (figure 1). When incorporated into the lignin polymer the subunits are referred to as *p*-hydroxyphenyl (H), guaiacyl (G) and syringyl (S) residues. The initial interaction between the phenylpropanoid subunits and the polysaccharides is thought to take place through the binding of single subunits to the phenolic residues, like ferulic acid, attached to the xylan chains. The subsequent growth of the amorphous lignin polymer is not completely unravelled but the hypothesis is that subunits are randomly oxidized and coupled by the action of laccase and peroxidase (Bowsher et al., 2008; Taiz & Zeiger, 2010). The different types of bonds found in the polymer can be seen in figure 2.

Depending on plant species the structure of the lignin polymer differs. This difference is explained by the varying proportions of the residues which can be seen both between taxa and within a single individual. While angiosperm hardwood contains roughly equal amounts of S and G residues, gymnosperm softwood contains mostly G residues and only minor amounts of H residues. Since the residues only differs in the number of methoxy substituents it would seem that these are affecting the bonding between the residues. The methoxy group restricts bonding which in S residues only leaves the C_4 position of the phenyl ring available while G and H residues can form bonds at both C_4 and C_5. The influence of the methoxy substituents is clearly seen when comparing the distribution of bond types between hardwood and softwood (table 1). The table clearly shows that ether bonds are more common than C-C bonds

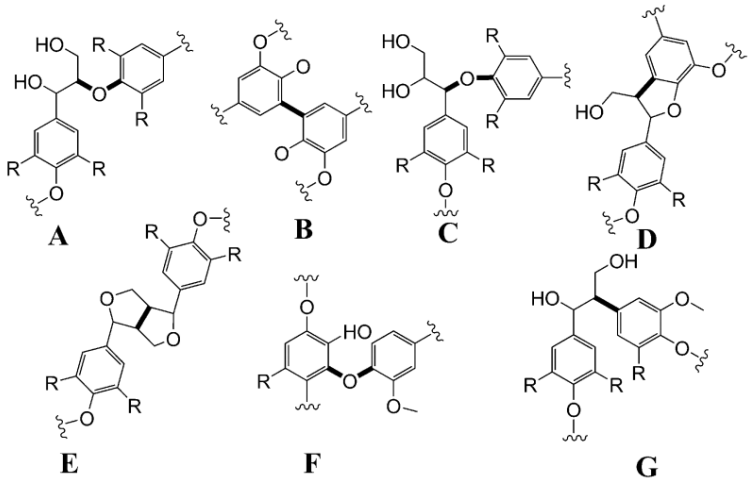


Figure 2: Common linkages found in the lignin polymer. **A**. β-*O*-4, **B**. 5-5, **C**. α-*O*-4, **D**. β-5, **E**. β-β, **F**. 4-*O*-5, **G**. β-1. Figure from Pandey & C. S. Kim (2011).

and that the β-O-4 bond is by far the most common ether bond. The difference in bond distribution between hardwood and softwood have a practical implication as the C-C bonds are more resilient towards degradation (Bowsher et al., 2008).

3.3 Lignin degradation in relation to oxygen concentration

As a part of the decomposition of wood and plant matter, lignin degradation has been noted in a number of environments. The first recorded scientific sighting stretches back to the late 19th century when it was observed that fungi degrades the recalcitrant constituents of wood (Spaulding, 1906). The ability of soil living litter-decomposing fungi to degrade aromatic compounds was investigated by Lindeberg who saw that the activity is due to two different extracellular enzymes, later identified as catechol oxidase and laccase (Lindeberg, 1948; McDonald et al., 2007). Since the findings of Spaulding and Lindeberg, the knowledge have grown to encompass not only fungi but also a diverse set of bacteria and other microorganisms from a number of environments. In later years much of the search for novel microorganisms and their enzymes has had the expressed goal of utilising the findings in industrial contexts, one of these being biorefineries. This means selecting and sampling environments where degradation of lignocellulosic

Table 1: Distribution of linkages in lignin in different wood types.

Type of linkage	Softwood lignin	Hardwood lignin
Arylglycerol-β-aryl ether (β-*O*-4)	46	60
Phenylcoumaran (β-5)	11	6
Noncyclic benzyl aryl ether (α-*O*-4)	7	7
Biphenyl (5-5)	10	5
Diaryl ether (4-*O*-5)	4	7
1,2-Diarylpropane (β-1)	7	7
Resinol (β-β)	2	3
Other	13	5

Table adapted from Dorrestijn et al. (2000).

material is taking place to maximize the probability of finding efficient candidate enzymes. One of these environments are the stomach of ruminant animals where a number of novel cellulases and xylanases have been found (Bashir et al., 2014). By comparing the community compositions of different gut-associated metagenomes, Scully et al. (2013) found that the datasets clustered according to the oxygen concentrations. The anaerobic gut of mammal herbivores housed obligate and facultative anaerobes while the aerobic insect-gut housed aerobes and facultative anaerobes. The prevalence of enzymes associated with lignin followed this pattern with very few candidates in the anaerobic gut-systems and a wide diversity in the aerobic systems.

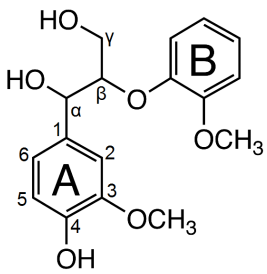
The termite represents a case where both oxygen conditions can be studied in the same system. Endogenous digestive enzymes are secreted in the oral cavity and works in cooperation with the enzymes of the residing microbiota to degrade the plant matter. The activity of the lignin-associated enzymes is correlated with the redox potential of the environment, showing high activity in the aerobic midgut and no activity in the anaerobic hindgut (Ke & Chen, 2013). A comparable environment is the soil of tropical forests which exhibits high rates of plant matter degradation but where the oxygen concentration varies temporally instead of spatially. It was shown that by alternating the oxygen concentration, the lignin degradation followed a periodic scheme with high degradation during the aerobic phase and low degradation during the hypoxic phase. Along with the degradation pattern, shiftings in the redox state of iron was observed. During the hypoxic phase Fe(III) was reduced to Fe(II) which returned to Fe(III) during the aerobic phase (Hall et al., 2015). Using soil from the same site, the two facultative anaerobes *Enterobacter lignolyticus* SCF1 (DeAngelis et al., 2013) and *Klebsiella* sp. strain BRL6-2 (Woo et al., 2014) were isolated based on anaerobic growth with alkali lignin as the only carbon source. Both of these strains showed the possibility of utilising the lignin degradation products as electron acceptors during anaerobic conditions.

However, there are evidence pointing to the ability of microorganisms associated with the stomach of herbivores, a supposedly anaerobic environment, to degrade or modify the lignin fraction of plant matter. In the rumen of cow the novel laccase RL5 was found and in the intestine of panda researchers found the laccase lac51, belonging to the facultative anaerobe *Pseudomonas putida* (Beloqui et al., 2006; Fang et al., 2012). Using microbes derived from the rumen of a cow, Kajikawa et al. (2000) showed that the benzyl ether bonds (α-O-4) in model compounds can be degraded under anaerobic conditions. However, the degradation seemed to be limited to phenolic units when the substrate increased in size. Extending the scope beyond the gut system, the ability to degrade or modify lignin anaerobically have been found in a number of relatively undisturbed environments and a few isolated bacterial species. Using lake sediment as inocolum Zeikus et al. (1982) found that the level of degradation is related to the size of the lignin polymer. While aromatic monomers, a dimeric model compound (figure 3a), low molecular weight alkali lignin and Kraft lignin were partly or completely degraded in between 16 and 30 days,

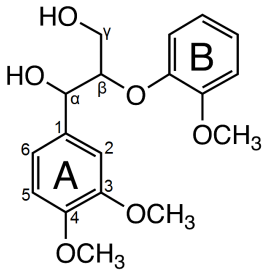
high molecular weight alkali lignin remained intact after 110 days. Similar results were reached using *Desulfovibrio desulfuricans* in pure culture, Kraft lignin as well as low molecular weight lignosulfonate were modified and degraded but the high molecular weight fraction of lignosulfonate remained untouched after 7 days (Ziomek & Williams, 1989). Using newspaper as the lignocellulose source as well as a dimeric model compound, Pareek et al. (2001) found that the substrates were degraded under sulfate reducing conditions by bacteria from an anaerobic bioreactor. While the level of degradation of the newspaper increased with time for more than 43 days, the model compound was completely degraded within 13 days in the presence of an additional complex carbon source.

Using samples from mangrove and paddy fields, Wu & He (2013) found that both sampling sites contained microorganisms able to degrade alkali lignin anaerobically over the course of 30 days, releasing both larger and smaller fragments. The majority of the species belonged to *Firmicutes* (*Clostridium*), *Bacteroidetes* (*Cytophaga* and *Bacteroides*) and δ-*Proteobacteria* (*Desulfovibrio* and *Geobacter*). The recalcitrance of high molecular weight lignin in the absence of oxygen was investigated by incubating insoluble Klason lignin together with Avicel cellulose under sulfate reducing conditions. When incubated with bacteria from the same source as Pareek et al. (2001), both the cellulose and lignin became partially degraded, albeit at a different rate and to a different extent, in a time frame of 50-100 days (J.-J. Ko et al., 2009). Through enrichment from a landfill sample, J.-H. Kim et al. (2009) found that *Desulfovibrio* sp. and *Thaurea* sp. could degrade 43% of the lignin in 19 days when the anaerobic cultures were amended with lactate and sulfate. The study also showed a two-phased degradation scheme with higher rates the initial 9 days and then lower rates the remaining 10 days.

Through the advances in sequencing in the later years it has become easier to capture both the identity and the potential activity of the organisms in an environment. When investigating the metagenome of a microbial community anaerobically degrading wood chips, van der Lelie et al. (2012) found that the major groups contributing to the degradation were *Clostridiales*, *Bacteroides*, *Magnetospirillum* and *Cyanobacteria*. In contrast to the large number of families of glycoside hydrolases, the lignolytic activity of these species was assigned to laccases, peroxidases and cellobiose dehydrogenase-type enzymes. While laccases only were found in *Cyanobacteria* and peroxidases only in *Cyanobacteria* and one of two groups of *Clostridiales*, genes coding the cellobiose dehydrogenase-type enzymes were widespread among the groups. However, as the lignin remained relatively intact after a year of incubation the authors believe the polysaccharides were liberates through local depolymerisation rather than widespread lignin degradation. To isolate bacteria without a culturing-step, single cell-sorting and sequencing was employed to capture the partial genome of a novel bacterium isolated from an anaerobic spring. The bacterium showed potential as a degrader of plant matter with genes coding for a laccase as well as glycoside hydrolases belonging to families 5 and 57. The gene coding for the laccase bears similarities to the gene *RL5* isolated from the cow rumen by Beloqui et al. (2006) and the authors speculates that this group of laccases have



(a) Guaiacylglycerol-β-guaiacyl ether



(b) Veratrylglycerol-β-guaiacyl ether

Figure 3: Two common dimeric model compounds representing the β-*O*-4 linkage in a phenolic and nonphenolic unit. Figures from Ulrich et al. (2008).

developed to function in anoxic/hypoxic environments (Youssef et al., 2011).

3.4 Degradation mechanisms

3.4.1 Chemical reference reactions

The paper and pulp industry have much experience in different processes for modifying and removing the lignin fraction from wood. This have accumulated to a considerable wealth of knowledge of the chemical principles behind the processes of pulping and bleaching. The deligninfication process can be divided into two processes where the "first involves degradation by cleavage of certain interunit linkages and the second entails introduction of hydrophilic groups into the polymer and its fragments" (Gierer, 1985). The author further rationalise the processes by two simplifications, the first reduces the complexity of the polymer by dividing the incorporated residues into phenolic and nonphenolic units, and the second reduces the reactions to either a nucleophilic or an electrophilic attack. These reactions require sites of partial charge, both positive and negative, which are introduced by electron rearrangements.

Electrophilic reactions takes place at sites of high electron density which can either be where oxygen atoms are attached or at double bonds (Gierer, 1985). Phenolic units are more reactive than nonphenolic ones due to the higher electron density the free hydroxyl group brings compared to the etherified, see figure 3 (McMurry, 2011). Nucleophilic reactions requires sites of partial positive charge which often means the α-carbon of the side chain. The main fragmentation of the lignin polymer is brought about through the cleavage of the β-ether linkage by the addition of a nucleophile to the α- or the γ-carbon. This withdraws charge from the β-carbon, enabling a second nucleophilic attack here (Gierer, 1985).

Pernemalm & Dence (1974) employed and extended the method outlined by Birch (1944) for reduction of aromatic compounds. Using sodium in liquid ammonia with ethanol as a proton source, a number of etherified phenyl methyl compounds with different substituents were reduced and their products identified. The general view was that, as was shown by Birch (1944), phenolic compounds resists reaction and that α- and β-ethers are cleaved through hydrogenolysis without any reduction of the ring. In addition, it was shown that the side chain influences the reactivity with an electron-withdrawing ability increasing the

reactivity. The Birch reduction system was also employed to degrade spruce lignin which had been treated to remove the phenolic units. The result showed that phenolic units were produced through hydrogenolysis of α- and β-ether cleavage but that these products were unreactive towards further reduction.

3.4.2 Enzymatic equivalent reactions

The ability of white-rot fungi, and especially *Phanerochaete chrysosporium*, to efficiently degrade lignin is well known and have been extensively investigated. The early work focused on the extracellular activity of lignin peroxidase (LiP) but manganese peroxidase (MnP) as well as laccase were also recognized (Kirk & Farrell, 1987). In addition to these, versatile peroxidase (VP) and dye-decolourising peroxidase (DyP) have recently been characterised as enzymes with lignin-degrading activity. All these enzyme classes were originally identified as fungal but homologs have been found in bacteria, mainly *Streptomyces* ssp. but also *Thermobifida fusca*, *Rhodococcus jostii*, *Bacillus lichenformis* and *Pseudomonas fluorescens* (Abdel-Hamid et al., 2013; Rahmanpour & Bugg, 2015). An additional system is the non-enzymatic Fenton-system found in the brown-rot and selective white-rot fungi where hydroxyl radicals ($OH^{\bullet}$) are generated through extracellular reactions (Jensen, Jr. et al., 2001; Arantes et al., 2011).

The success of the fungal enzyme systems can be viewed in the light of the structure of the lignin polymer. Kirk & Farrell (1987) states that the "structural features of lignin dictate unusual constraints on biodegradative systems responsible for initial attack: They must be extracellular, nonspecific, and nonhydrolytic". These terms are met by all of the systems above, their mechanisms involves either extracellular enzymes in an active state or diffusible redox mediators that attack the polymer. The mechanisms of the peroxidases and laccases differs with regards to the active state. While the peroxidases becomes oxidised by two electrons by hydrogen peroxide which subsequently enables two one-electron oxidation reactions (Abdel-Hamid et al., 2013), the laccases bypasses the activation by hydrogen peroxide and catalyses the direct one-electron oxidation of a suitable substrate (Morozova et al., 2007). The substrate range is decided by the redox potential of the system, both laccase and LiP were able to oxidise a range of phenols and aromatic amines but only LiP was able to oxidise high-potential methoxybenzenes (Kersten et al., 1990). The different redox potentials of the systems in table 2 should thus reflects differences in substrate suitability.

There are other systems displaying an ability to attack the bonds in the lignin polymer. These multi-component systems relies on the introduction of electron-withdrawing entities which enables a nucleophilic attack at sites with a partial positive charge. Strict aerobic species belonging to the genus *Sphingomonas* (Balkwill et al., 2006) was shown to possess a system utilising this principle for the cleavage of the β-ether bond in both dimeric and polymeric model compounds. However, there was a distinct difference as the specific activity towards the polymeric compound were more than 1000 times lower than towards the dimeric (Picart et al., 2014). The key step in this system involves the oxidation of the hydroxyl group

at the α-carbon, thereby forming a double bond. This withdraws electrons from the β-carbon, making a nucleophilic attack possible by reduced glutathione (GSH) (Masai et al., 1999). Using a model compound lacking the double-bond, Picart et al. (2014) showed that the β-ether linkage remained intact.

As was shown in the previous section, there are bacteria that have the ability to break the linkages in different kinds of lignins. But these studies generally lacks information on potential enzymes responsible for the breaking of the linkages, making it hard to develop tentative reaction mechanisms. In the pure culture-study using *D. desulfuricans*, Ziomek & Williams (1989) found that the activity was extracellular and only achieved by growing cultures. Based on an increase in sulfur content and evidence of ring cleavage in treated Kraft lignin, the authors speculates in a reductive mechanism involving insertion of thiol groups. The changes observed in low-molecular weight lignosulfonate were mostly related to an increase in phenolate hydroxyl groups, such as increased solubility and mercury-binding capacity. This points to an ether-cleaving *O*-demethylase ability. Certain anaerobic bacteria have the ability to cleave the methyl group from the aromatic ring using cofactors like corrinoid and tetrahydrofolate (Gibson & Harwood, 2002). Acetogenic bacteria as well as the sulfate-reducing bacterium *Desulfitobacterium hafniense* DCB-2 possess this kind of corrinoid-dependent *O*-demethylase system (Studenik et al., 2012). If this is the case for *Desulfovibrio* as well it might explain both the results in the study of Ziomek & Williams (1989) and the finding of species in the genus in a number of anaerobic, biomass-degrading environments (J.-H. Kim et al., 2009; van der Lelie et al., 2012; Wu & He, 2013; Dai et al., 2015).

Another sulfate-reducing bacterium found to degrade lignin was identified as *Thauera* sp. and together with *Desulfovibrio* sp. it degraded 43% of the lignin in 19 days under optimal conditions (J.-H. Kim et al., 2009). However, the study did not give any indication to a mechanism. But it has been shown that the facultative anaerobe *Thauera aromatica* possess a system for aromatic degradation using a dearomatizing reductase (Boll, 2005), which can explain the degradation. The system is designated as an ATP-dependent benzoyl-CoA reductase which transfers two electrons from reduced ferredoxin to benzoyl-CoA, reducing it to cyclohex-1,5-diene-carbonyl-CoA. The process is described as a biological Birch reduction on the basis of aromatic ring reduction in a water-free environment. The reaction is facilitated by the CoA thioester-functionality which both stabilizes the radical intermediates by additional resonance structures and acts as an electron-withdrawing substituent (Fuchs et al., 2011). An analog ATP-independent system exists in several obligate anaerobes with different physiologies like Fe(III)-reducing *Geobacter*, fermenting *Syntrophus aciditrophicus* and different sulfate-reducing bacteria (Löffler et al., 2011). Both *Thauera* sp. and *Azoarcus* sp., another facultative anaerobe possessing an ATP-dependent benzoyl-CoA reductase Boll (2005), were found in a consortia degrading lignin (Wang et al., 2013), as was a species beloning to *Geobacter* (Wu & He, 2013). Species of *Magnetospirillum*, found in the community anaerobically degrading wood chips (van der Lelie et al., 2012), have homologs to the ATP-dependent system of *Thauera* which can explain its existence in this environment (Boll, 2005).

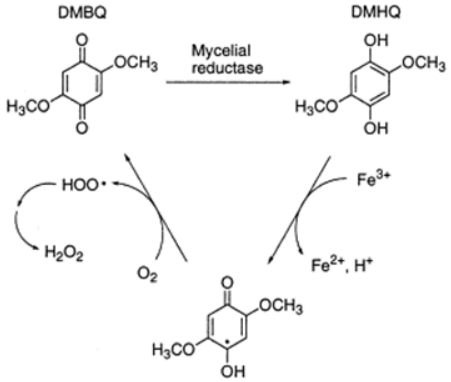


Figure 4: Hydroquinone redox cycle. Figure from Kerem et al. (1999).

The role of Fe(III)-reduction have been implicated in several lignin-degrading environments. The reason is its potential participation in the non-enzymatic Fenton reaction involving $OH^{\bullet}$ as oxidants. In *Gloeophyllum trabeum* the reaction is cyclic and driven by hydroquinone metabolites that acts as redox mediators, shuttling electrons to and from iron-complexes in different redox states (figure 4). A key aspect of this system is the extracellular generation of hydrogen peroxide from which the hydroxyl radicals are generated (Jensen, Jr. et al., 2001). The existence of both Fe(II) and hydrogen peroxide was noted in the termite gut (Ke & Chen, 2013), and Hall et al. (2015) speculates that fluctuating redox conditions might be the driving force in nonspecific lignin oxidation in soil. Both species of bacteria isolated by DeAngelis' group showed the ability to transfer electrons from the cell to lignin-derived quinones during anaerobic growth, possibly providing a mechanism for dissimilatory lignin degradation (DeAngelis et al., 2013; Woo et al., 2014).

3.5 Metagenomic expression libraries

While the advent of large-scale sequencing have made the finding of new genes much easier, the results are heavily dependent on the database used for comparison. Since the identification is based on homology it may preclude potentially interesting genes that lacks any similarity to previously identified genes, making the discovery of truly novel genes and enzymes a hard task. One way around this is an expression library

Table 2: Lignin degradation systems in fungi.
The reference in the first column is valid for the whole row unless anything else is stated.

System	Type organism	Mediator	E°, V
Lignin peroxidase (LiP)[1]	*P. chrysosporium*	Veratryl alcohol	+1,36 [2]
Manganese peroxidase (MnP)[1]	*P. chrysosporium*	Chelated Mn^{3+}	+0.55-0.65 [3]
Versatile peroxidase (VP)[1]	*Pleurotus eryngii*	VA & chel. Mn^{3+}	+0.55-1.36
Dye-decolorising peroxidase (DyP)[4]	*Bjerkandera adusta*	-	-
Laccase[5]	*T. versicolor*	-	+0.43-0.78
Fenton chemistry[6]	*Gloeophyllum trabeum*	$OH^{\bullet}$	+2.32 [7]

[1] Abdel-Hamid et al., 2013, [2] Khindaria et al., 1996, [3] López et al., 2007, [4] Linde et al., 2015, [5] Morozova et al., 2007, [6] Jensen, Jr. et al., 2001, [7] Wood, 1988.

which deals with activity rather than homology. The concept of metagenome and the treatment of all the genetic material in a sample as a single entity emerged in the late 90s. The first metagenomic expression library was made by ligating DNA extracted from soil samples to BAC vectors and using *E. coli* as the expression host. In this way, whole pathways could be obtained in a single clone (Handelsman et al., 1998). A number of different environments have since been probed in search for novel enzymes, using different expression systems (Coughlan et al., 2015). The first example of a metagenomic library obtained from rumen came from Ferrer et al. (2005) who discovered several different novel enzymes with a variety of lignocellulolytic activities. Using the same library, the group later found a novel laccase (Beloqui et al., 2006).

While Handelsman et al. (1998) used large-insert BAC vectors, the library of Ferrer et al. (2005) was created by using λ-bacteriophages with an average insert size of 5,5 kb. Since then three main vector systems have developed: plasmids (<15 kb), fosmids (<40 kb) and BAC (<200 kb). The systems have different merits depending on the goal of the screening, if it is single genes or complete pathways. The plasmids comes in a higher copy number than the other two which are usually kept as a single copy but can be induced to multiply within the host. The higher expression is advantageous when screening the library to be able to detect positive transformants. The downside to the smaller insert system is that the number of clones necessary for a good representation of the metagenome increases. All three systems can be used with *E. coli* as the host but the resulting library may not be complete as all genomes are not equally expressed leading to bias (Leis et al., 2013). An example of this are species belonging to the actinomycetes whose unrecognised promoters and high G+C-content makes it hard for *E. coli* to express the genes. To solve this, a sub-library can be made with a more suitable host, e.g. *Streptomyces* (Rajendhran & Gunasekaran, 2008).

In an activity-based plate screening, the activity of interest is sought by using a selective agent in the medium. In the search for enzymes active against lignocellulosic biomass it is common to add polysaccharides (cellulose, xylan, β-glucan) to the medium and stain the plates to find positive transformants degrading the added polysaccharide. The staining, e.g. with Congo red, creates a halo surrounding the colony of the positive transformant. Alternatively, dyed analogs to the polysaccharides can be used for easier recognition (Leis et al., 2013). In the search for lignolytic enzymes, the research have mostly focused on oxidative enzymes like peroxidases and laccases. These can be found using a number of compounds, such as syringaldazine and catechol, which changes colour upon oxidation (Pointing, 1999). A prerequisite of the plate assay is that the gene product and the substrate is located in the same place, either inside or outside of the cell. The intracellular expression in the host poses a problem in the cases when the membrane is impermeable to the substrate. One way to avoid these potential false negatives is to perform the screening after cell lysis, provided the lysis leaves the expressed proteins intact (Ekkers et al., 2012).

4 Method

4.1 DNA extraction

The content of the rumen of three sheep were removed at the time of slaughter and directly separated into solid and liquid fractions. The separation were made by filtering the content through three layers of cheese cloth. The liquid fraction was aliquoted into samples of 200 µl and the solid fraction was divided into 15 and 50 ml tubes before the samples were put on dry ice. Upon arrival to the laboratory the samples were transferred to -80°C and stored. The solid samples were cryomilled as per Roume et al. (2013) in liquid N_2 using a Retch MM400 with 35 ml steel cylinders and 16 mm steel balls. The cylinders and steel balls were pre-cooled with liquid N_2 prior to the transfer of the solid samples. The samples were homogenised by shaking for 1 minute at 30 Hz.

4.1.1 Method 1: Bead beating

The DNA from both solid and liquid fraction was extracted using Norgen All-in-One Purification Kit. 100 µl TE-buffer was added to both liquid (200µl) and solid sample (∼ 100 mg) which were mixed and thawed by vortexing for 10 s. 400 µl Buffer SK and 4 µl β-mercaptoethanol were added to the samples before transfer to 2 ml tubes with two 4 mm steel balls. The tubes were put in a pre-cooled (-20°C) tube adapter and then lysis were performed by shaking for 1 min at 30 Hz using a Retch MM400.

The lysed samples were moved to 1.5 ml tubes and centrifuged for 5 min, 10000 rpm, 4°C to pellet the cell debris. The supernatants were transferred to new 1.5 ml tubes and 100 µl 96% EtOH was added as per Roume et al. (2013). 5 s of vortex to mix and the samples were then loaded to the spin columns provided with the kit and the protocol was then followed with the exception of elution volume for DNA where only 50 µl was used. The DNA was eluted from the column twice into different tubes to maximize the yield. Following the extraction, the DNA was aliquoted and fragmented by pipetting 30, 40, 50 and 60 times up and down according to the manual from the CopyRight v2.0 Fosmid Cloning Kit (Lucigen) to obtain fragments of optimal size for fosmid cloning. The integrity of the DNA was checked by running it on a 0,7% agarose gel at 2 V/cm for 16 h.

4.1.2 Method 2: Enzymes and SDS

A milder extraction method based on Zhou et al. (1996) and K.-C. Ko et al. (2013) was employed to ensure the integrity of the DNA. An extraction buffer was made containing 2% CTAB, 20 mM EDTA, 1.4 M NaCl and 0.1 M Tris-HCl. 200 µl buffer was added to 200 µl of the liquid sample and 300 µl buffer to ∼100 mg solid sample. The samples went through a freeze-thaw-cycle three times (-20°C 30 min + 65°C 10 min). 100 µl lysozyme (100 mg/ml) was added to the samples which were incubated at 37°C, 225 rpm for 2.5 hour. 130 µl 10% SDS was added together with 50 µl proteinase K (10 mg/ml) and the

incubation continued for 30 min. The samples were then centrifuged for 10 min, 6000 rpm, 4°C and the supernatants were transferred to new 1.5 ml tubes. 1 vol. of isopropanol was added to precipitate the DNA and the samples were centrifuged again for 2 min, 6000 rpm, 4°C. The supernatants were poured of and the pellets were washed in 300 µl 70% EtOH by inversion followed by centrifugation for 2 min, 6000 rpm, 4°C. The supernatants were poured off and the pellets air dried before resuspension in 100 µl dH_2O.

A second purification was performed to remove impurities from the DNA. 300 µl chloroform:isoamyl alcohol (24:1) was added to the samples which were mixed by inversion and centrifuged for 2 min, 6000 rpm, 4°C. The top phase was transferred to a new 1.5 ml tube and 0.7 vol. of isopropanol was added. The content was mixed and the tube centrifuged again for 2 min, 6000 rpm, 4°C. The supernatant was poured off and the pellet washed in 300 µl 70% EtOH and left on the bench for 10 min. Centrifugation for 2 min, 6000 rpm, 4°C followed and the supernatant was poured off and the pellet was allowed to dry before resuspension in 100 µl dH_2O. Following the extraction, the DNA was aliquoted and fragmented by pipetting 30, 40, 50 and 60 times up and down according to the manual from the CopyRight v2.0 Fosmid Cloning Kit (Lucigen) to obtain fragments of optimal size for fosmid cloning. The integrity of the DNA was checked by running it on a 0,7% agarose gel at 2 V/cm for 16 h.

4.2 Ligation and phage transfection

Before the blunt end ligation was performed, the DNA was end-repaired to phosphorylate the ends. This was done by mixing 20 µl DNA, 10 µl 5X DNATerminator End Repair Buffer, 18 µl H_2O and 2 µl DNATerminator End Repair Enzyme and incubate at 37°C for 30 min at followed by inactivation at 70°C for 15 min. The end-repaired DNA was run on a 0.7% agarose gel at 2 V/cm for 16 h to separate the fragments. The fragments in the size range 30-70 kb was excised from the gel and extracted from the gel using QIAEX II Gel Extraction Kit (Qiagen).

A number of ligations were performed using the ligase included in the fosmid cloning kit (Lucigen) with varying incubation conditions (table 3). In addition to this, overnight ligations in 4°C with varying vector:insert-ratios were performed using a T4 ligase (New England Biotech) (table 4). To analyse the insert pattern, the ligation reactions were digested with the restriction enzyme *Bam*HI according to the conditions in table 5. The ligated fosmids were packed into the head of λ-phages using the Gigapack III Plus Packaging Extract (Agilent Technologies). 3 µl of the ligation reaction was added to one tube of phage packaging extract and left in room temperature for 2 hours after which 500 µl SM buffer (Lucigen) and 20 µl chloroform was added to stop the reaction. The reaction tube was centrifuged for 10 s to separate debris and then stored in the fridge until transfection of *E.coli*-cells.

To grow the *E. coli*-cells of the Replicator FOS strain (Lucigen) for transfection, cells were streaked from a glycerol stock on a YT-agar plate without antibiotics. The plate was incubated at 37°C overnight

Table 3: Ligations using the components included in the kit (Lucigen).

	Trials			
Components	1	2	3	4
CloneDirect 10X Ligation Buffer	1	1	1	1
pSMART FOS blunt vector (500 ng/µl)	1	1	1	1
PEG 6000 (50%)	0	0	0.6	0.6
DNA	1	2	2	3
dH_2O	6	5	4.4	3.4
CloneSmart DNA Ligase (2 U/µl)	1	1	1	1
Total volume (µl)	10	10	10	10
Conditions				
Incubation	22°C, 3 h	22°C, 2 h	4°C, o/n	4°C, o/n
Inactivation 70°C, 15 min	Yes	No	No	Yes

Table 4: Ligations using a T4 ligase and associated buffer (NEB).

	Vector:insert ratio		
Components	1:5	1:1	5:1
T4 Ligase Reaction Buffer (with ATP)	2	2	2
pSMART FOS blunt vector (50 ng/µl)	1.5	1.5	1.5
PEG 6000 (25%)	0.6	0.6	0.6
DNA	5	1	0.2
dH_2O	9.9	13.9	14.7
T4 DNA Ligase (400000 U/µl)	1	1	1
Total volume (µl)	20	20	20

and then stored in 4°C. A single colony was picked to 5 ml of LB-medium supplemented with maltose and $MgSO_4$ at final concentrations of 0.2% (w/v) resp. 10 mM. The culture was incubated overnight in 37°C at 225 rpm. 0.5 ml of the turbid overnight culture was used to inoculate 45 ml of maltose and $MgSO_4$-supplemented LB-medium. This culture was subsequently grown to an OD_{600} of 0.8-1.0 in 37°C at 225 rpm. The culture was then centrifuged for 10 min at 2000 rpm to pellet the cells, the supernatant was poured off and the pellet was resuspended in 15 ml fresh maltose and $MgSO_4$-supplemented LB-medium. 100 µl of the resuspended cells were transferred to a 15 ml tube and 10 µl of packaged λ-phages were added. The tube was incubated in 37°C, 225 rpm for 20 min before the cells were spread on YT-CXIS plates and incubated in 37°C.

Table 5: Conditions for treatment of ligated fosmids with restriction enzyme.

	Vector:insert ratio		
Components	1:5	1:1	5:1
Fast Digest 10X Buffer	2	2	2
Ligation reaction	0.5	2	5
dH_2O	16.5	15	12
*Bam*HI	1	1	1
Total volume (µl)	20	20	20

5 minutes of incubation in 37°C + 15 minutes in 80°C.

4.3 Bioinformatical searches

When reviewing the litterature, species belonging to *Desulfovibrio* and *Clostridium* were encountered in a number of environments exhibiting lignin degradation. Based on this, bioinformatical searches targeting these genera were conducted to find clues for their presence.

4.3.1 Benzoyl-CoA reductase system

The availability of free benzoyl-CoA is a necessity for the benzoyl-CoA reductase pathway as it serves as the initial substrate in the aromatic ring cleavage. Bacteria possessing the benzoyl-CoA reductase system also have the ability to produce the substrate. To find homologs to benzoyl-CoA forming enzymes, the protein sequence for the enzyme benzoyl-CoA ligase from *T. aromatica* (GenBank id AAN32623.1) was used as query in a BLASTp search at NCBI (ncbi.nlm.nih.gov). In addition to this, homologs to proteins in the actual pathway were also searched for. Due to the lack of sequences of both genes and proteins in the benzoyl-CoA reductase-pathway in the GenBank database, an *in-silico* PCR (Bikandi et al., 2004) was performed using the primers developed by Löffler et al. (2011) to find the coding sequence in the obligate anaerobe *G. metallireducens*. This sequence was subsequently used as query in a BLASTn search at NCBI (ncbi.nlm.nih.gov).

4.3.2 *O*-demethylase homologs

In the study of Ziomek & Williams (1989), evidence pointed to demethylation and ring cleavage. Both the genes and the translated proteins from the *O*-demethylase system from *D. hafniense* (Studenik et al., 2012) were used as query in nucleotide and protein-BLAST at NCBI.

5 Results

5.1 DNA extraction

Following the cryomilling in liquid N_2, two different methods were employed to extract DNA from a sample from the rumen of sheep. In the first method the sample was lysed through bead beating in buffer and the DNA was subsequently separated using the All-in-One Purification Kit (Norgen) following the instructions from Roume et al. (2013). This method resulted in a good concentration according to the NanoDrop 1000 (table 6) but showed a continuous smear when separated on an agarose gel (figure 5a). As the protocol was developed to obtain DNA as well as RNA and protein, RNAse was not included in any of the buffers which may have lead to RNA-contamination of the DNA fraction. The poor integrity is probably the result of the harsh lysis treatment.

To obtain DNA of larger size a second method was employed using milder lysis and extraction conditions (Zhou et al., 1996; K.-C. Ko et al., 2013). An extraction buffer was initally added to the sample which then went through 3 freeze-thaw cycles. After this the sample was incubated with lysozyme, SDS and proteinase K to release the DNA which then was separated from the debris through centrifugation. The supernatant containing the DNA was purified twice to remove impurites, first using isopropanol and then chloroform:isoamyl alcohol (24:1). The resulting DNA was less fragmented compared with the previous method (figure 5b) but still contained impuritied according to the 260/280 and 230/260-values from the NanoDrop 1000 (table 6).

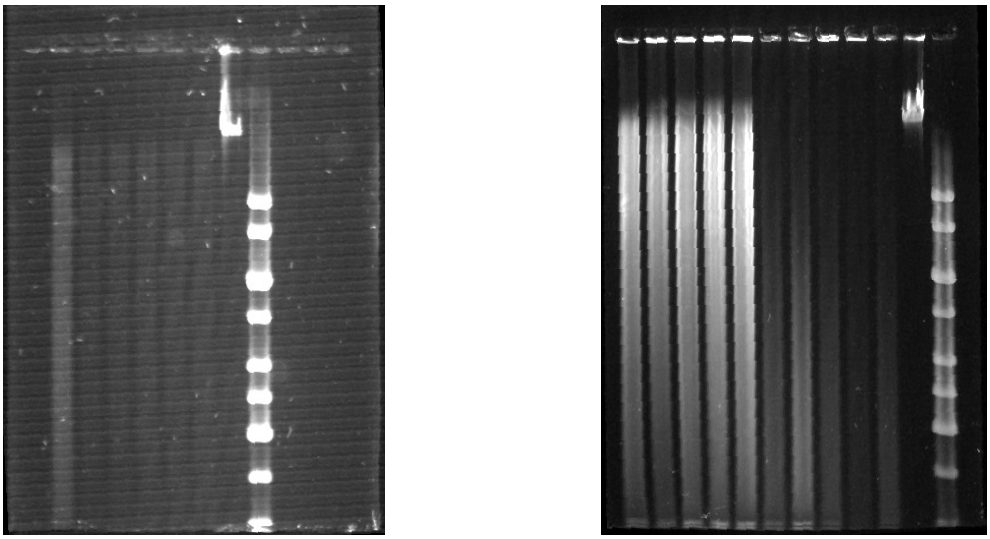
5.2 Ligation and phage transfection

As the extraction method using enzymes and SDS resulted in more intact DNA, this was used for subsequent ligations and transfections. To obtain fragments suitable for ligation to the fosmids, the DNA was first enzymatically end-repaired and size-selected through separation on an agarose gel. The concentration of the end-repaired DNA extracted from the gel was very low, around 5 ng/µl, which did not permit it to be used for ligations. Based on this, the agarose size separation-step was not used and end-repaired DNA of all sizes was used in the ligations to the fosmid vector.

However, using the CloneSmart DNA Ligase included in the fosmid cloning kit (Lucigen) did not result in any successful ligations. Upon treatment of the ligation reaction with the restriction enzyme *Bam*HI only the vector backbone was visible when separated on an agarose gel (figure 6a). As the vector contains

Table 6: Results of DNA extractions.

	Values		
Sample	Conc. (ng/µl)	260/280	230/260
Solid sample, method 1	595	2.18	1.30
Solid sample, method 2	550	1.77	1.30
Liquid sample, method 2	1060	1.32	0.51



(a) **(b)**

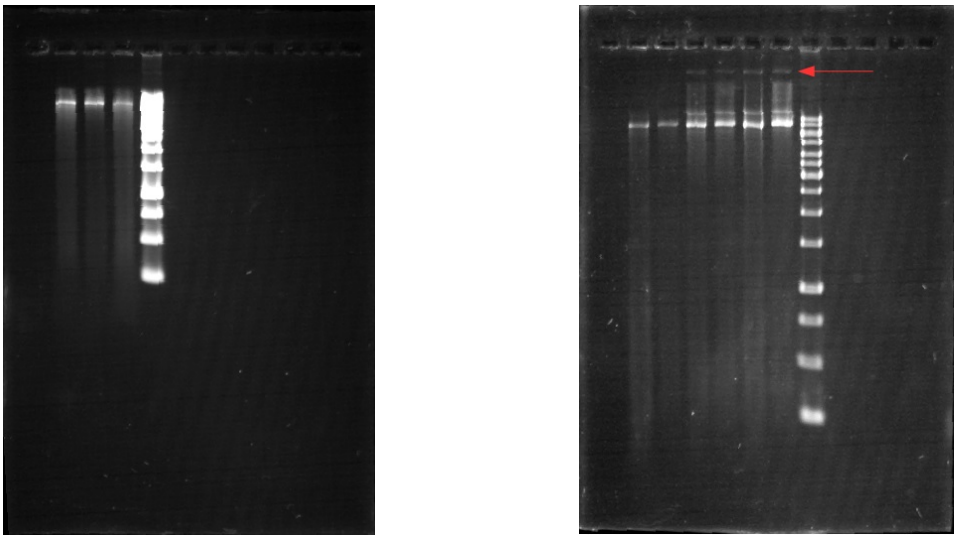
Figure 5: Pictures of the gels following the two DNA-extraction methods and the results of the fragmentation of the DNA by pipetting. The original sample was divided and pipetted 30, 40, 50 and 60 times up and down. **(a)** DNA extraction of solid sample using the bead beating method. Lane 1: empty, lane 2: unfragmented DNA, 1st elution, lane 3: unfragmented DNA, 2nd elution, lane 4: 2nd elution 30x, lane 5: 2nd elution 40x, lane 6: 2nd elution 50x, lane 7: 2nd elution 60x, lane 8: λ-bacteriophage DNA (48,5kb), lane 9: Thermo Scientific GeneRuler 1kb DNA Ladder. **(b)** DNA extraction of samples using lysozyme, proteinase K and SDS. Lane 1: Unfragmented DNA, solid sample, lane 2: 30x, lane 3: 40x, lane 4: 50x, lane 5: 60x, lane 6: unfragmented DNA, liquid sample, lane 7: 30x, lane 8: 40x, lane 9: 50x, lane 10: 60x, lane 11: λ-bacteriophage DNA (48,5kb), lane 12: Thermo Scientific GeneRuler 1kb DNA Ladder.

cutting sites for *Bam*HI on both sides of the insert, a successful ligation should after cleavage result in at least two bands on the gel, the backbone and the insert. A second attempt was performed using a high-activity T4 ligase (NEB) with different vector:insert-ratios which resulted in successful ligations. The different patterns and intensities between samples treated and untreated with *Bam*HI reveals that ligation took place with at least some efficiency (figure 6b). An increase in the amount of insert compared to vector appeared to result in a higher number of ligation events. Both the vector:insert-ratios 1:1 and 1:5 resulted in a single large fragment (red arrow in figure 6b) as well as a range of fragments >10 kb.

The transfections using the ligations from trial 1-4 did not result in any recombinant cells which, looking at figure 6a, is not surprising.

5.3 Bioinformatical searches

The collected findings in the reviewed literature pointed to two main activities associated with lignin degradation, aromatic ring cleavage and the removal of the etherified methyl groups. The search for enzymes related to these activities was limited to the genera *Desulfovibrio* and *Clostridium*. This was due to the fact that these taxa were repeatedly implicated as having a part in the lignin degradation, either alone (Ziomek & Williams, 1989) or as a part of a consortia (J.-H. Kim et al., 2009; van der Lelie et al., 2012; Wu & He, 2013; Dai et al., 2015).



(a) (b)

Figure 6: Analysis of ligations, treated with *Bam*HI.
(a) Ligations using CloneSmart DNA Ligase. Lane 1: empty, lane 2: trial 2, lane 3: trial 3, lane 4: trial 4, lane 5: marker. **(b)** Ligations with different vector:insert-ratios using T4 ligase. Lane 1: empty, lane 2: 5:1 untreated, lane 3: 5:1 treated, lane 4: 1:1 untreated, lane 5: 1:1 treated, lane 6: 1:5 untreated, lane 7: 1:5 treated, lane 8: marker

Desulfovibrio To find enzymes producing benzoyl-CoA, the initial substrate of the benzoyl-CoA reductase-pathway, the protein sequence for benzoyl-CoA ligase from *T. aromatica* was used as query in a BLASTp search at NCBI (ncbi.nlm.nih.gov). Many hits annotated as CoA ligases returned when searching within the genus *Desulfovibrio*. However, only the species *D. magneticus* and *D.* sp. X2 contained homologs annotated as benzoyl-CoA ligases which indicates that if homologs exists in *Desulfovibrio* they are not widespread. No homologs to genes coding for the benzoyl-CoA reductase pathway were found and the activity of *Desulfovibrio* towards lignin can thus not be explained by a class II benzoyl-CoA reductase homolog.

To find possible explanations to the demethylation of Kraft lignin seen by Ziomek & Williams (1989), searches for homologs to the *O*-demethylase system from *D. hafniense* were performed. The nBLAST did not return any relevant hits as the e-value was too high (>0.5) in all cases. The pBLAST search found a number of potential homologs to the ferredoxins relevant to the system but not any relevant hits to the three subunits of the enzyme system. Only two very weak hits were returned when methyltransferase I, the subunit responsible for the substrate specificity (Kreher et al., 2010), was used as query which points to the absence of an *O*-demethylase system in species belonging to *Desulfovibrio*.

Clostridia When searching for homologs to the benzoyl-CoA ligase from *T. aromatica* both in the genus *Clostridium* and the larger phylum *Firmicutes*, very few hits with a similar annotation were returned. The majority of hits were instead identified as 4-hydroxybenzoate-CoA ligase, indicating an alternative pathway in *Firmicutes* for benzoyl-CoA production and subsequent aromatic degradation.

Table 7: Homologs to *D. hafniense* *O*-demethyltransferase system found through BLAST

	Protein		
Species	Methyltransferase I	Corrinoid protein	Methyltransferase II
C. ljungdahlii	ADK13822	ADK13821	ADK13825
	ADK13827	ADK13826	ADK13829
C. autoethanogenum	AGY77052	AGY77051	AGY77050
	AGY77057	AGY77056	AGY77055
C. carboxidivorans	EET84205	EET84206	EET84207
C. scatologenes	AKA72173	AKA72174	AKA72175
C. drakei	WP_032075386	WP_032075387	WP_032075388

The nBLAST search for homologs to class II benzoyl-CoA reductases resulted in a number of hits, most however from uncultured bacteria from environmental samples. Except from the strains beloning to *Firmicutes* from the study of Löffler et al. (2011), the most well defined strain containing a homolog was the Clostridia bacterium enrichment culture clone BF. The hit was identified as a putative aldehyde ferredoxin oxidoreductase in locus GU357914.

Shifting focus to the *O*-demethylase system from *D. hafniense* resulted in a number of relevant hits. The large majority of the species belonged to the taxonomic subgroup *Clostridiales*, including five species *Clostridium* containing homologs to all three subunits (table 7). The gene numbering points to a clustering of the genes, possibly indicating an operon. This finding motivates the occurrence of the genus in environments experiencing lignin degradation since the methyl groups offers an energy source for the bacteria. And as the methyl groups are replaced by hydrogen the solubility increases and the steric hindrance decreases, enabling further degradative actions by other microorganisms.

6 Discussion

Biorefinery designs and degradation mechanisms Based on the general picture offered by the literature reviewed in this study, there seems to be two main options when considering lignin degradation in an industrial setting. These options affects the make up of the biorefinery and are therefore central to the overall design. The first option is to aim for a widespread degradation of the polymer as a whole without targeting any specific bonds. The other option is to target the bonds between the lignin and the polysaccharides which would liberate the two fractions for further individual processing. While the local cleavage of linkages was seen in the degradation of wood chips and in the gut of the termite (van der Lelie et al., 2012; Ke & Chen, 2013), the global degradation strategy is represented by the fungal systems and their homologs (table 2). If the aim is to process the polysaccharides as well as the lignin fraction, then the strategy involving local cleavage seems more suitable. To reach an efficient process using this strategy, one of the key aspects is to identify and target the different types of ether and ester-linkages that cross-links the lignin and the polysaccharides (Iiyama et al., 1994).

If the aim simply is to gain access to the polysaccharides at the expense of the lignin, the literature shows two different fragmenting mechanisms, either targeted at the side chain or at the aromatic ring. While the former principally is a nucleophilic process, the latter is electrophilic (Gierer, 1985). All of the systems in table 2 are of the electrophilic type while the nucleophilic type is represented by the GST-dependent β-etherase system of *Sphingomonas*. The β-etherase system does not have any requirements related to oxygen but shows on the other hand very poor performance against polymeric lignin (Picart et al., 2014). The perhaps most promising alternative are the different laccases found in environments exhibiting low oxygen concentration (Beloqui et al., 2006; Youssef et al., 2011; Fang et al., 2012; van der Lelie et al., 2012). These enzymes may be adapted to the conditions, perhaps by a more relaxed need for electron acceptors and not being limited to oxygen. If coupled to a diffusible mediator the efficiency can be increased by both a wider substrate range as well as better penetration into the polymer (Morozova et al., 2007).

Another field to investigate further is the role of sulfate reducing bacteria in lignin degradation. While the role of *Clostridium* partly can be understood by the finding of homologs to an *O*-demethylase system, species of *Desulfovibrio* did not contain any homologs to the two systems surveyed. One possible explanation to its presence in a number of studies cited in this work can be found in its physiology. Sulfate reducing bacteria uses SO_4^{2-} and produces the reduced sulfur compound H_2S (Hogg, 2005) which, under physiological condition, mostly is dissociated into the HS^- ion (Pietri et al., 2011). The HS^- ion can act as a nucleophile and add to sites of partial positive charge, which is the case in the Kraft pulping process (Gierer, 1985). It was observed that the lignin-degrading ability of *D. desulfuricans* was limited to the growth phase and that the activity was extracellular (Ziomek & Williams, 1989). As the HS^- ion only is produced as long as the bacterium have SO_4^{2-} available for consumption (J.-H. Kim et al., 2009),

and therefore growth, it would explain the growth-aspect on the lignin degradation. This hypothesis represents a true biological equivalent of the chemical pulping which, if included in an industrial context, would be dependent on SO_4^{2-} as well as ensuring continuous growth of the bacteria.

Organisms, their environments and how to obtain them It would seem that the number of feasible strategies for lignin degradation are narrowed down to two, the established laccase-mediator system and the hypothesis presented here involving sulfate reducing bacteria. Both of these methods are applicable to the conditions described by Kirk & Farrell (1987). The strategies requires different conditions which affects the sampling and screening procedure when looking for new species amenable for integration in an industrial context. To find new laccases, functional screenings using different substrates of varying redox potentials under different oxygen conditions can be used. An alternative method is to use electrodes to measure the direct electron transfer rate of the enzymes (Abdellaoui et al., 2013). The electrodes can cover a wide potential range, enabling functional screenings irrespective of the structure of the substrates. The enzymes can either be found by the creation of metagenomic libraries or by direct culturing of samples and using the crude supernatant.

If the library route is taken it is of highest importance that the DNA-extraction is as mild as possible. The extraction methods in this work were too damaging to the DNA which created fragments in a variety of sizes, most of them too small for optimal fosmid-cloning. If the attempts at the creation of a library would have been successful, the library would probably not have been representative and thus missing many potentially interesting genes. A good extraction procedure is therefore important as it affects a number of subsequent steps, all contributing to a reduction of the representability. An easier way would have been to make a plasmid-based library instead of an fosmid-based. The length of the inserts can be much smaller which makes the extraction process less sensitive, increasing the chance for recombinant clones down the line. The downside is that more colonies needs to be screened to reach the same coverage (Leis et al., 2013). The sequencing alternative would also be a viable route to take based on the large database available (Levasseur et al., 2013).

While organisms harbouring suitable laccases seems to be widespread in terms of environments, the sulfate reducing bacteria have a stricter niche. They require sulfate for growth and a lack of oxygen which competes as electron acceptor in the respiratory chain (Hogg, 2005). The screening for candidate strains of sulfate reducing bacteria would ideally be based on both the ability to produce H_2S and a thermophilic physiology. A high temperature would not only increase the reactivity of the HS^- ion against lignin (Gierer, 1985), it would also exclude contaminating organisms due to the harsh environment. Based on this, species isolated from hydrothermal vents may be a good starting point as they are adapted to both high temperatures as well as high pressure (Frank et al., 2013), another important pulping-aspect (Gierer, 1985).

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