Lignocellulose-degrading microbial consortia
Cortes Tolalpa, Larisa

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Chapter 1

General introduction

Larisa Cortés Tolalpa
Lignocellulose biomass composition

During the past century, world energy consumption has mostly depended on the utilization of fossil fuels, which has provoked negative changes in our climate and increased the emission of greenhouse gases in the atmosphere. An important emerging trend in the 21th century is the switch from non-renewable fossil resources to renewable ones for the production of biofuels and other valuable compounds. The use of lignocellulose materials has emerged as an attractive and sustainable source of carbon for this. Recycling of carbonaceous materials is also important considering the actual scarcity of arable land. Khoo et al (2016) indeed support the use of lignocellulosic biomass (LCB), for example for the production of biofuels, as it can reduce the dependency on fossil fuels and contribute to climate change mitigation.

LCB is the most abundant source of reduced carbon on earth, being wheat (*Triticum aestivum*) the major LCB source. Wheat is a major food crop in the world, next to rice and maize. Around 75% of the total agricultural residues are derived from these three crops at global level (Xie and Peng 2011). Other lignocellulose residues include maize, sugar cane bagasse, switch grass, decaying wood and miscanthus, next to most of the waste produced by the food industry, see Table 1 (Dyk and Pletschke 2012; Dyk et al. 2013).

<table>
<thead>
<tr>
<th>Sourceset</th>
<th>Classification</th>
<th>Type</th>
<th>Cellulose (%)</th>
<th>Hemicellulose (%)</th>
<th>Lignin (%)</th>
<th>Ash (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>Crop residues</strong></td>
<td><strong>Agriculture</strong></td>
<td>Wheat straw</td>
<td>37–41</td>
<td>27–32</td>
<td>13–15</td>
<td>11-14</td>
</tr>
<tr>
<td></td>
<td></td>
<td>Maize</td>
<td>38-40</td>
<td>6.1-28</td>
<td>7-21</td>
<td>3.6.7</td>
</tr>
<tr>
<td></td>
<td></td>
<td>Rice straw</td>
<td>28-36</td>
<td>23-28</td>
<td>12-14</td>
<td>14-20</td>
</tr>
<tr>
<td></td>
<td></td>
<td>Barley straw</td>
<td>31-45</td>
<td>5-20</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td></td>
<td></td>
<td>Cotton stalk</td>
<td>80–95</td>
<td>27-38</td>
<td>14-19</td>
<td>2-7</td>
</tr>
<tr>
<td></td>
<td></td>
<td>Sugarcane bagasse</td>
<td>32-48</td>
<td>19-24</td>
<td>23-32</td>
<td>1.5-5</td>
</tr>
<tr>
<td></td>
<td></td>
<td>Sorghum Straw</td>
<td>32-35</td>
<td>24-27</td>
<td>15-21</td>
<td>-</td>
</tr>
<tr>
<td></td>
<td></td>
<td>Switchgrass</td>
<td>31-35</td>
<td>24-28</td>
<td>17-23</td>
<td>-</td>
</tr>
<tr>
<td></td>
<td><strong>Herbaceous energy crops</strong></td>
<td><em>Miscanthus giganteus</em></td>
<td>37-45</td>
<td>19-25</td>
<td>17-21</td>
<td>1-3</td>
</tr>
<tr>
<td></td>
<td></td>
<td>Grasses</td>
<td>25-40</td>
<td>25-30</td>
<td>15-20</td>
<td>-</td>
</tr>
<tr>
<td><strong>Industry</strong></td>
<td><strong>Forest</strong></td>
<td>Poplar (hardwood)</td>
<td>4-55</td>
<td>24-40</td>
<td>18-25</td>
<td>1-4</td>
</tr>
<tr>
<td></td>
<td></td>
<td>Pine (softwood)</td>
<td>25-42</td>
<td>21-30</td>
<td>18-26</td>
<td>0.3-2</td>
</tr>
<tr>
<td><strong>City</strong></td>
<td><strong>Food waste</strong></td>
<td>Nut shell</td>
<td>25-30</td>
<td>25-30</td>
<td>30-40</td>
<td>-</td>
</tr>
</tbody>
</table>

Information adapted from the following references (Wei et al. 2009; Kumar et al. 2015; Ravindran and Jaiswal 2016; Cai et al. 2017)
Generally speaking, lignocellulose biomass consists of about 30-44% cellulose, 23-50% hemicellulose and 7.7-15% lignin (Figure 1). The remaining fraction in the LCB is made of pectin, proteins, ash, salt minerals and silica Table 1 (Ravindran and Jaiswal 2016; Cai et al. 2017). Below I discuss each of them separately.

**Cellulose.** Cellulose consists of linear chains of glucose molecules linked by β-1-4 glycosidic bonds. There are two types of cellulose structures, i.e. amorphous and crystalline. The amorphous form is soluble and is easily digested by enzymes, while the crystalline structure is formed by cellulose chains that are strongly linked by hydrogen bonds, forming microfibrils. This type of cellulose is very recalcitrant to degradation and solubilization (Figure 1) (Ravindran and Jaiswal 2016; Cai et al. 2017).

**Hemicellulose.** Hemicellulose has a more variable configuration than cellulose. It is a heterogeneous polymer that is composed of short polysaccharide chains, such as xylan, mannan, galactan and arabinan. In these, the monomers are mainly five monosaccharides: D-xylose, L-arabinose (pentoses), D-galactose, D-mannose and D-glucose (hexoses). Xylan, the most abundant molecule, is formed by β-D-xylopyranosyl residues linked by β-1,4-glycosidic bonds. Its most abundant form is heteroxylan, which is comprised of xylose residues in the backbone, also carrying acetate, arabinose and glucose residues (Dyk and Pletschke 2012).

*Figure 1. Schematic representation of lignocellulose distribution in plant material and composition.*
**Lignin.** Lignin is an aromatic polymer formed by lignols within a three-dimensional structure. Its chemical arrangement provides the rigid structure of plants. The three lignol monomers are hydroxyphenyl alcohol, coniferyl alcohol and synapyl alcohol (Ravindran and Jaiswal 2016; Khoo et al. 2016).

In the LCB, hemicelluloses and cellulose are linked by hydrogen bonds, whereas both moieties are linked to lignin by covalent bonds (Ravindran and Jaiswal 2016; Khoo, Ee, and Isoni 2016), forming a highly complex and heterogeneous structure.

**Benefits of lignocellulose degradation and factors affecting its bioconversion**

It has been estimated that, globally, LCB in agricultural waste amounts to about \(1.5 \times 10^{11}\) ton per year (Guerriero et al. 2016). The utilization of this biomass for production purposes can be considered as an environmentally-friendly process that can mitigate greenhouse gas emission. In the past, studies have mainly focused on the conversion of cellulose to simpler monomers for subsequent transformation to, for instance, ethanol. There are increasing research efforts focused on the bioconversion of hemicellulose, which means the production and subsequent utilization of pentose and hexose molecules (Gírio et al. 2010; Ji et al. 2011). Overall, the production of diverse commodities by industrial applications of compounds derived from the hemicellulose, cellulose and also lignin parts of lignocellulose biomass is widely expanding (Figure 2) (Guerriero et al. 2016).

In spite of the great promise of the utilization of LCB in waste, it is still necessary to develop methods to overcome its inherent recalcitrant nature. Lignocellulose materials, from an evolutionary point of view, have evolved to a complex chemical structure in order to resist microbial degradation or animal assault. At the molecular level, the recalcitrance of LCB is related to the following: The lignin content, the degree of crystallinity of cellulose, the polymerisation degree of the polysaccharides and the available surface area of the biomass (d’Errico et al. 2015; Sun et al. 2016). Recently, it was found that the degree of ester linkage between the lignin and the carbohydrate moieties of the LCB also influence its degradability (Rabemanolontsoa and Saka 2016). The methods used to reduce the recalcitrant nature of LCB can be divided in pre-treatments or enzymatic hydrolysates.
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Figure 2. Products that can be derived from the main components of lignocellulose substrate, like sugar cane, wheat straw and maize. Products from the main component (blue arrows) and those obtained from the biotransformation of the monomers (orange arrows). Cellulose can be transformed into pharmaceutical, cosmetic, printing ink and paper products. Xylan can serve as a source for plastic additives, strengthening agents in paper and textile printings. Lignin can be used as a cement additive, and in the production of resins and flocculants. The monomer of cellulose, glucose, can be transformed into biofuel, whereas xylose, from hemicellulose, can produce furfural (a polymer precursor or solvent), xylitol or ethanol. Monomers of lignin such as humic acids are used in agriculture; monolignols are used as precursors of cresol, catechols, resorcinols, quinones, vanillin and guaiacols. Figure made based on Guerriero et al. (2016).
Pretreatments

Usually, LCB is not easily accessible for enzymes or microorganisms. Thus, the biomass needs to be mechanically, biologically or physicochemically pretreated to make the cellulose and hemicellulose moieties accessible for efficient enzymatic depolymerisation (Talebnia et al. 2010). Ideally, an effective pretreatment process should adhere to five criteria: 1) Increasing sugar yield and minimizing sugar loss; 2) Maximize exposure contact surface by reducing level of intertwining and particle size, 3) Maximize conversion rate by increasing hydrolysis rate; 4) Reduce the production of inhibitory compounds that affect the downstream process, and finally 5) Reduce cost by reducing energy consumption. Figure 3 shows the classification of selected major pretreatment strategies.

**Physical pretreatments** are aimed at increasing the accessible surface area of LCB by reduction of the particle size and pores and disrupting the regular structure. It includes chipping, grinding and milling. Alternatively, possible physical pretreatment are pyrolysis, gamma radiation, microwave treatment, infrared heating and sonication (Kumar et al. 2015).

**Chemical pretreatments** involve the use of diluted acid or alkali, oxidation agents and organic solvents and ionic liquids (ILs). The most commonly applied methods are acid and alkaline hydrolysis. Acid hydrolysis is applied on raw LCB to improve downstream enzymatic hydrolysis, while alkaline treatment increases
the digestibility of the polysaccharides and facilitates enzymatic attack, as it increases porosity and incites an enhanced surface area. In both cases the main disadvantage is the formation of inhibitory compounds, next to high costs of waste disposal and a large environmental footprint (Sun et al. 2016) (Table 2).

**Physicochemical processes** use a combination of pretreatments such as hydrothermal treatment, under which liquid hot water (LHW) and steam explosion (SE), with chemical treatment, e.g. ammonia fiber explosion (AFEX) (Sun et al. 2016). **Steam explosion** is one of the most expensive pretreatments, nevertheless, it is one of the most effective and it is especially applied on lignocellulose waste materials. This method uses high-pressure saturated steam at between 0.69 and 4.83 MPa with temperatures of 160 - 260°C for several seconds to a few minutes (Jönsson and Martin 2016). **Ammonia fiber explosion (AFEX)** is an alkaline thermal pre-treatment, where the lignocellulose biomass is exposed to liquid ammonia at high temperature and pressure for a short period, which is followed by a rapid pressure release.

**Biological pretreatments** are environmentally friendly and economical alternatives for the disruption of the lignocellulose complex matrix. Differently from physical and chemical pretreatments, biological pretreatments are done under mild conditions of pressure and temperature. Various organisms have been used in biological pretreatments, mainly white rot fungi, that attack both cellulose and lignin, next to brown rot fungi that attack only cellulose (Sindhu and Pandey 2016). Two examples illustrate the effect of this type of pretreatments over the plant biomass. In one case a fungal consortia was used on raw maize, the result was the reduction of the 43% of the lignin content, thus increased the hydrolysis rate in seven folds (Song et al. 2013). While, in other example, *Punctualaria* sp. TUFC20056 was applied on bamboo culms achieving the reduction of lignin in 50% (Suhara et al. 2012). Because no chemicals are used, there is no need for recycling or recovering chemicals, there is no release of toxic compounds to the environment and energy is saved. On the other hand, biological pretreatments have a very low rate of hydrolysis resulting in a time-consuming process (Vasco-Correa et al. 2016).

At the present time, pretreatments are mandatory for utilization of lignocellulose biomass, but the main disadvantages are the increase in the production cost, as well as the generation of toxic compounds that not only interfere with downstream bioprocess, but also negatively affect the environment (Sun et al. 2016).
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In Table 2 I show a review of different types of pretreatments, as well as advantages and disadvantages of each. Nowadays, the development and application of effective pretreatment to lignocellulose biomass is clearly urgent. Thus, efforts should go into developing an efficient method that breaks up the substrate and does not inhibit microbial activity as well as enzymatic degradation of the substrate. Such a method may be substrate- and even climate-specific.

**Enzymatic hydrolysis**

The hydrolysis of lignocellulose, because of its complexity, requires numerous enzymes with different specificities working in a synergistic manner. The variation in structure between substrates from different sources and the effect of different types of pretreatments further increase the complexity of developing a standard method. In Figures 4-6 I show an overview of the types of enzymes that are required to degrade complex lignocellulose substrates. In a generic sense, microorganisms produce two types of enzyme systems for lignocellulose degradation: (1) freely released enzyme systems, which are mostly produced by many aerobic bacteria and fungi, and (2) multi-enzyme complexes named cellulosomes, which are mostly found in anaerobic bacteria such as *Clostridium* (Dyk and Pletschke 2012; Bayer et al. 2013)

**Cellulose degradation**

To degrade cellulose, three major groups of enzymes are required to work synergistically: endoglucanases, exoglucanases (cellbiohydrolases) and β-glucosidases (Figure 4). Endoglucanases (endo-β-(1,4)-glucan hydrolases) are characterized by their hydrolysis of internal β-(1,4)-glucosidic linkages. These enzymes attack low-crystallinity regions of the cellulose fibers. Exoglucanases (exo-β-(1-4)-glucanases) remove the cellobiose units from the free chain ends. They have a preference for attacking longer chain substrates than β-glucosidases. In addition, β-glucosidases hydrolyse cellobioses and other short-chain β-1,4-oligosaccharides, realising glucose monomers. Most β-glucosidases are active on a range of β-dimers of glucose (Kumar et al. 2008; Gupta and Verma 2015).

**Hemicellulose degradation**

Due to its more complicated composition compared to cellulose, hemicellulose requires a larger number of different enzymes to be hydrolyzed effectively. Enzymes involved in the degradation of hemicellulose can be divided into depolymerising enzymes (cleaving the backbone) and those that remove substituents (Dyk and Pletschke 2012).
<table>
<thead>
<tr>
<th>Type</th>
<th>Pretreatment</th>
<th>Advantage</th>
<th>Disadvantage</th>
<th>By-product formation</th>
</tr>
</thead>
<tbody>
<tr>
<td>I. Physical</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Mechanical size reduction</td>
<td>• Chipping (10-30 mm)</td>
<td>• Increase accessible surface area</td>
<td>• High energy consumption, problems at industrial scale</td>
<td>• No inhibitor formation</td>
</tr>
<tr>
<td></td>
<td>• Grinding</td>
<td>• Reduce crystallinity</td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td>• Milling (0.2-2mm)</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td>• Gamma irradiation by gamma</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>II. Chemical</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Acid hydrolysis</td>
<td>Diluted acid: H₂SO₄, HCl, H₃PO₄, and HNO₃</td>
<td>• Reduction hemicellulose content</td>
<td>• Problems for recovery the chemical</td>
<td>• Aliphatic carboxylic acids</td>
</tr>
<tr>
<td></td>
<td></td>
<td>• Hydrolysis of hemicellulose to monosaccharides</td>
<td></td>
<td>Furans phenolic compounds</td>
</tr>
<tr>
<td>Alkaline</td>
<td>• Diluted NaOH (complemented H₂O₂)</td>
<td>• Reduces lignin content</td>
<td>• Pollution</td>
<td>• Acetic acid, dicarboxylic acid phenolic compounds</td>
</tr>
<tr>
<td></td>
<td>• Ca(OH)₂</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Organic solvent</td>
<td>• Methanol, ethanol, acetone, ethylene glycol and their mixture</td>
<td>• Almost total hydrolysis of hemicellulose</td>
<td>• High price of organic solvents</td>
<td>• Specific to the solvent used</td>
</tr>
<tr>
<td></td>
<td></td>
<td>• Separation and recovery of high quality lignin</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Ionic liquids</td>
<td>• Organic solvent, liquid at room temperature</td>
<td>• Reduce crystallinity efficiently</td>
<td>• High cost ILs</td>
<td>• Inhibition of enzymes by high salt concentration</td>
</tr>
<tr>
<td>III. Physico-chemical</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Steam explosion</td>
<td>• Hot water</td>
<td>• Solubilize hemicellulose</td>
<td>• Acetic acid</td>
<td></td>
</tr>
<tr>
<td></td>
<td>• High pressure</td>
<td>• Affect lignin structure</td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td></td>
<td>• Cost effective</td>
<td>• High equipment cost</td>
<td></td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td>• Minor amount of furans</td>
<td></td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td>• Aldehydes</td>
<td></td>
</tr>
<tr>
<td>Ammonia fiber explosion (AFEX)</td>
<td>• Liquid ammonia (NH₄OH)</td>
<td>• Cellulose swell</td>
<td>• High equipment cost</td>
<td>• No inhibitor formation</td>
</tr>
<tr>
<td></td>
<td>• High pressure and temperature</td>
<td>• Increase accessible surface area</td>
<td>• Ammonia cost</td>
<td></td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>IV. Biological</td>
<td>• Brown, white and soft fungi</td>
<td>• Degrade lignin and hemicellulose</td>
<td>• Long incubation period</td>
<td>• Possible consumption of carbon source</td>
</tr>
</tbody>
</table>

Table 2. Physical, chemical and biological pretreatments used on lignocellulose biomass.

With information from the following references: Talebnia et al. (2010); Kumar et al. (2015); Ravindran and Jaiswal (2016); Sun et al. (2016); Jönsson and Martin (2016).
Xylan is the most abundant component in the hemicellulose part of wheat straw. For its degradation it is necessary the action of endo-xylanases (endo-1,4-β-xylanases), which cleave the xylan backbone into shorter oligosaccharides is required, followed by the activity of β-xylosidase, which cleaves short xylo-oligosaccharides into xylose. For the degradation of glucomannan, the action of endo-mannanase and β-mannosidase is needed. Generally, xylans and mannans have several different substituents linked to the main backbone, such as arabinose, acetyl, galactose and glucose groups. Some of the key enzymes involved in hemicellulose degradation are α-L-arabinofuranosidases, α-glucuronidases, α-galactosidases, feruroyl esterases, acetyl xylanesterases and acetylmannan esterases (Gupta and Verma 2015) (Figure 5).

Enzymes with hemicellulolytic activity are generally classified into glycosyl hydrolase (GH) families GH2, GH10, GH11, GH16, GH26, GH30, GH31, GH39, GH42 and GH43. Among these families, family GH43 is particularly interesting. This is one of the largest families of GHS in the carbohydrate active enzymes (CAZy) database, having 4555 members. The family comprises a range of debranching enzymes that may act in the degradation of hemicellulose, particularly arabinoxylans. Moreover, an action on pectin is supposed. Family GH43 has emerged as an important CAZy enzyme family for biodegradation of complex substrates such as biopolymers.

Evidence for the importance of GH43 family comes from genomics studies of
LCB degrading microorganisms (Bayer et al. 2013) as well as the human gut microbiome (El et al. 2013), where enzymes of the GH43 family were identified as one of the most abundant CAZy enzymes present. In addition, in a metagenome study of a soil-derived degrader consortia bred with wheat straw showed a significant enriched of the families GH43, next to GH2, GH92 and GH95 (Jiménez et al. 2015a). Moreover, a proteomic study has shown that GH43 family proteins were synthesized exclusively when the degrader bacteria grew on wheat straw, but not on cellulose or other less complex carbon sources (López-Mondéjar et al. 2016). In the same line, a metasecretome analysis, from three different microbial consortia fed with wheat straw, xylose and xylan, revealed that only the wheat straw selected-consortia synthesized glycosyl hydrolases belonging to family GH43 (Jiménez et al. 2015b). This was revealing, as it pointed to a unique role of this CAZy family in the degradation of such type of complex polysaccharides and specifically related with the decomposition of hemicellulose. In the light of these results, the study of GH43 has recently become fundamental in our understanding of lignocellulose degradation context.

Figure 5 Hemicellulose degradation. Molecular structure of hemicellulose and sites of action of the most common endoglucanase, endo-β-xylanase, α-glucuronidase, β-xylosidases and α-arabinofuranosides. Other enzymes needed in degradation of hemicellulose are acetyl xylan esterase, endo-mannanase, β-mannosidase, ferulic acid esterase, α-galactosidase and p-coumaric acid esterase.
Lignin degradation

The degradation of lignin is catalysed by two main classes of enzymes: Peroxidases (lignin and manganese) and laccases. Working together, these enzymes lead to the complete degradation of lignin. Lignin peroxidase is a heme-containing glycoprotein, which requires hydrogen peroxide as the oxidant. This enzyme degrades non-phenolic lignin units (Figure 6).

Manganese peroxidases act on phenolic and non-phenolic lignin units through lipid peroxidation reactions. They oxidize Mn$^{2+}$ to Mn$^{3+}$ which then oxidizes phenol rings to phenoxy radicals, leading to decomposition of the compound. On the other hand, laccases catalyse the oxidation of phenolic units, phenolic compounds and aromatic amines to radicals. The lytic activity of laccases can be increased by the addition of 3-hydroxyanthranilic acid, 2,2 P-azino-bis (3-ethylthiazoline-6-sulfonate) which will act as a redox mediator (Sindhu and Pandey 2016).

Accessory enzymes

There are indications that many other enzymes contribute to lignocellulose degradation in ways that are not yet clearly understood. These so-called accessory enzymes act on less abundant linkages found in lignocellulosic biomass and include carbon binding modules (CBM), arabinases, lyases, pectinases, galactanases, several types of esterases and polysaccharide monooxygenases (PMOs) (Dyk et al. 2013). Among these auxiliary enzymes, the most relevant groups are CBM, PMOs and glucuronyl esterase family-15 class (CE15).
CBM are autonomously folding and functioning protein domain that lack catalytic activity but are able to bind to carbohydrate chains through their active site (Brumm 2013). CBM are important because they allow the hydrolytic enzymes to remain bound to the substrate while they perform their hydrolytic activity (Cantarel et al. 2009). CBM confer high selectivity in the binding, being able to target different substrate forms depending on different structural characteristics (Dyk and Pletschke 2012).

PMOs are enzymes that enhance the degradation of recalcitrant biopolymers by hydrolytic enzymes. They work synergistically with glycoside hydrolases (GHs) in the degradation of cellulose and chitin. PMOs enable a rather unusual depolymerisation of crystalline cellulose through an oxidative mechanism (Beeson et al. 2015). PMOs are intriguing enzymes, not only because they oxidize C-H bonds but also because their chemistry does not require separation of the polysaccharide chain from the crystalline matrix backbone for bond cleavage. The PMOs are very recent additions to the known polysaccharide degradation machinery, as their oxidative chemistry was reported for the first time in 2010 (Vaaje-Kolstad et al. 2010).

CE15 are accessory enzymes that present an interesting function: They break down bonds between hemicellulose and lignin, which is one of the factors of the recalcitrance of the lignocellulose biomass, hence the CE15 represent an incredible potential for industrial lignocellulose degradation processes. Thus type of enzymes hydrolyse the ester bond between lignin alcohol and the 4-O-methyl-D-glucuronic acid side chain of xylan in plant cell walls (Sunner et al. 2015). The first CE15 enzyme was purified from the fungus *Schizophyllum commune* (Špániková and Biely 2006) and the majority of CE15 studies derive from saprotrophic fungi despite the fact that many bacterial species have genes encoding homologues of fungal enzymes (De Santi et al. 2016). The presence of these genes represent an opportunity to investigate the CE15 family enzymes and their potential participation in lignocellulose degradation.

**Microbial communities and ecology-based biotechnology**

Microbial communities dominate almost every habitat on the planet. From oceans to soil, plants and animal/human bodies, systems are populated with diverse microbial communities that play very important roles in global processes. These range from the key life supporting biogeochemical cycles, to processes involved in plant/animal/human health. The systems on Earth thus offer a rich reservoir of
microbial functions that can be harnessed to serve human needs, such as in the environmentally-friendly biotechnological approaches. As described by Jimenez et al. (2016) and Maruthamuthu et al. (2016) the application of selected microbial consortia, over single strains, for such biotechnological purposes presents important advantages.

One of them is that microbial consortia are able to perform complex tasks that single strains struggle to perform in, for example, lignocellulose degradation. The reason is that the deconstruction of complex substrates requires many different types of enzymes and diverse chemical reactions, all at the same time or at short time intervals, in the same system. Moreover, division of labor (Jimenez et al 2017), resilience to environmental fluctuations (resistance in variation substrate composition) and resistance to microbial invasion play major roles (Pandhal and Noirel 2014; Mallon et al. 2015).

**Ecology-based biotechnology**

Usually, biotechnological processes that produce compounds and/or enzymes employ pure cultures of microorganisms that have been industrially bred for efficiency of growth and production. However, the use of single strains has clear limitations with respect to versatility. Hence, an alternative approach is offered by the application of ecology-based biotechnology (eco-biotechnology). Eco-biotechnology is the combination of the principles of microbial ecology with the purposes of industrial biotechnology. Hence, it is based on the application of ecological principles for the construction of cultures that are optimized for the production of metabolites and/or enzymes. The fundamentals of this approach lie in the natural selection – via competition – leading to optimized efficiency of growth and production, rather than on genetic or metabolic engineering. In this, selective pressure for the desired metabolism is applied to an initially diverse microbial inoculum by choosing the substrate and operating conditions. Thus, the ecosystem conditions ‘reign organisms’ evolution towards an optimized process, via the Darwinian ‘survival-of-the-fittest’ principle (Johnson et al. 2009). Here, in the light of the complexity of the LCB, survival of the fittest may encompass the fittest collaborating group rather than single organism.

Thus, agricultural waste residues, for example wheat, grass or sugarcane are posited to be better degraded by consortia rather than pure cultures. Moreover, the use of mixed cultures may overcome problems of substrate specificity, product inhibition and low yields. Eco-biotechnology has already been used for
the optimal production of cellulases applying a mix culture of *Bacillus aerius*, *Bacillus anthracis*, *Cellvibrio japonicus* and *Klebsiella pneumoniae* to a mix of lignocellulose substrate (palm oil and rice straw) (Oke et al. 2016). Moita et al. (2014) demonstrated the feasibility of production of polyhydroxyalkanoates by applying a microbial consortia and using glycerol, a waste substrate without any pretreatment, as a sole carbon source. The results allow the development of an more efficient production process.

**Habitat biasing and enrichment cultures**

Enriched microbial consortia have already been employed in LCB degradation, and good efficiencies have been achieved (Wongwilaiwalin et al. 2010). In addition to the number of enzymes produce and greater microbial stability, a key advantage of microbial consortia lies on the fact that strain isolation by using conventional plating, which represents a complicated and time-consuming process, is avoided. Moreover, given that only a small fraction of existing microbes in complex habitats like soil and sediment can be cultured, the isolation of LCB degrading microbes is not always successful. Some of the strategies used to develop microbial consortia rely on habitat biasing and enrichment cultures.

**Enrichment cultures** – also called habitat biasing (Ekkers et al. 2012) – is a strategy in which a deliberate bias is introduced in an environmental sample to modulate the microbial community structure in a particular direction (*in situ* or *ex situ*). For instance, adding a selective medium will result in an enrichment of a target microbial community that is able to use the specific substrate present in the medium. This will increase the required functions in the selected microbiome, and thus the genes or operons of interest (Cretoiu et al. 2012; Jiménez et al. 2014). Haruta et al. (2002) obtained a stable and complex lignocellulolytic microbial consortium from successive enrichment cultures on rice straw compost. The consortium had a high activity on various cellulosic materials, including rice straw, paper and cotton. Along the same line, Wongwilaiwalin et al. (2010) presented an analysis of a structurally stable lignocellulose degrading microbial consortium together with the characterization of its lignocellulolytic enzyme systems. The consortium was deemed to be applicable for biomass degradation and conversion in the biotechnological industry. In our group, Jimenez et al. (2014), by using dilution stimulation-approach, obtained a microbial consortia capable to consume torrefied wheat straw as single carbon source, as well as, grow in presence of 5-hydroxymethylfurfural.
There are several approaches to produce LCB-grown enrichment cultures, and the selection of the enrichment technique depends on the specific purpose and conditions of the experiment. Some approaches and examples can be found in Table 3. Lee et al. (2013) provide an in-depth discussion of the enrichment approaches, their challenges and further work.

### Table 3. Different enrichment approaches yielding different microbial consortia.

<table>
<thead>
<tr>
<th>Enrichment</th>
<th>Approach</th>
<th>Example</th>
<th>Reference</th>
</tr>
</thead>
<tbody>
<tr>
<td>Dilution-to-extinction</td>
<td>The original cultures is diluted until the desire activity is eliminated</td>
<td>Cellulose conversions to hydrogen</td>
<td>Wang et al. (2010)</td>
</tr>
<tr>
<td></td>
<td>Increase of the concentration of the particular compound for selection of desired functional strains.</td>
<td>Degradation of contaminant</td>
<td>Chen et al. (2010)</td>
</tr>
<tr>
<td>Concentration-to-extinction</td>
<td>A high concentration of a compound is added as a selective force, which will cause a long lag period until adaptation of the microbial community</td>
<td>Obtaining minimal functional consortium from activated sludge, the resulted consortium was able to remove individually meta-, para-, and ortho-cresol</td>
<td>Ho et al. (2010)</td>
</tr>
<tr>
<td>Heat-to-extinction</td>
<td>The selective pressure is the temperature.</td>
<td>Obtain a functional consortium from soil from hydrogen production</td>
<td>Zhang et al. (2008)</td>
</tr>
<tr>
<td>Hydrodynamics-to-extinction</td>
<td>Hydrodynamic selection pressure is applied through step-wised decrease of the hydraulic retention (HRT).</td>
<td>The microbial community had not varied significantly with the acclimation time but was highly affected to the HRT</td>
<td>Zhang et al. (2006)</td>
</tr>
<tr>
<td>Dilution-to-stimulation</td>
<td>Dilution the inoculum in succession, avoiding lose the function by checking along the enrichment process.</td>
<td>Selection of (hemi)cellulolytic bacteria. Lignocellulose degradation</td>
<td>Ho et al. (2012), Jiménez et al. (2014)</td>
</tr>
</tbody>
</table>
Dilution-to-stimulation approach
In this approach, an inoculum is introduced into an appropriate substrate in fresh medium. Following incubation, aliquots are sequentially transferred to new fresh medium with the substrate. Thus, in each transfer presumably the most efficient members of the consortium would be selected. The final culture would contain the most functional organisms while non-functional ones are presumably eliminated or reduced. The result would be an optimized consortium of reduced diversity capable to efficiently consume the substrate (Ho et al. 2012; Lee et al. 2013).

To use this type of enrichment technique, two main criteria have to be fulfilled. First, the original inoculum should be highly effective with respect to the target function to incorporate sufficient strains of interest. Second, the key functional organisms should be enriched to high densities and the constituent cells should be in a well-dispersed state so that serial dilution can preserve them, whilst removing non-functional strains.

The main advantage of the dilution approach is the reduction of the complexity of the community and the increased abundance of microorganisms capable to utilize the substrate of interest. Thus, analyses of the population composition and metabolic pathways can be carried out with a dramatic impact on the time length of the experiment. Moreover, this approach also lead to microbial communities that are highly efficient and stable – demonstrated by their tolerance to being sub-cultured several times in medium with and without cellulosic material (Haruta et al. 2002). Table 4 shows examples of microbial consortia able to degrade lignocellulose biomass. This high efficiency and stability is a reflection of the interactions between microbial members of the consortium.

Microbial interactions
Microbes in nature live in large communities, in these, their behavior is characterized by complex interactions with abiotic factors (environment) and biotic ones (other organisms). These interactions can be intraspecific, involving individuals of the same species, and/or interspecific, i.e. between individuals of different species (Ghosh et al. 2016). Dependency between the members within the community (or interdependency) can be large and dominating a system. A presumably large interdependency of microorganisms might be reflected in the fact that, very often, less than 1% of microbial life can be cultivated in the laboratory, presumably due to many bacteria depending on others for growth. (Pandhal and Noirel 2014).
Table 4. Examples of lignocellulose degrader microbial consortia, enriched from a variety of sources and substrate

<table>
<thead>
<tr>
<th>Source</th>
<th>Substrate</th>
<th>Final microbial composition</th>
<th>Condition</th>
<th>Reference</th>
</tr>
</thead>
<tbody>
<tr>
<td>Feces mixtures</td>
<td>Filter paper, printing paper, cotton, and rice straw</td>
<td><em>Clostridium</em> sp., uncultured <em>Betaproteobacterium</em>, <em>Brevibacillus</em> sp., <em>Pseudoxanthomonas</em> sp.</td>
<td>Facultative anoxic</td>
<td>Haruta et al. (2002)</td>
</tr>
<tr>
<td>Soil</td>
<td>Raw maize powder and filter paper</td>
<td><em>Ralstonia</em> sp., <em>Clostridium</em> sp., uncultured <em>Firmicutes</em>, <em>Propanibacterium acnes</em>, uncultured <em>Betaproteobacterium</em>, <em>Pantoena</em> sp.</td>
<td>Facultative anoxic</td>
<td>Feng et al. (2011)</td>
</tr>
<tr>
<td>Bagasse compost</td>
<td>Bagasse, rice straw, and corn stover</td>
<td>8 major groups including <em>Clostridium</em> sp., <em>Thermoanaerobacterium</em> sp., <em>Rhodocyclaceae</em> sp.</td>
<td>Facultative anoxic</td>
<td>Wongwilaiwalin et al. (2010)</td>
</tr>
<tr>
<td>Variety of lignocellulosic</td>
<td>Bermuda grass</td>
<td><em>Pseudoxanthomonas byssolorax</em>, <em>Microbacterium oxydans</em>, <em>Bacillus</em> sp., <em>Ochrobactrum anthropi</em>, <em>Klebsiella trevisanii</em></td>
<td>Oxic</td>
<td>Okeke and Lu (2011)</td>
</tr>
<tr>
<td>substrates</td>
<td></td>
<td>Species from <em>Raoultella</em>, <em>Klebsiella</em>, <em>Kluyvera</em>, <em>Citrobacter</em>, <em>Enterobacter</em>, <em>Pseudomonas putida</em>, <em>Acinetobacter calcoaceticus</em>, <em>Flavobacterium johnsoniae</em> and <em>Arthrobacter intermedia</em></td>
<td>Oxic</td>
<td>Jiménez et al. (2014)</td>
</tr>
<tr>
<td>Forest soil</td>
<td>Wheat straw/wheat straw torrified</td>
<td><em>Sphingobacterium kitaharashimense</em>, <em>Raoultella terrigena</em>, <em>Pseudomonas putida</em>, <em>Stenotrophomonas rhizophila</em>, <em>Coniochaeta ligniaria</em> and <em>Acremoniun sp.</em> were present in at least three treatments.</td>
<td>Oxic</td>
<td>Brossi et al. (2015)</td>
</tr>
<tr>
<td>Forest soil</td>
<td>Wheat straw, corn stover, switch grass</td>
<td><em>Sphingobacterium multivorum</em>, <em>Citrobacter freundii</em>, <em>Acinetobacter calcoaceticus</em>.</td>
<td>Oxic</td>
<td>Cortes-Tolalpa et al. (2016)</td>
</tr>
</tbody>
</table>
Chapter 1. General Introduction

Types of interactions
Microbes present two main basic types of interactions: Cooperative (positive) and non-cooperative (negative). Cooperative interactions include mutualism and commensalism. In mutualism, both organisms benefit from the interaction. There are two types of mutualism, i.e. obligatory and facultative mutualism. In facultative mutualism, although both organisms benefit from the interaction, they can exist in separate pure culture (Jagmann and Philipp 2014). In obligatory mutualism, the organisms involved are not able to perform a specific action when separated from the interaction partner. In commensalism, an organism benefits from the interaction with another one, while the other one is neither positively nor negatively affected. For example, in biodegradation commensals may feed on compounds that are released by the indifferent partner organism (Germerodt et al. 2016).

In non-cooperative interactions, parasitism, amensalism and predation are included. A parasitic interaction is established if the recipient (the ‘beneficiary’) forces the producer to release a metabolite that it can use (Jagmann and Philipp 2014). In predation, an organism (the prey) is consumed by another one (the predator). For example, ciliates feeding on bacteria (Mitri and Foster 2013). In competition, two or more organisms occupy the same niche, which means that both are able to consume the same resource (Dolinšek et al. 2016) and so – according to ecological theory – one ultimately outcompetes the other one (Gause 1934). Competition for resources is probably the most frequent interaction in the microbial world, and it has strong consequences for enrichment or habitat biasing approaches.

However, in a generic sense, all of the aforementioned interactions may play roles in nature and they generally occur together in any ecosystem, among prokaryotic and eukaryotic organisms coexisting there. In mechanistic terms, interactions in the microbial world may occur by the transfer of molecular (and/or genetic) compounds or information. Examples are seen in the exchange of metabolites, metabolite conversion, signaling compounds, resulting in chemotaxis. Moreover, genetic exchanges can also take place (Ghosh et al. 2016). The exchange of compounds is key in microbial communication, in particular quorum sensing (QS), which enables a population to collectively regulate the gene expression in response to host and/or environmental signals, produced by the same or even by different species. Examples of microbial communication like QS are exchanges of secondary metabolites, production of siderophores, biofilm formation and cellular transduction signaling (Mesquita Braga et al. 2016).
Interactions in selected microbial consortia

As established in the previous sections, the use of microbial consortia for biotechnological purposes represents advantages over the classical use of monocultures. To be able to explore these advantages, it is important to consider the interactions between the microorganisms in question.

However, identification of specific interactions between microbes in a complex consortium is a difficult task, as it is highly likely that different interactions happen simultaneously (Pandhal and Noirel 2014). Application of synthetic microbial consortia could be a solution to this problem, as such consortia are usually composed of few well-identified organisms. Sometimes, those species do not co-inhabit the same environment, but their metabolic activities may be combined (Jagmann and Philipp 2014). Determination of nutrient utilization, metabolite production, exchange of metabolites, production of signal molecules, spatial distribution, genome sequence and population dynamics are fundamental to enhance our understanding of the interactions. The knowledge could be applied to extend the stability of the consortia and improve the process efficiency (Ghosh et al. 2016).

Microbial consortia are ideal for the conversion of LCB substrates by providing the complex metabolic functions necessary for efficient polymerization. In previous work, microbial consortia capable to efficiently consume wheat straw have been produced (Jimenez et al. 2014). In these studies, bacteria members of the final specialized communities were isolated and identified, like *Raoultella/Klebsiella*, *Enterobacter amnigenus*, *Arthrobacter intermedia*, *Citrobacter*, *Pseudomonas putida*, as well as the fungi species *Penicillium citrinum* and *Coniochaeta ligniaria*. However, although their metabolic degradation capacity was tested, the main drivers of the selection remain unknown. Also, we do not know how efficient the process was in the end and it is likely that the efficiency was low, leading to the accumulation of remaining parts of hemicellulose and lignin.

Despite the large number of studies focusing on developing microbial consortia for LCB degradation, a few questions remain unsolved, and answering them might be key to improving LCB degradation. First, a key question pertaining is related to the paradigm of Martinus Beijerinck (1922): ‘Everything is everywhere and the environment selects’ – i.e. to what extent different inocula when bred on the same substrate under similar conditions will yield a similar final consortium make-up? Furthermore, would the outcome of such enrichment be driven by phylogenetic or functional attributes? In other words, will similar or highly...
different collaborating organisms appear in final consortia bred from different inocula on the basis of highly similar enrichment conditions? Alternatively, if the same inoculum is used to develop consortia on different LCB are the selective forces acting on the same inoculum be different across the suite of LCB materials – i.e. will the microbial consortia bred be similar? Second, what would happen with the composition of consortia if we add an extra stress factor like high salinity or increased recalcitrance substrate? Lastly, the complexity of the lignocellulose substrates makes synergistic actions of the degraders indispensable, thus the key question relates to understanding the mechanisms driving microbial interaction during LCB degradation.

**Aim of this thesis**

The aim of this thesis is to generate a better understanding of the key driving forces in the selection of specialized lignocellulolytic microbial consortia. Moreover, I aimed to gain fundamental knowledge about microbial cooperative interactions in the LCB degradation process. Such information is indispensable for the design, optimization and future application of degrader synthetic consortia.
**General research questions of this thesis**

- Do different lignocellulose sources (wheat straw, switch grass and maize) influence the composition of the degrader consortia as well as the degradation capacity? (Chapter 2)

- How does microbial source influence the final lignocellulose degrading consortia? (Chapter 3)

- What organisms are key to the degradation process and how do they function? (Chapters 2 and 3)

- Which is the influence of high salinity levels on lignocellulose degrader consortia? Is it possible to obtain a viable degrader consortium using a highly recalcitrant substrate (pre-digested wheat straw) as the single carbon and energy source? How will the complexity of the substrate influence the composition of the final consortia? (Chapter 4)

- Is there any cooperative relationship between selected abundant lignocellulose degraders from the consortia? Is the cooperative interaction depending of the compositional structure of the carbon source? (Chapter 5)

- What main genome features can be highlighted to illustrate the cooperative behaviour of microbial degraders? (Chapter 6)

**General hypotheses underlying this thesis**

1. Given the overall similarity in the composition of the lignocellulose substrates, consortia built on different substrates but using the same microbial inoculum will present a very similar microbial composition.

2. Different microbial sources used as inocula on the same LCB substrate will, under highly similar conditions, generate phylogenetically-different but functionally similar enriched consortia, due to microbial functional redundancy.

3. In order to develop salt tolerant LCB degrader consortia, salt marsh soil can be used as inoculum source.

4. Substrate made highly recalcitrant to degradation by pre-digestion will allow the selection of highly-specialized microbial consortia that are capable of transforming the most recalcitrant part of the substrate into utilizable resources.

5. Given its spatial complexity, wheat straw promotes cooperative relationships between microbial degrader strains. Such cooperation is built on exchanges of compounds or enzymes.

6. The analysis of the genomes of two synergistic degrader strains provides fundamental knowledge that fosters our understanding of the cooperative relationships in degrader consortia. Such interactions are observed exclusively in organisms growing on complex carbon sources.
Figure 7 Work flow of this thesis. The diagram shows the interconnection between the chapters in this thesis. Each chapter corresponds to one general hypothesis.
Thesis outline

Chapter 2 explores the effect of the use of different lignocellulose substrates (wheat straw, switch grass and maize) and the same inoculum source (forest soil) on the selection of enriched LCB degrading microbial consortia. To determine whether the consortia is phylogenetically or functionally similar, I characterized the structure and composition of selected consortia obtained across the enrichment by using 16S rRNA and ITS based PCR-DGGE; I also identified potential lignocellulose degrading strains in the final bacterial consortia and determined the degradation potential of the consortia using infrared fourier-transform infrared spectroscopy (FT-IR). Microbial strains were recovered, identified and tested for potential lytic activity. A group of bacteria were presented in three of the four enrichments, they were *Sphingobacterium kitahiroshimense*, *Enterobacter amnigenus*, *Raoultella terrigena*, *Pseudomonas putida* and *Stenotrophomonas rhizophila*, as well as the fungi strains *Coniochaeta ligniaria* and *Acremonium* sp. All the strains in the core presented CMC-ase and xylanase activity. The data revealed that the substrate type importantly determine the final composition of the consortia.

Chapter 3 explores the importance of the inoculum source (forest soil, canal sediment and decaying wood) in the selection of microbial degrader consortia. I addressed this question by verifying (1) the structures of the lignocellulose degrading consortia produced after ten sequential enrichments on raw wheat straw, next to (2) the degradation potential of the substrate by these microbial consortia. Across the enrichments, the selected consortia were characterized by analyses of the structure of bacterial and fungal communities by using 16S rRNA gene and ITS based PCR-DGGE, respectively. I also evaluated the bacterial community composition of the final consortia by using 16S ribosomal RNA amplicon sequencing. Similarly, to Chapter 2, microbial strains were recovered, identified and tested for potential lytic activity. Identification of the most abundant members of the community reveled a bacteria core (common between the three final consortia) which was composed by species of *Sphingobacterium*, *Citrobacter*, *Acinetobacter* and *Flavobacterium* or *Chryseobacterium*. The fungal strains were consortia-derived specific, however, as the Chapter 2, *Coniochaeta ligniaria* and *Acremonium* sp. were also found. Determination of lignocellulose degradation by the microbial consortia revealed that hemicellulose was the most consumed part of the substrate. I further obtained isolates of the most abundant and potential most relevant organisms in the consortia, i.e. *Sphingobacterium*
multivorum, Flavobacterium ginsengisoli, Chryseobacterium taihuense, which lytic activities were remarkably high. The data revealed that the final composition was strongly influence by the initial inoculum.

Taking into account the importance of the substrate composition (Chapter 2) and the relevance on inoculum selection (Chapter 3) in the selection of a grader microbial consortia, in Chapter 4 I explored the potential of using salt-marsh soil as inoculum for the production of microbial consortia capable of using wheat straw (as the sole carbon source) under highly saline conditions. Furthermore, I studied the possibility of increasing the recalcitrance of the substrate and how this increment in recalcitrance affects the composition of the microbial consortia. This was achieved by feeding fresh substrate to the consortia in the first part of the enrichment (transfers 1-6), whereas from transfers 7 onwards, I replaced fresh by pre-digested substrate, i.e. substrate that was previously degraded by the consortia. Briefly, the pre-digest substrate caused a notorious shift in the bacteria composition with a more strike effect on the fungi communities. Analysis of degradation capacity of the microbial consortia showed that enriched consortia bred with pre-digested substrate could degrade better cellulose and lignin than those selected in fresh substrate. Key cultivable degrader bacteria and fungi were also recovered, identified and tested for lytic activity. The most dominant bacteria in the consortia were Joostella marina, Flavobacterium beibuense, Algoriphagus ratkowskyi, Pseudomonas putida and Halomonas meridiana. The selected final consortia are a potential source of hydrolytic enzymes specialized on recalcitrance lignocellulose substrate and capable to work under saline condition.

Considering the potential role of microbial interactions in the degradation of complex polysaccharides, in Chapter 5 I explored the collaboration capacity of selected microbial degrader strains recovered in chapter 3. First, the strains were screening for their ability to growth on wheat straw as a single carbon source as well as the production of hydrolytic enzymes. Then, using minimal synthetic consortia with selected degrader strains, I examined their interactivity on carbon sources with different levels of “recalcitrance”. Monocultures and co-cultures were tested for growth and secretion of lytic enzymes, when growing in: (1) the simplest carbon source (glucose), (2) synthetic lignocellulose substrate (carboxymethyl cellulose, xylan-beechwood and lignin) and (3) wheat straw. Recalcitrance of the substrate was positively related to the microbial interactions, indicating that recalcitrance increases the cooperative relationship between the microbial species.
The synthetic community developed in Chapter 5 showing the highest level of synergistic interaction was used, in Chapter 6, as model the study of microbial interactions triggered by the complexity of the substrate. In order to understand the functional complements of the two collaborating bacterial species, I sequenced the genomes of *Sphingobacterium multivorum* w15 and *Citrobacter freundii* so4. I then compared the genomes by focusing on the lignocellulolytic arsenal across these two strains. I joined the genome analyses and the physiological data and used them propose a possible mechanism for lignocellulose degradation in the context of this collaborative pair, which is influenced by the complexity of carbon source.

Chapter 7 discusses the findings of this thesis and explores the avenues to future work.
References


Chapter 1. General Introduction


Chapter 1. General Introduction


