Identifying Novel Lignocellulosic Processing Enzymes from *Cellulomonas fimi* using Transcriptomic, Proteomic and Evolution Adaptive Studies

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Abstract

The declining reserves of fossil fuel twined with an increasing concern about the environmental consequences of burning these fuels and rising carbon dioxide levels, means that a more sustainable replacement is required. Lignocellulosic biomass is an attractive candidate that has been shown to be the best sustainable alternative source to produce bioethanol for liquid transportation fuels. It has enormous availability, is renewable and cost-effective. As an agricultural residue, it does not compete with food production. However, lignocellulosic biomass of plant cell walls is composed mainly of cellulose, hemicellulose and lignin, which are extremely resistant to digestion. Converting this biomass to useful products of fermentable sugars for bioethanol production has met with little success as harsh pretreatment and costly enzyme applications are required. An arsenal of enzymes and a synergistic mechanism are required to deconstruct recalcitrant lignocellulosic biomass for an efficient production of lignocellulosic bioethanol. To achieve this goal, this study used transcriptomic and proteomic approaches with the objective of identifying new genes and enzymes involved in lignocellulose degradation. This revealed that the only one AA10 of *Cellulomonas fimi* was among the highest enzymes identified during the degradation of cellulose. Another other 20 hypothetical proteins co-expressed with CAZymes have been identified including a potentially exclusively new *C. fimi* β-glucosidase (PKDP1) that contains a PKD-domain and oxidoreductase predicted function of PQQ-domain. A naturally mutagenized *C. fimi* population also was screened from an adaptive evolution experiment involving exposure to a wheat straw environment. One of the strains in the adaptive population (Strain-6) showed a higher association with wheat straw biomass, which may be an indication of the strategy that being used by the adapted strain to tackle obstinate substrates to sustain growth. These results show many new enzymes would be revealed from the *C. fimi* repertoire in order to have a better enzymatic cocktails for lignocellulose breakdown. For the future, this encourages a deeper understanding of lignocellulose deconstruction mechanisms by an orchestra of multiple enzymes in a bacterial system.

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Dedication

This thesis is dedicated to my beloved children,
who being my buffering system, my backbone,
during the ups and downs and the bittersweets
of this PhD journey,

Harith Hayyan & Sophea Insheera;

This is for both of you and for our brighter future.

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In the name of God, the Most Beneficent the Most Merciful

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Most of all, I'm gratefully praise to the Greatest God, who make the impossible, possible.

Verily, with hardship, there is relief.

(Al-Insyirah [The Consolation], 94:6)

Author's declaration

I declare that the work presented in this thesis is my own original work of research and I am the sole author, except where due reference has been given to collaborators and co-workers.

This work has not previously been presented for an award at this, or any other, University.

All sources are acknowledged as References.

1 Introduction

1.1 WORLD'S TREND ON WATER-FOOD-ENERGY DEMAND

The demand for water, food, and energy are predicted to increase by 40, 35, and 50 percent, respectively in the coming decades (1). This leads to the debate on 'resource scarcity' where the scientific findings suggest that humanity has exceeded the planetary boundaries and is threatening its own safety (2). Water, food and energy resources are tightly interconnected, forming a policy nexus (3,4) that is being discussed all over the globe by policy makers and scientists looking for solutions for sustainable development planning (Figure 1.1).

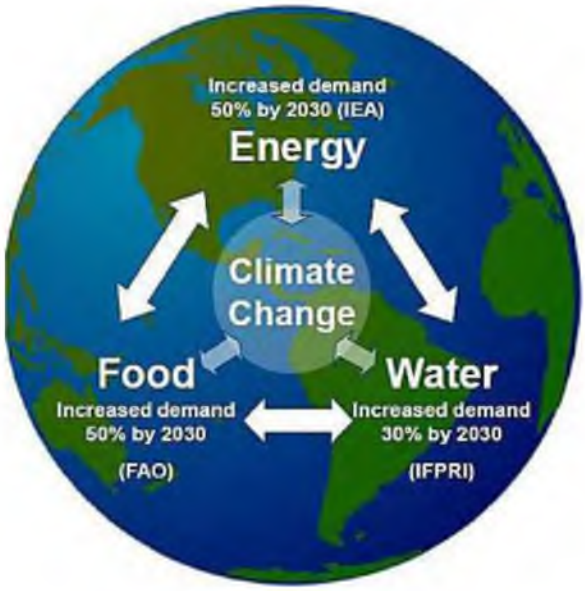

Figure 1.1: The world's trend demand and policy nexus.

It is predicted that by 2030 the world will need to produce around 50 per cent more food and energy, together with 30 per cent more fresh water, whilst mitigating and adapting to climate change. Illustration is reproduced from Beddington (5).

1.1.1 Global resources insecurity and climate change

Water is important for life and is a vital resource for the economy. It is also plays a fundamental role in the climate regulation cycle. Therefore, the management and protection of water resources is one of the keystones of environmental protection (4). Water insecurity caused by unmonitored development and environmental stress such as climate change may have a material impact on the economy. Climate change is the change in climate parameters such as regional temperature, precipitation, or extreme weather caused by increase in the greenhouse

effect. It is significantly impacted by agriculture due to increasing water demand, limiting crop productivity and reducing water availability in areas where irrigation is most needed (4). Several other sectors can also cause climate change; e.g. from burning activities by the release of CO_2 from fossil fuel combustion and an underestimated source of greenhouse gases (GHG) emissions that is anticipates from tropical deforestation (6–8). The term Greenhouse gases refers to gases that contribute to the effect by absorbing infrared radiation (heat). The greenhouse effect is the process where the greenhouse gases (water vapors, CO_2, methane, etc.) in the atmosphere absorb and re-emit heat being radiated from the Earth, hence trapping warmth that causes global warming (9). Global climate change is linked to the accumulation of greenhouse gases which causes concerns regarding the use of fossil fuels as the major energy source. To mitigate climate change while keeping energy supply sustainable, one proposal solution is to rely on the ability of microorganisms to use renewable resources for biofuel synthesis. Figure 1.2(A) shows the percentage of global greenhouse gas emissions from a study conducted by Intergovernmental Panel on Climate Change (IPCC) from 2010 (10). Electricity, heat production (25%) as well as agriculture, forestry and other land use (24%) contributed the most greenhouse emissions followed by transportation sector (14%). The increment production of GHG and black carbon emission are among the disadvantages of human activities that create a serious environmental concern. A study showed that the emission of CO_2 is projected to be increased since 1750 towards 2050 (Figure 1.2(B)).

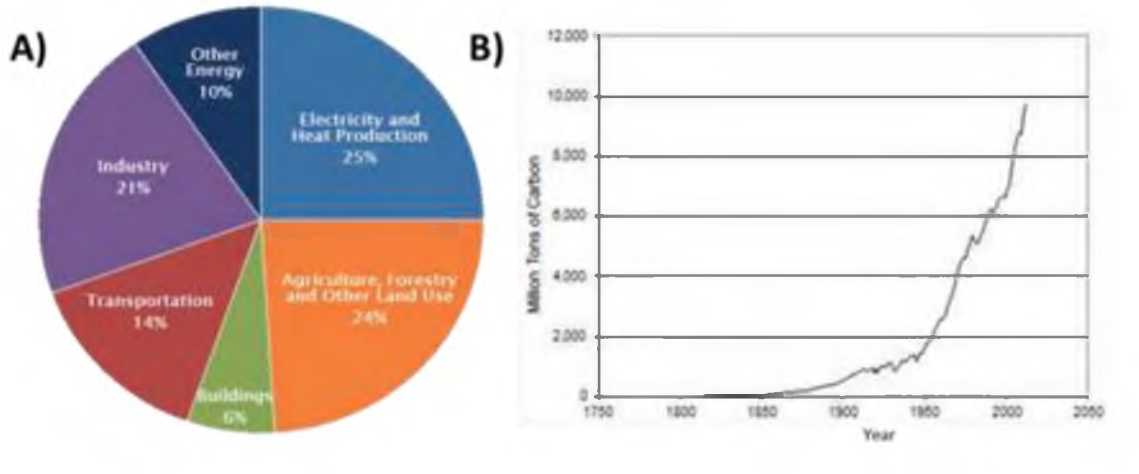

Figure 1.2: Proportions of global greenhouse gas emissions by economic sectors (A) and the projection of CO_2 emission (B).

A) Six major economic sectors that use energy and produce GHG based on a global emissions study from 2010 reported in IPCC report, 2014 (10). B) Projection of global carbon dioxide emissions from fossil fuel burning since 1751 to 2012 (11).

Large scale tropical deforestation caused by burning activities in countries such as Indonesia as well as the burning of agricultural residues which occur particularly in developing countries e.g. in India and Vietnam cause toxic and severe air pollution (8). The National Aeronautics and Space Administration (NASA) revealed the severity of these activities from the satellite images taken from space (Figure 1.3). The maps revealed that stubble-burning was not widespread in 2000. However, the problem had grown alarmingly by 2002 and continues to be a major health hazard.

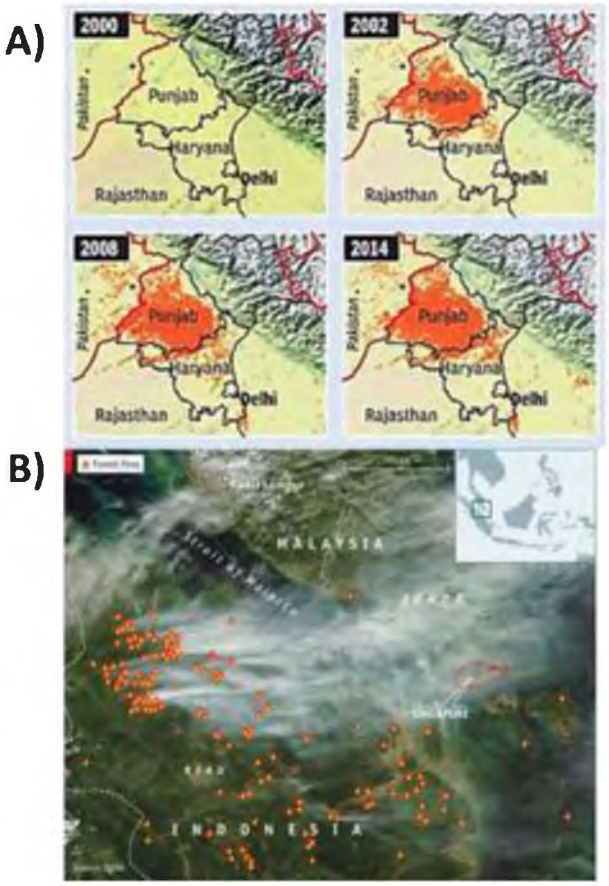

Figure 1.3: Fires from open burning in India and Indonesia detected by NASA satellites.

A) NASA satellite pictures reveal the evolution of paddy-stubble burning problem in Punjab, India since 2000. Each orange dot represents 1 sqkm^2 area where significant fires were observed. Each map shows cumulative farm fires from Oct 1 to Nov 10, each year**.** Images are reproduced from an article in 2015 by Amit Bhattacharya (12). **B)** Fires in Sumatra, Indonesia detected by NASA's Moderate Resolution Imaging Spectroradiometer (MODIS) sensor in 2013. Fires set for deforestation/land clearing in Indonesia triggered health warnings in Singapore and most parts of Malaysia. Images courtesy of Google Earth and NASA, reproduced from an article from The Economist (13).

The NASA images are clear proof that widespread crop burning has become a major contribution to air pollution and GHG emission specifically in Asian countries. These are among the examples that emission of CO_2 and GHG contribute to the net carbon change in the atmosphere which trigger the climate instability and result in global warming (6). Black carbon emissions are a potential danger to human health and may cause premature deaths (14). The real scenarios are happening on the ground when the farmers burn fields to clear crop stubble left after harvest. However, as many farmers cannot afford to spend extra money to use a tractor and plough stubble into the earth to be decomposed, open-burning of the stubble became the fastest and cheapest option. To decompose the stubble, the farmers have to further invest in watering systems. As this process takes time, it is not favorably practiced. Furthermore, the quality of the stubble after being harvested using a machine is not usable as fodder and could not be recycled into cardboard (15).

The International Energy Agency (IEA) are targeting a 50% reduction of greenhouse gasses by 2050 (16). Several technologies for generating bioenergy to produce heat and power already exist, ranging from conventional solid wood heating installations for buildings to biogas digesters for power generation, to large-scale biomass gasifications, as well as the production of biofuels especially for transportation sector (16). Renewable sources for the generation of electricity and heat and can be produced from tidal and wind energies. However, these resources cannot be utilized as fuels; particularly liquid fuel for transportation. Therefore, the only way to produce sustainable renewable liquid fuels is through the use of renewable biological products to create biofuels.

1.2 BIOETHANOL AS A BIOFUEL

Biofuels are produced by the conversion of biomass into liquids or gases, such as ethanol, lipids as biofuel precursors, biogas, or hydrogen, via biological or thermal processes. Bioethanol (CH_3CH_2OH) is a liquid biofuel which can be produced from several different feedstocks. Bioethanol can be used as a chemical in industrial applications or as fuel for energy generation; neat or blended with gasoline or diesel fuels. Biofuels can be broadly divided into first generation and second generation. Briefly, first generation bioethanol is mainly produced from edible crop feedstock by fermenting starch or sugars. The issue with first generation fuels is that their use of food commodities adds stress to world food security in an unsustainable manner. Second generation biofuels are produced from woody, non-food (lignocellulosic) plant biomass

such as crop residues or dedicated biomass crops. This is achieved via several pretreatments steps of the biomass, enzymatic hydrolysis and fermentation of the resulting sugars.

1.2.1 First generation of biofuel

Currently, first generation biofuels are sourced from crops such as starch, sugar, vegetable oil as energy-containing molecules, or even animal fats processed by conventional methodologies. First generation biofuels offer benefits for reducing CO_2 emission and can aid to improve domestic energy security. Biodiesel (bio-esters), bioethanol and biogas are the examples of the first generation biofuels that have been categorized by its ability to be blended with petroleum-based fuels and combusted in existing internal combustion engines (17,18). The production of first generation biofuels is now commercially competitive with the largest ethanol producing countries, United States of America (USA) and Brazil being responsible for the production of 54 x 10^6 and 21 x 10^6 m^3 in 2011, respectively (19). However, the source of feedstock raised concerns on the possible impact on biodiversity and land use; besides the competition with food crops (17). The disadvantage with these first generation biofuels is that they compete for resources with food commodities, adding to the stress on world food security brought about the growing global human population. This apparent conflict greatly limits the amount and sustainability of the biofuels that can be produced. One way in which the food security issue can be avoided is by producing biofuels from the woody non-food parts of crops and other residues.

1.2.2 Second generation of biofuel

Second generation bioethanol can be produced by fermenting sugars from the lignocellulosic biomass of dedicated bio-energy crops e.g. miscanthus, or those from co-products such as cereal straw (20). Three major steps are involved in biomass-to-ethanol process; 1) biomass pretreatment and fractionation, 2) enzymatic hydrolysis of cellulosic fraction, and 3) fermentation of the derived sugars to ethanol. Many factors contribute to the overall costs of producing biomass derived ethanol, however, the feedstock cost has been reported to be among the highest (21). To reduce these costs, one of the possible ways forward is by making use of underutilised biomass materials such as wheat straw from agricultural farms. In England, there is a potential cereal straw supply of 5.27 million tons (Mt) from arable farm types; 3.82 Mt are currently used and 1.45 Mt currently chopped and incorporated (22). Approximately 10 Mt of cereals straw was generated from 3 million hectares of wheat, barley, and oats in 2015 (23,24). Of this, 75% of straw is used for animal bedding, 23% is chopped and recombined into

the soil, and 2% is used for the mushroom compost. Wheat straw is an example of lignocellulosic biomass which comes from an agricultural by-product by harvesting the cereal grains. There could be up to 1.4 million tons of wheat straw per annum available for the potential sectors such as the biofuel industry in the UK alone (25). The variations in regional straw yields (t ha^{-1}) have a great effect on the England supply of straw and the potential amount of bioethanol that can be produced. This shows that commercially competitive substrates are available. However, biomass digestibility is still a major challenge. Thus, a few approaches still need to be improved; 1) to make biomass more digestible without compromising crops yield, and 2) to apply more effective pretreatments and enzymes for bioethanol conversion (26).

1.3 LIGNOCELLULOSIC BIOMASS

Lignocellulosic biomass is an attractive resource for fuel and biochemical production due to its abundance in nature. Waste biomass, the stalks of agriculture crops such as wheat and paddy, corn stover and wood can serve as raw materials. One of the attractions of woody plant biomass, or lignocellulose, is that it is rich in polysaccharides that can be converted into sugars for fermentation. However, one of the reasons that lignocellulose is abundant is because it is hard to break down into simple sugars due to its naturally durable structure. The plant cell wall is a structure characterized by a network of polysaccharides, structural proteins, and phenolic compounds. This network of polymers protects the plant against external stresses and provides structural and mechanical support to plant tissues. It is biochemically resistant mainly due to the presence of polyphenols called lignin that serve as protection and natural barrier of the plant against hydrolytic enzymes produced by microorganism in nature (27). The chemical composition and mechanical properties make plant cell walls a rich source of chemicals and fermentable sugars for the production of biofuels as it is comprised of roughly 70% polysaccharides that can potentially serve as a source of fermentable sugars (28). Plant cell walls are classified as primary and secondary cell walls. Both are different in their physiological roles as well as their chemical composition. Primary cell walls are located around dividing and elongating cells which consists of a large proportion of polysaccharides (cellulose; 40-50%, hemicelluloses; 20-40%, and pectin; 20-30%). Secondary cell walls are made up of cross-linked hetero-matrix of cellulose, hemicelluloses, and lignin and are laid down on the interior of the primary cell walls (Figure 1.4). The relative abundant of these three polymers varies depending on the type of biomass (29).

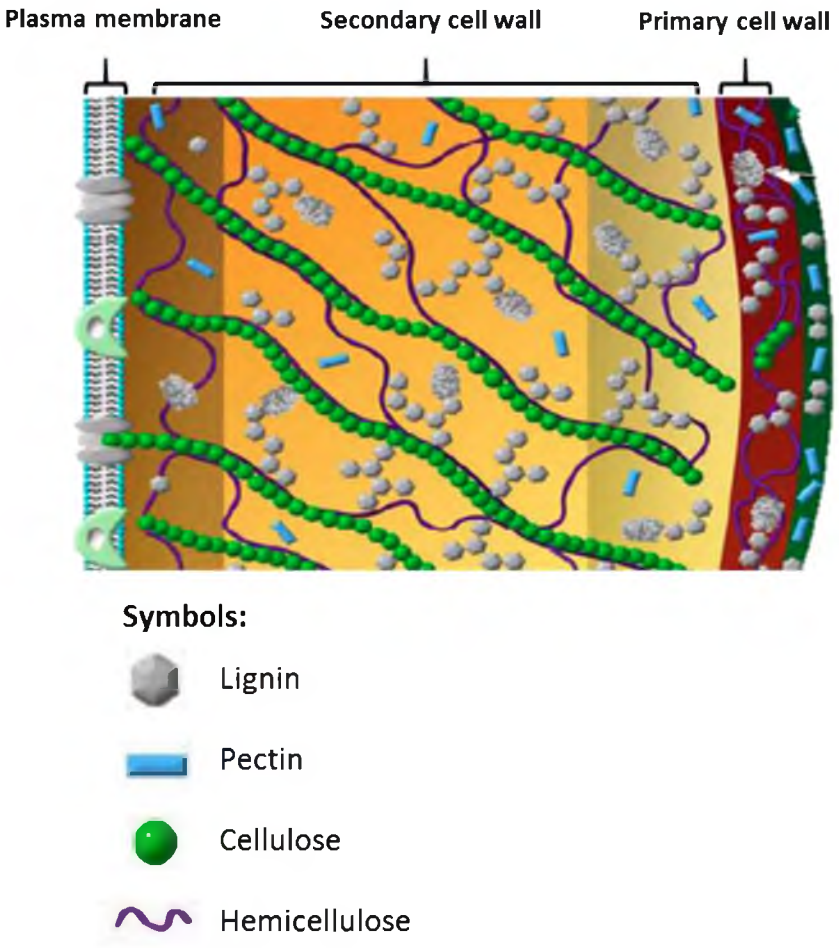

Figure 1.4: Illustration of a plant cell walls.

The features of the plant cell wall are shown. Relative thickness of the cell wall layers, relative abundance and specific localization of the various cell wall components, such as pectin, cellulose, hemicellulose, lignin and protein are illustrated. Image is reproduced from Achuyathan *et al.*, 2010 (30).

1.3.1 Cellulose

Cellulose is the main component of plant cell walls and the most abundant organic compound in terrestrial ecosystems. A linear cellulose polysaccharide consists of hundreds to over ten thousand β-1,4 linked glucose units (Figure 1.5A). The cellulose chains aggregate into microfibrils via hydrogen bonding and van der Waals interactions shows in Figure 1.5B (31,32). These microfibrils are crystalline, non-soluble, and challenging for enzymatic saccharification. Consecutive sugars along chains in crystalline cellulose are rotated by 180 degrees, meaning that the disaccharide (cellobiose) is the repeating unit. Cellulose tends to contain both well-ordered crystalline regions and disordered, more amorphous regions. While its recalcitrance to

enzymatic degradation may contribute problems, one big advantage of cellulose is its homogeneity. Complete depolymerization of cellulose yields just one product, glucose. Cellulose deconstruction is critical to ecosystem functioning and the global carbon cycle. Only selected lineages of fungi and bacteria have evolved the ability to efficiently degrade this highly recalcitrant substrate (33).

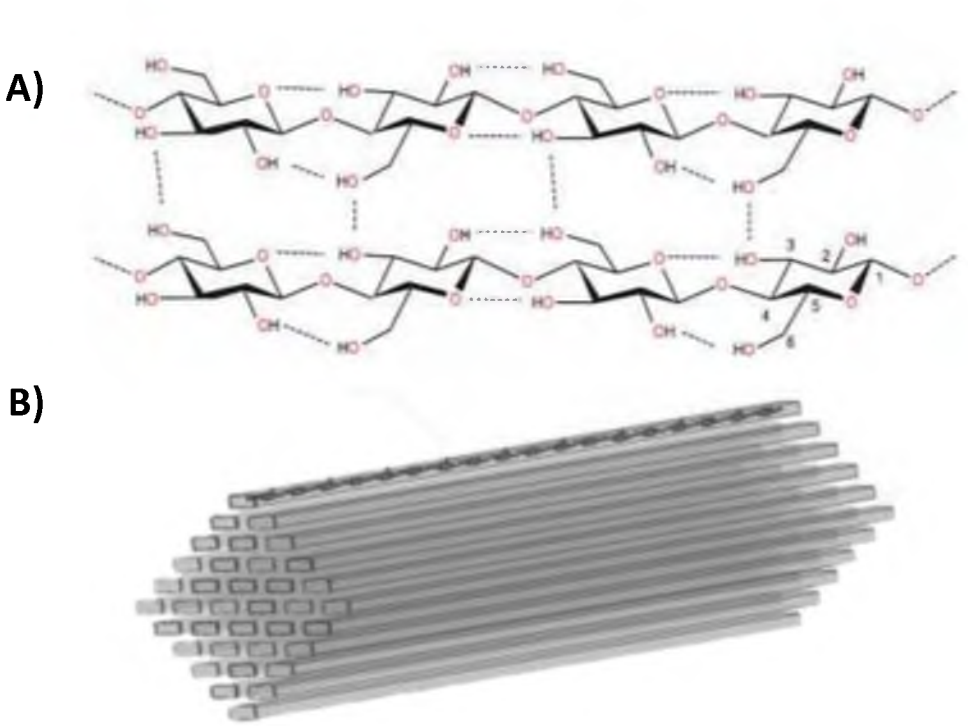

Figure 1.5: Overview of cellulose structure.

A) Cellulose chain (partial structure) consists of glucose monomers depicting an internal network of hydrogen bonds. The carbon numbering scheme is depicted on one glucosidic unit. Image is reproduced from Hemsworth *et al.*, 2013 (34). B) Simplistic sketch of a β-cellulose microfibril. Parallel cellulose chains aggregate into crystalline structures called microfibrils. Illustration is reproduced from Horn *et al.* (35).

1.3.2 Hemicellulose

Hemicelluloses are a large group of polysaccharides found in the primary and secondary cell walls. Hemicelluloses are built up by pentoses (D-xylose, D-arabinose), hexoses (D-mannose, D-glucose, D-galactose) and sugar acids (36). These are including β-glucan, xylan, xyloglucan, arabinoxylan, mannan, galactomannan, arabinan and so on. The hemicelluloses found in cereal straws are largely represented as complex heteropolysaccharides with various degrees of branching of the β-1,4-linked xylopyranosyl main chain structure (37). Softwood contains mainly

glucomannans, while in hardwood xylans are most common. Hemicelluloses interconnect with other cell wall components through covalent bonds and secondary forces (38). Both the cellulose and hemicellulose can be broken down enzymatically into the component sugars which may be then fermented to ethanol. Multiple classes of enzymes are required for effective degradation of cellulose and hemicelluloses (39). The break down process involves enzymes like glycoside hydrolases, carbohydrate esterases, polysaccharide lyases, endo- hemicellulases and others, the concerted action of which hydrolyze glycosidic bonds, ester bonds and remove the chain's substituents or side chains. These include endo-1,4-β-xylanase, β,-xylosidase, β-mannanase, β-mannosidase, α-glucuronidase, α-L-arabinofuranosidase, acetylxylan esterase and other enzymes (40).

1.3.3 Lignin

While cellulose and hemicellulose are built from carbohydrates, the random structure in the tridimensional network inside the cell which consists of lignin is built up by oxidative coupling of three major C_6-C_3 (phenypropanoid) units, namely syringyl alcohol (S), guaiacyl alcohol (G), and *p*-coumaryl alcohol (H) (41,42). Lignins are highly branched, substituted, mononuclear aromatic polymers in the cell walls of certain biomass, especially woody species, and are often bound to adjacent cellulose fibers to form a lignocellulosic complex (Figure 1.6). This complex and the lignins alone are often quite resistant to conversion by microbial systems and many chemical agents. The lignin-hemicellulose complex surrounds the cellulose with which it is bound through extensive hydrogen bonding to form a supramolecular structure that protects the cellulose and is the reason for biomass recalcitrance (30). Lignin is one of the most abundant natural polymers expected to play an important role in the near future as a raw material for the production of bio-products. Large amounts of lignin are produced each year by the pulp and paper industry as by-products of delignification. The amount of lignin in plants vary widely, and is normally in the range of 20-30% by weight (43). Lignin is an aromatic hetero-biopolymer role as the constituent of an internal cell wall in all vascular plants including the herbaceous varieties. In the plant cell wall, hemicelluloses serve as a connection between lignin and cellulose and gives the whole cellulose-hemicelluloses-lignin network structure more rigidity besides 20 different types of bonds present within the lignin itself (44). Owing to its cross linking, lignin *in-situ* is usually insoluble in all solvents, unless it is degraded by physical or chemical treatments.

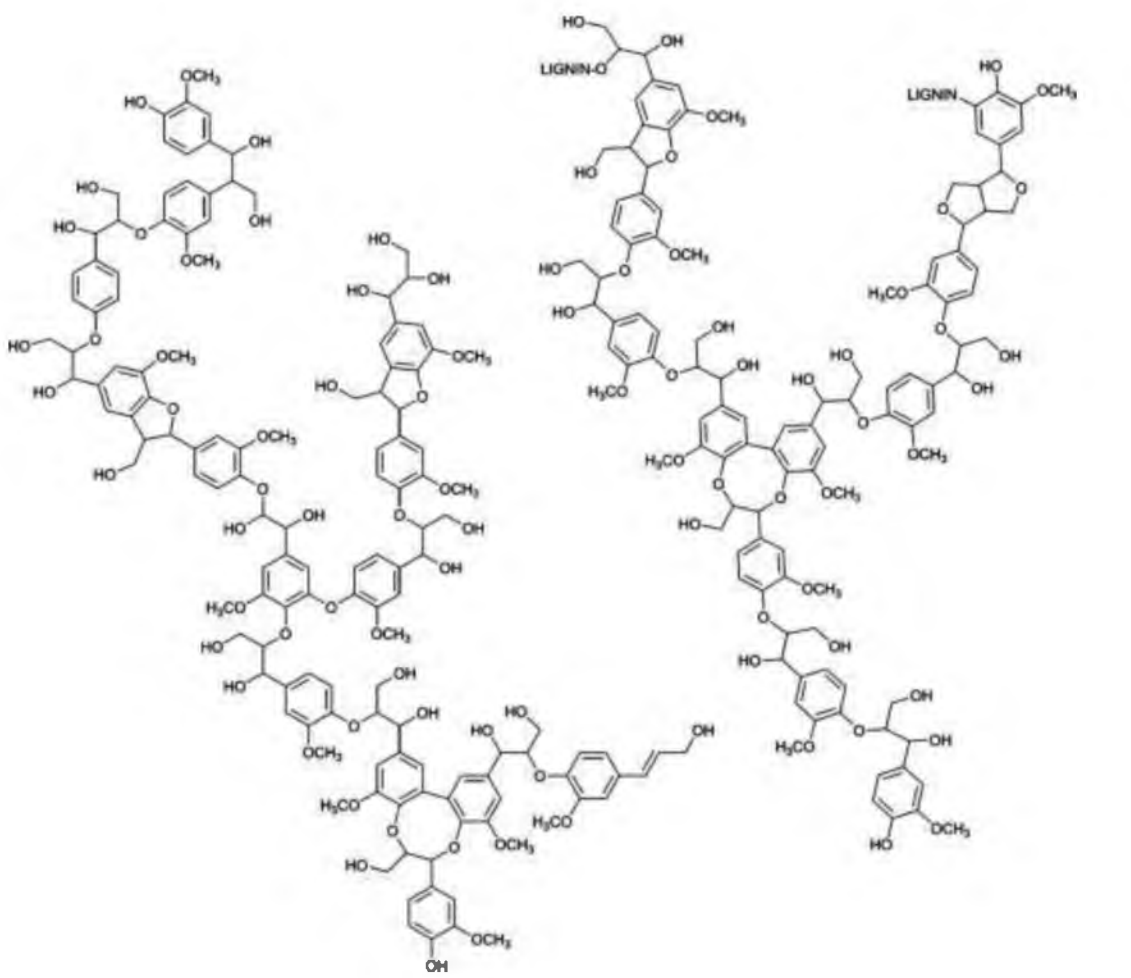

Figure 1.6: Structure of lignin.

Unlike most natural polymers, such as cellulose and starch, which consist of a single monomer and intermonomeric linkage, lignin is a network polymer made up by oxidative coupling of three major C6-C3 (phenylpropanoid) units with many carbon-to-carbon and ether linkages, such as β-*O*-4, 4-*O*-5, β-β, β-1, β-5, and 5-5' (45). It is covalently linked to polysaccharides, forming a lignin-hemicellulose network made up of benzyl-ether, benzyl-ester, and phenyl-glycoside bonds (44). Image is reproduced from Crestini *et al.*, 2011 (46).

1.4 LIGNOCELLULOSE DIGESTION IN NATURE

Despite lignocellulose being a hard-to-digest structure, a range of animals and microbes can digest lignocellulosic biomass in nature. Animals such as termites (47,48), beetles (49,50) and marine wood borers (51,52) have evolved to live on a diet of lignocellulose. Microbes are the main source of lignocellulose digestion in these animal systems and also serve to turn over woody biomass in the environment. Filamentous fungi are major degraders of lignocellulosic biomass in the environment due to their ability to degrade lignin. This is mostly achieved

through the action of enzyme-mediated oxidative free radical attack of the lignin, exposing the polysaccharides for hydrolytic enzyme attack. Many biomass-degrading organisms secrete synergistic cocktails of individual enzymes with one or several catalytic domains per enzyme, whereas a few bacteria synthesize large multi-enzyme complexes (cellulosomes) which contain multiple catalytic units per complex (39,53). The cellulosomes present in obligate anaerobic microbes contain many catalytic units per individual complex, linked to a single carbohydrate binding module (CBM) bearing scaffoldin via cohesin–dockerin interactions (54,55). Although lignocellulolytic fungi such as *Aspergillus*, *Penicillium*, *Schizophyllum*, *Trichoderma*, *Phanerochaete* and *Sclerotium* species can secrete industrial quantities of extracellular enzymes, bacterial enzyme production can be more cost-efficient (56).

1.4.1 Aerobic lignocellulolytic bacteria

The rapid growth and multi-enzyme complexes with increased functionality and specificity ensure that the lignocellulolytic bacteria tolerate larger and more diverse environmental stresses during lignocellulose decomposition and occupy wider niches than filamentous fungi (57). A few bacterial species are currently known to degrade both cellulose and lignin. Among them are member of the genera *Pseudomonas* (order *Pseudomonadales*), *Streptomyces*, as well as *Cellulomonas* (order *Actinomycetales*) which are likely to employ extracellular laccases and peroxidases to attack lignin (42,58,59). With respect to recent trends in lignocellulose decomposition research, the broad studies conducted by scientists on laccases and peroxidases have identified that aerobic lignocellulolytic microbes exhibit free and complex enzymes synergy which require terminal or intermediate electron acceptors to support the decomposition under limited carbon source conditions (60).

1.4.2 Glyosyl Hydrolases (GHs)

In Nature, the enzymatic deconstruction of cellulose and hemicellulose is achieved by the orchestrated action of various carbohydrate-active enzymes (CAZymes), typically acting together as a cocktail with synergistic activities and modes of action (61) (see Figure 1.7). GHs are important enzymes that cleave glycosidic bonds that exist in cellulose and hemicellulose. The capacity of GHs are aided by polysaccharide esterases that remove methyl, acetyl and phenolic esters, permitting the GHs to break down hemicelluloses (62). Additionally, polysaccharides are depolymerised by the activity of polysaccharide lyases (PL) (63).

More recently, the action of lytic polysaccharide monooxygenases (LPMOs) has been shown to be critical for efficient cellulose hydrolysis by the oxidative cleavage of difficult to access glucans on the surface of crystalline cellulose microfibrils (64,65). Across the Tree of Life, the GH cocktail composition varies significantly in composition depending on the kingdom of the cellulolytic organism, the evolutionary pressure, and the environmental niche of the cellulolytic habitats (61). Lignocellulose-utilising creatures secrete some GHs, however most benefitted from a mutualism relationship with their enzyme-secreting gut microflora, a particular example in termites. However, in shipworms the system consists of GH-secreting and LPMO-secreting bacteria that separate from the site of digestion, whereas, the isopod *Limnoria* solely relies on endogenous enzymes (51,52).

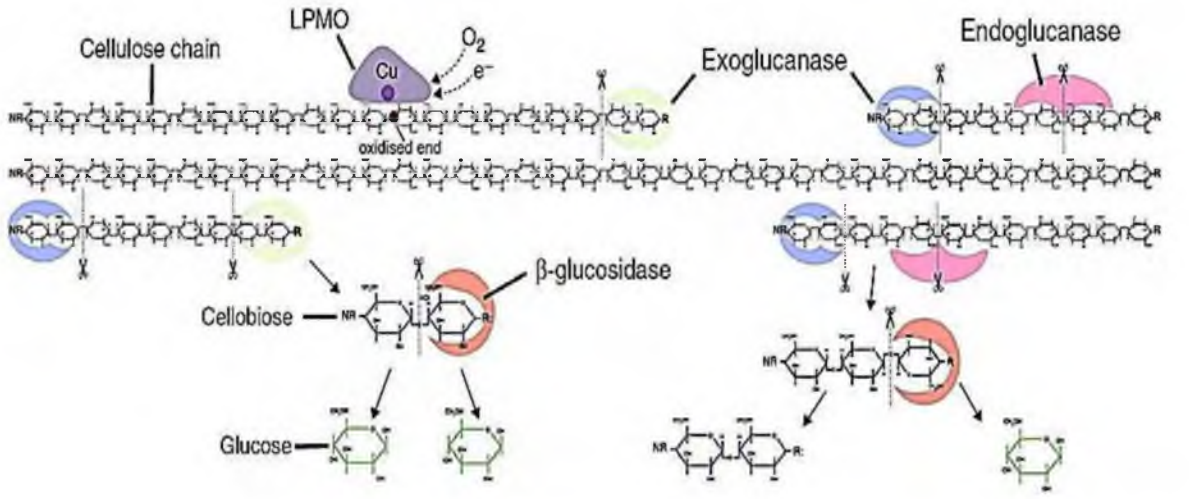

Figure 1.7: Schematics of microbial mechanisms of lignocellulose degradation.
Aerobic cell-free cellulase system employed by most of bacteria and fungi. Cellulose is digested via the synergistic interaction of individual GH and LPMO secreted enzymes. NR-, non-reducing ends; -R, reducing ends. Image is reproduced from Cragg *et al.* (51).

1.4.3 Lytic polysaccharide Monooxygenase (LPMO)

Lytic polysaccharide monooxygenases (LPMOs) are a type of enzyme which requires a reducing agent (either a small molecule reducing agent or cellobiose dehydrogenase), oxygen, and a copper (Cu) ion bound in the active site for activity (57,66,67). The glycoside hydrolases, pectate lyases, esterases and the new LPMOs are all often found as parts of multi-modular enzymes that contain substrate-targeting carbohydrate-binding modules (68). These enzymes are important for the decomposition of recalcitrant biological macromolecules such as chitin and plant cell wall

polymers (61,69). Since their discovery, LPMOs have become integral factors in the industrial utilization of biomass, especially in the sustainable generation of cellulosic bioethanol (70). LPMOs were originally designated as GH61 and CBM33, but now classified as Auxiliary Activity (AA) 9, AA10 and AA11 in the CAZy database (69,71,72). The reclassification of GH61 to AA family was based on the findings that although some GH61s appeared to have weak endoglucanase activity, enzymes from this group could enhance enzymatic depolymerization of cellulose into soluble sugars by GHs. The AA9 contains fungal enzymes and AA10 predominantly bacterial enzymes (73). 3D structural analyses of lytic polysaccharide monooxygenases of both bacterial AA10 (previously CBM33) and fungal AA9 (previously GH61) enzymes uncovered structures with b-sandwich folds containing an active site with a metal coordinated by an N- terminal histidine (68). LPMO are copper-containing enzymes (metalloenzymes) that depolymerize recalcitrant polysaccharides by breaking down the glycosidic bonds and direct oxidative attack on the carbohydrate polymer chains through a flat site with a centrally located copper atom (65). LPMOs cleave the polysaccharide chain by utilising the oxidative capacity of molecular oxygen to scission a glycosidic C-H bond. To break these bonds, LPMOs activate oxygen, in a reducing agent dependent manner, at a copper-containing active site known as the histidine brace (34,74,75). Working together with both canonical polysaccharide hydrolases and other electron transfer compounds, these enzymes significantly boost the deconstruction of polysaccharides into oligosaccharides. Consequently, they have real potential for improving the production of biofuels from lignocellulose sustainable sources.

1.4.4 *Cellulomonas fimi* ATCC® 484™

At a biochemical level, one of the best understood cellulose-degrading bacterial systems is derived from *Cellulomonas fimi. C. fimi* is a Gram-positive coryneform bacterium, a group which includes a range of cellulolytic facultative anaerobes. The *C. fimi* genome encodes an array of glycosyl hydrolases (GHs) and Carbohydrate Active Enzymes (CAZymes) with similar numbers (176 CAZymes) to those found in other cellulomonads (*Cellulomonas Uda*, *Cellulomonas flavigena* and *Cellulomonas* sp. CS-1) but it has a slightly lower number of CAZymes compared to other cellulase-secreting bacteria such as *Fibrobacter succinogenes* (190 CAZymes), *Streptomyces coelicolor* (268 CAZymes), *Streptomyces bigichenggensis* (276 CAZymes), and *Streptomyces davawensis* (337 CAZymes) (76). Despite the lesser number of CAZymes, previous studies reported its proficiency and capability to utilize cellulose by expressing extracellular cellulases which include exoglucanases (39,77–80), and endoglucanases (80,81) towards digestion of diverse set of carbohydrates including crystalline cellulose, *in vitro.* From the reported studies, 30 structures of proteins from *C. fimi* are available in the Protein Data Bank (PDB) and 10 well-characterized enzymes have been fully reviewed in Universal Protein Resource (UniProt KB) database (see Table 1.1) regarding to the mode of action of their catalytic and carbohydrate-binding module of actions towards various of polysaccharides (82–85). *C. fimi* is still of interest due to significant gaps in knowledge with regard to its ability to digest recalcitrant lignocellulose. Interestingly, the *C. fimi* genome not reveal any homology to typical cellulosome components such as scaffoldins, dockerins or cohesins which exists commonly in facultative anaerobes enzymatic systems (86). This is in contrast with other reports where *C. fimi* was reported to have a mutually exclusive approach by using both "secreted-enzyme" and "surface-enzyme" strategies during cellulose digestion other than the reported carbohydrate-binding proteins (87,88). This characteristic was only found in two cellulolytic facultative anaerobes bacteria including *C. fermentans* (86).

Table 1.1: Characterized *C. fimi* proteins involving in polysaccharide degradation as listed in the UniProt KB database.

Reviewed *C. fimi* characterized proteins as curated in the UniProt KB database accessed in April, 2017.

UniProt ID	UniProt Entry name	Length	Protein name	Gene names	Catalytic activity	Protein family/CAZy	References
P14090	GUNC_CELFA	1,101	Endoglucanase C	cenC Celf_1537	Endohydrolysis of (1->4)-β-D-glucosidic linkages in cellulose, lichenin and cereal β-D-glucans.	**CBM4.** Carbohydrate-Binding Module Family 4. **GH9.** Glycoside Hydrolase Family 9.	(89–92)
P50899	GUXB_CELFA	1,090	Exoglucanase B	cbhB cenE, Celf_3400	Hydrolysis of (1->4)-β-D-glucosidic linkages in cellulose and cellotetraose, releasing cellobiose from the non-reducing ends of the chains.	**CBM2.** Carbohydrate-Binding Module Family 2. **GH48.** Glycoside Hydrolase Family 48.	(77,93,94)
P50400	GUND_CELFI	747	Endoglucanase D	cenD	Endohydrolysis of (1->4)-β-D-glucosidic linkages in cellulose, lichenin and cereal β-D-glucans.	**CBM2.** Carbohydrate-Binding Module Family 2. **GH5.** Glycoside Hydrolase Family 5.	(93)
P07984	GUNA_CELFI	449	Endoglucanase A	cenA	Endohydrolysis of (1->4)-β-D-glucosidic linkages in cellulose, lichenin and cereal β-D-glucans.	**CBM2.** Carbohydrate-Binding Module Family 2. **GH6**. Glycoside Hydrolase Family 6.	(39,89,95,96)

...continued

UniProt ID	UniProt Entry name	Length	Protein name	Gene names	Catalytic activity	Protein family/CAZy	References
P26255	GUNB_CELFI	1,045	Endoglucanase B	cenB	Endohydrolysis of (1->4)-β-D-glucosidic linkages in cellulose, lichenin and cereal β-D-glucans.	**CBM2.** Carbohydrate-Binding Module Family 2. **CBM3**. Carbohydrate-Binding Module Family 3. **GH9.** Glycoside Hydrolase Family 9.	(81,97)
P50401	GUXA_CELFA	872	Exoglucanase A	cbhA Celf_1925	Hydrolysis of (1->4)-β-D-glucosidic linkages in cellulose and cellotetraose, releasing cellobiose from the non-reducing ends of the chains.	**CBM2.** Carbohydrate-Binding Module Family 2. **GH6.** Glycoside Hydrolase Family 6.	(93,98)
Q7WUL4	HEX20_CELFI	496	β-N-acetylhexosa-minidase	hex20 hex20A	Hydrolysis of terminal non-reducing N-acetyl-D-hexosamine residues in N-acetyl-β-D-hexosaminides.	**GH20.** Glycoside Hydrolase Family 20.	(99)
P54865	XYND_CELFI	644	Bifunctional xylanase/ deacetylase	xynD	Endohydrolysis of (1->4)-β-D-xylosidic linkages in xylans.	**CBM2**. Carbohydrate-Binding Module Family 2. **GH11.** Glycoside Hydrolase Family 11.	(100–103)

...continued

UniProt ID	UniProt Entry name	Length	Protein name	Gene names	Catalytic activity	Protein family/CAZy	References
P07986	GUX_CELFI	484	Exoglucanase/ xylanase	cex xynB	Hydrolysis of (1->4)-β-D-glucosidic linkages in cellulose and cellotetraose, releasing cellobiose from the non-reducing ends of the chains. Endohydrolysis of (1->4)-β-D-xylosidic linkages in xylans.	**CBM2.** Carbohydrate-Binding Module Family 2. **GH10.** Glycoside Hydrolase Family 10.	(39,85,96,97,104–109)
Q7WUL3	NAG3_CELFI	564	β-N-acetylgluco-saminidase/b-glucosidase	nag3 nag3A	Hydrolysis of terminal non-reducing N-acetyl-D-hexosamine residues in N-acetyl-β-D-hexosaminides. Hydrolysis of terminal, non-reducing β-D-glucosyl residues with release of β-D-glucose.	**GH3.** Glycoside Hydrolase Family 3.	(99)

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