DIVERSITY AND ENZYME PRODUCTION FROM LIGNOCELLULOSE-DEGRADING BACTERIA IN SOIL FROM PENANG LANDFILL

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by

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LIST OF SYMBOLS

μ	Growth rate
°C	Degree Celsius
g	Gram
cm	Centimeter
R^2	Coefficient of determination
k	Growth rate
w/v	Weight per volume
Bp	Base pair
ln	Neparian logarithm
%	Percentage
U	Enzyme unit
nm	Nanometer
pI	Isoelectric Point

LIST OF ABBREVIATIONS

CBP	Consolidated Bioprocessing
CH4	Methane
C/N	Carbon Nitrogen
CLEA	Cross-linked Enzyme Aggregates
CMC	Carboxymethylcellulose
CO2	Carbon Dioxide
CPW	Citrus Peel Wastes
DyP	Dye- decolourising peroxidase
FVW	Fruit Vegetable Waste
GH	Glycoside Hydrolase
GHG	Green House Gas
H2O2	Hydrogen Peroxide
LiP	Lignin Peroxidase
MnP	Manganese Peroxidase
MSW	Municipal Solid Wastes
TS	Total Solid
VP	Versatile Peroxidase
VS	Volatile Solid

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DIVERSITI DAN PENGHASILAN ENZIM DARIPADA BAKTERIA PENGURAI LIGNOSELULOSA DALAM TANAH DARI TAPAK PELUPUSAN SAMPAH DI PULAU PINANG

ABSTRAK

Biomass lignoselulosa menjadi sangat menarik untuk menghasilkan tenaga berbanding dengan menggunakan tanaman makanan. Kajian difokuskan untuk mengembangkan proses dan teknologi yang dapat menggunakan biomassa ini untuk menghasilkan polimer berharga sebagai ganti sumber fosil yang mahal dan terhad. Enzim lignoselulotik bertindak sebagai biokatalis yang menguraikan lignoselulosa menjadi komponennya untuk dihidrolisis menjadi produk yang berguna. Kajian ini dilakukan untuk memberikan pencirian yang meluas dan mengenal pasti bakteria lignoselulotik di dalam sesuatu persekitaran yang jarang dikaji. Objektif lain adalah untuk menapis bakteria bagi kesesuaian multi-enzim dalam pengilangan berasaskan lignoselulosa, dan kemudian menghasilkan maklumat dan cadangan untuk merekabentuk konsortium masa depan untuk penguraian lengkap lignoselulosa. Kajian bebas kultur telah dilakukan menggunakan kaedah metagenomik untuk menentukan kepelbagaian bakteria dalam endapan Sisa Pepejal Perbandaran selepas pengekstrakan DNA dan tindak balas rantai Polimer. Kaedah bergantung kepada kultur merupakan asas pemencilan dan penapisan bakteria dari persekitaran yang sama untuk mengenal pasti bakteria berpotensi yang menunjukkan aktiviti multi-enzim. Kajian kinetik kemudian dijalankan dengan menggunakan kadar pertumbuhan dan masa penggandaan isolat bakteria dalam lignin dan kanji sebagai kriteria pemilihan. Kajian kualitatif menggunakan pelbagai media selulolitik digunakan untuk penapisan. Analisis filogenetik dijalankan dengan menggunakan urutan 16S rRNA isolat bakteria.

Isolat telah dikaji secara kuantitatif untuk pengungkapan selulase dan dua konsortium telah direka untuk menguji pengungkapan enzim yang lebih baik. Pengoptimuman dilakukan untuk menentukan keadaan terbaik bagi setiap konsortium. Kajian fisikokimia menunjukkan tapak pelupusan ini mempunyai julat suhu mesofilik dan termofilik serta campuran nilai pH asidik dan alkali. DNA komuniti bakteria mempunyai 357,030 urutan efektif dan 1891-unit taksonomi operasi (OTUs). Empat puluh filum ditemui, dengan filum dominan Proteobacteria, Firmicutes, Actinobacteria, dan Bacteroidota, manakala Aerococcus, Stenotrophomonas, dan Sporosarcina merupakan spesies dominan. Pemeriksaan terhadap fungsi metabolik komuniti untuk enzim lignoselulolitik menunjukkan bahawa fungsi gen selulase, xilanase, esterase, dan peroksidase dapat dibuktikan di dalam data yang diperoleh. Dapatan keputusan pengurutan gen 16S rRNA menunjukkan isolat bakteria Bacillus proteolyticus, Bacillus Sanguinis, Bacillus spizizeni, Bacillus paramycoides, Bacillus paranthracis dan Neobacillus fumarioli. Aktiviti selulase untuk isolat ini berada dalam julat 0.8-1.7U/ml. Consortia MRE menunjukkan kecondongan termofilik yang lebih tinggi berbanding dengan Consortia SPR. Kajian ini menunjukkan bakteria mampu menghasilkan enzim lignoselulolitik dan penguraian biojisim lignoselulosa yang hadir dalam sisa pepejal komunal.

DIVERSITY AND ENZYME PRODUCTION FROM LIGNOCELLULOSE-DEGRADING BACTERIA IN SOIL FROM PENANG LANDFILL

ABSTRACT

Lignocellulosic biomass has become a very appealing energy source to produce energy when compared to using food crops. Research is focusing on developing processes and technologies that can use this biomass to produce valuable polymers and energy instead of using expensive and exhaustible fossil sources. Lignocellulolytic enzymes act as biocatalysts that breakdown lignocellulose into its components for further hydrolysis into useful products. The study was done to provide extensive characterization and identification of lignocellulolytic bacteria in an environment that has been sparing studied for the purpose of discovering bacteria with lignocellulolytic potential for future applications. Another objective was to screen the bacteria for multi-enzymatic suitability in lignocellulose-driven refinery, and then generate information and recommendations for designing future consortia for the complete degradation of lignocellulose in the future. Culture independent study was carried out using metagenomic methods to determine the bacterial diversity of municipal solid waste sediments after DNA extraction and Polymerase chain reactions. Culture dependent methods formed the basis of isolating and screening bacteria from the same environment to identify potential bacteria that exhibit multi-enzymatic activity. Kinetic studies were then carried out using the growth rate and doubling time of bacterial isolates in lignin and starch as the criteria for selection. Qualitative studies using various cellulolytic media were used to further screen candidates. Phylogenetic analysis was conducted using the 16S rRNA sequences of the bacterial isolates.

Promising isolates were studied for cellulase expression quantitatively and two consortia were designed to test for improved enzyme expression. Optimisation was done to determine the best condition for the most promising consortia. Physicochemical studies revealed the landfill's environment has both mesophilic and thermophilic temperature ranges and a mixture of acidic and alkaline pH values. The bacterial community DNA was revealed to have 357,030 effective sequences and 1891 operational taxonomic units (OTUs) assigned. Forty phyla were found, with the dominant phyla identified to be *Proteobacteria, Firmicutes, Actinobacteria*, and *Bacteroidota*. The dominant species were identified as *Aerococcus*, *Stenotrophomonas*, and *Sporarcina*. A look into the community's metabolic function for lignocellulolytic enzymes' function specifically showed that cellulase, xylanase, esterase, and peroxidase gene functions were present in the studied environment. 169 isolates were gotten, screened and kinetically examined. Enzymes assays revealed promising isolates which 16S rRNA gene sequencing showed as *Bacillus proteolyticus, Bacillus Sanguinis, Bacillus spizizeni, Bacillus paramycoides, Bacillus paranthracis* and *Neobacillus fumarioli*. The cellulase activity for these isolates ranged from 0.3 - 0.8U/ml. Consortia MRE reflected higher thermophilic inclination compared to Consortia SPR and had cellulase activity of 1.7U/ml. The study shows promising bacteria capable of expressing lignocellulolytic enzymes and degrading lignocellulosic biomass present in municipal solid waste.

CHAPTER 1

INTRODUCTION

1.1 Introduction and Background

Worldwide, the large-scale production of a wide range of chemicals and synthetic polymers depends on fossil resources (Tsegaye et al., 2019). However, our fossil resources are dwindling, and their use poses devastating environmental effects. This has led to the search for alternative and viable sources which can replace fossil fuels and still provide valuable end products. One of the identified sources is lignocellulosic biomass, the most abundant renewable biomass on earth, which contains cellulose which is the most available organic polymer (Hongyan Chen et al., 2017; Sukumaran et al., 2005). Biomass is a foreseeable renewable source of energy which promises environmental sustainability (Singhania, 2011).

Some characteristics that make it attractive are its worldwide availability, non-competitiveness with food supplies, being significantly cheaper than crude oil and the ability to be produced quickly. It can also be gotten at a lower cost than other agriculturally important feedstocks, and these are the reasons it is considered as an ideal renewable feedstock to produce biofuels, commodity chemicals and polymers. It clearly exhibits great economic significance and environmental friendliness (Hongyan Chen et al., 2017). Lignocellulosic biomass is identified as an affordable, abundant, and green resource, a vital source for clean and alternative energy production which minimize our reliance on fossils (Ge et al., 2018; Isikgor & Becer, 2015; Qian, 2013; Zhou et al., 2011). Lignocellulose biomass contains lignin and polysaccharides such as cellulose, hemicellulose, pectin, ash, minerals, and salts (Tsegaye et al., 2019). Cellulose and hemicellulose comprise of different sugars while lignin is an aromatic

polymer. Cellulose and lignin are the two most abundant polymers (Allen et al., 2016; Jaramillo et al., 2015). Lignocellulose represents a valuable resource of largely unexplored sustainable carbon (Jaramillo et al.,2015).

One of the most important goals when lignocellulose is used in biorefinery is to break lignocellulose down into these significant components cellulose and lignin. Various pretreatment approaches have been developed to increase accessibility and biodegradability of these components for enzymatic or chemical action. Valuable compounds can be obtained through chemo catalytic or microbial production processes. This has driven the development of methods for the valorization of lignocellulosic biomass which has in turn favored improvements in the fields of chemical and microbial synthesis (Isikgor & Becer, 2015).

Using lignocellulosic biomass as feedstock to produce energy has become very appealing as it eliminates competition with food crops, and they are available in abundance naturally. Sources of lignocellulosic feedstock include agricultural residues and even municipal sludge, and these contain high organic contents (Den et al., 2018).

Studies have shown that lignocellulosic biomass can be broken down by several microorganisms (Kumar et al., 2008) since diverse fungal and bacterial genera produce cellulolytic and hemicellulolytic enzymes (Chukwuma et al., 2020) under aerobic and anaerobic conditions (Wongwilaiwalin et al., 2010). The use of enzymes from microbial sources is being preferred to others because they are not difficult to nurture and manipulate for desired yields when compared to other sources (Prakash et al., 2013). Bacterial and fungal organisms are considered the available biological organisms present in nature, with the ability to breakdown both manmade and natural polymers (Pathak & Navneet, 2017).

In lignin breakdown, fungi, especially white rot, have been extensively studied (Muaaz-Us-Salam et al., 2020). Despite the large amount of research that has established fungi as primary decomposers, their genetic manipulation capabilities for genetic engineering still remain very low as opposed to other organisms of interest. Fungal enzymes also do not show significant specificity and are costly to manufacture industrially. They also do not do well in extreme conditions as they cannot tolerate or adapt to altered environmental conditions (DeAngelis et al., 2013). Bacteria and the enzymes they produce have shown that they can adapt better to pH and temperature changes as opposed to fungi (Jones et al., 2018). Recent analysis of a synthetic microbial community structure revealed that the bacteria in the consortia are more important for lignocellulolytic enzyme activity than the fungi (Hu et al., 2017). Bacterial Glucuronoyl esterases (GEs) from carbohydrate esterase family 15 were biochemically characterized and structurally determined on model substrates to deepen the knowledge on their biological roles and functions. The results revealed few enzymes with higher catalytic efficiencies than previously characterized fungal GEs (Arnling Bååth et al., 2018). A halotolerant lignocellulose degrading microbial consortia was produced from salt marsh soil microbiome using wheat straw as carbon and energy source. The consortium showed bacteria have a prime role in the degradation of recalcitrant lignocellulose under saline conditions, as opposed to fungi. The final consortia showed greater lignocellulolytic haloenzymes than the initial one with the results affirming bacteria's central role in lignocellulose degradation in saline environment, as compared to fungi (Cortes-Tolalpa et al., 2018).

In recent times, research focus is turning to bacteria for lignocellulose conversion to useful products as a result of their ability and versatility (López-Mondéjar et al., 2019). To achieve success in the degradation of lignocellulose as a part of the production of

biofuels, it is necessary to have novel and efficient enzyme mixtures, consortia of microorganisms and appropriate use of bioengineering to improve promising strains and create microbial communities that can use synergistic relationship to breakdown the biomass. It is interesting that in nature bacterial abundance increases when simple carbon sources are diminished leaving their complex counterparts and higher lignin levels. These abilities to form relationships with other organisms, thrive in an environment where nutrient is a complex or limiting source, as well as a functional diversity, a wide range of terminal electron acceptors and lignin degrading abilities are responsible for the increased bacterial interest in future biotechnological strategies (Rosnow et al., 2017).

A lot of lignin degrading bacteria has come from guts of insects. Alphaproteobacteria, Gammaproteobacteria and Actinomycetes are some of the recognized species for breaking down lignin. Discovering novel bacterial lignin degrading enzymes is vital to industrial production of next-generation biofuels. This is largely due to bacteria's potential as engineered organisms involved in biofuel generation, their flexible oxygen demands and ability and range in extreme environmental conditions (DeAngelis et al., 2011). Time's effect on the synergism between lignocellulolytic enzymes during the hydrolysis showed aerobic bacteria are one of the groups with the most potential for breakdown of lignocellulosic material via a free enzyme system. Anaerobic bacteria on the other hand have a different type of lignocellulolytic system that involves complex protein structures like cellulosomes and xylanosomes (Malgas et al., 2017).

Bacteria also possess a handful of characteristics making them more advantageous in the production of hydrolytic enzymes, which are vital to the degradation of lignocellulosic biomass (Nilsson, 1998). Research findings from several researchers

shows bacteria-driven breakdown of lignocellulosic biomass with some identified bacteria including *Clostridium, Cellulomanas, Bacillus, Thermomonospora, Ruminococcucs, Bacteroides, Erwinia, Acetovibrio, Microbispora, and Streptomyces* (Vijayalaxmi et al., 2013). In the process of breaking down lignocellulose, bacterial cellulases and hemicellulases are known to have several advantages and is more effective when it comes to ease to culture, possibility of speeding up production and boosting expression (Taha, Shahsavari, et al., 2015). Bacterial strains generally have short generation times which means they can be grown with ease for further use in biofuel production. They can withstand environmental stress better as they are biochemically versatile with the ability to adapt to changes in temperature, salinity, pH, oxygen availability (Nilsson, 1998; Muaaz-Us-Salam et al., 2020)

1.2 Problem Statement

Lignocelluloses have received a lot of attention globally, in an attempt to develop technologies based on natural biomass and reduce dependence on expensive and exhaustible substrates fossil fuels (Isikgor and Becer, 2015; Saini et al., 2015). Agricultural, forest, and agro-industrial activities generate tons of lignocellulosic wastes annually, which present readily procurable, economically affordable, and renewable biomass for various lignocellulose-based industrial and biotechnological applications (Binod et al., 2010; Limayem & Ricke, 2012; Ravindran & Jaiswal, 2016b).

Unfortunately, the full potential of such biomass is normally underutilized and there is a large amount of waste involved. Waste generated in pre- and post-harvest agricultural losses and the food-processing industry end up in the landfills (Adrio &

Demain, 2014). Further limiting its mass utilization despite its promising outlook, is lignocellulose's physicochemically recalcitrant nature and costliness to process (Ezeilo et al., 2017; Woo et al., 2014a). Lignin removal is still a major issue to overcome as existing pre-treatment methods are costly and cumbersome. This prevents access to useful metabolites such as bio ethanol and enzymes which can be derived from Lignocellulosic materials (Limayem and Ricke, 2012; (Ravindran & Jaiswal, 2016a).

The landfill has a heterogenous nature that can contain large amounts of lignocellulosic materials which can be used for biomass conversion. Despite this, landfills microbial population have been barely explored and studied for this potential and possible resource (Yang et al., 2021). There is little data on biomass degrading communities in landfill sites as focus for identifying lignocellulose degraders has been on other communities (Huang et al., 2005; Yao et al., 2020). There is a need to provide taxonomic and functional characterization of microbial communities in landfills to help future planning of sustainable solutions to meet energy needs of an ever growing planet (Ransom-Jones et al., 2017)

In addition, lignocellulolytic enzymes are important in sustainable hydrolysis of lignocellulosic biomass for use in various applications (Deswal et al., 2012). To successfully breakdown lignocellulose biomass to produce biofuels, it is imperative to have novel and efficient enzyme mixtures, consortia of microorganisms, and appropriate use of bioengineering to improve promising strains and create microbial communities with synergistic relationship (Rosnow et al., 2017). Bacterial communities show great promise for lignocellulolytic breakdown but have been sparingly studied for this purpose.

1.3 Objective of the Study

The main objective of the study was to study the bacterial diversity of the landfill with a focus on possible lignocellulolytic potential. This was achieved by carrying out with the following specific objectives:

- To study the microbial diversity of lignocellulose degraders using culture independent approaches.
- To screen isolated microorganisms for ligninolytic and cellulolytic abilities and select the best based on their kinetic activity.
- To characterize and identify selected microorganisms in terms of enzyme production.
- To optimise and compare enzymatic activity between promising microorganisms.

1.4 Significance of the Study

This research was expected to provide a new insight on the bacterial diversity in the landfill and their potential in lignocellulose bioconversion. The findings in this work were aimed at finding the most prevalent bacterial groups which could be of biotechnological importance in the degradation of lignocellulosic waste. The significance of the study include:

I. extensive characterization and identification of lignocellulolytic bacteria present in the Pulau Burung landfill site of Malaysia.

II. screening for multi-enzymatic bacteria to find those that can express more than one enzyme and be useful in lignocellulose driven refinery.

III. generating information and recommendations for designing future consortia for complete degradation of lignocellulose.

1.5 Thesis Organization

The thesis contains five chapters that includes the Introduction, Literature review, Materials and Methodology, Results and discussion, and Conclusion. The introductory chapter gives an insight on the use of lignocellulose in biorefinery and the objectives and significance of the study. Chapter two is the literature review which details previous studies on lignocellulose driven refinery carried out by past researchers. It also covers lignocellulolytic bacteria and enzymes that have been documented in literature. Chapter three covers the materials and methods including the various scientific and data analysis used in the course of the study such as metagenomics and culture dependent methods of bacterial analysis. In chapter four the research findings are narrated with key bacterial strains discovered that showed potential for breakdown of lignocellulose into useful products. The thesis ends with the conclusions and future recommendations in Chapter five.

CHAPTER 2

LITERATURE REVIEW

2.1 Background

Energy is a necessity for civilization and also used to indicate a community's social and economic situation. A large part of the world's population is unable to access advanced energy sources and still use traditional methods such as burning wood and coal to meet their daily energy. Using these less advanced options for energy needs poses environmental and health problems and, hence, more sustainable and greener alternatives are needed (Surendra et al., 2014). The realisation that fossil resources are finite has led to increased research in finding to find alternatives to secure the future of humanity's energy needs (Matsakas et al., 2017).

Bioenergy is an emerging and feasible energy source tasked with meeting emerging and pressing energy demands that have come about as a result of fossil fuels' damaging effects and their imminent scarcity (Singh et al., 2012). With the steady incline of the global population (Dar et al., 2021; Rena et al., 2020) there is more pressure on the scientific community to proffer solutions to meet the enormous demands for fuel as people move from place to place (De Bhowmick et al., 2019). Interestingly, bioenergy is fed by a diverse range of readily available feedstock, most of whom are wastes, thus increasing their appeal (Singh et al., 2012).

Lignocellulose biomass represents a cheap renewable raw material whose entire biomass component can be used to produce economically viable end products. Bioenergy is geared to contribute to social and economic development in communities globally (Fazio & Barbanti, 2014) with a projection that bioenergy's contribution to the global energy pile will be between 20-30% by 2035. This has led to more countries embracing biomass from agricultural and forestry activities. The use of lignocellulose

biomass in second generation fuels is being hailed as a preferred option because it reduces land use and food supply concerns. First generation biorefineries may look to replace their key substrates with lignocellulosic wastes, to help reduce environmental and food concerns and lower cost because the existing infrastructures can be used for biofuel production as opposed to purchasing new set ups. Lignocellulosic biomass which is made up largely of crop residues, energy crops, animal manures and municipal solid wastes (MSW) can be converted and then utilised for bioenergy production (Dar et al., 2021). Lignocellulosic biomasses can be used for biofuel production (Rodríguez-Valderrama et al., 2020), or as a source of raw materials for producing natural and synthetic products and chemicals (Esparza et al., 2020).

Biodegradable biomass can be obtained in huge quantities across the entire food production line from harvesting, transportation, and storage to marketing and processing (Ramanujam, 2015). It is a carbohydrate rich biomass, highly degradable with high moisture content, and thus holds great appeal for sustainable bioenergy production. They contain high amounts of cellulose and hemicellulose but their lignin and protein contents are quite low in comparison (Ugwuanyi et al., 2009).

The disposal of these waste further adds cost to processors, and when improperly disposed of, it leads to major environmental problems (Sharma et al., 2016). The vast amounts of MSW being generated globally cause severe problems as the existing conventional disposal methods (landfills, incineration, composting) are fa from satisfactory. In addition to pollution, these wastes are usually disposed of and discarded in water bodies and on land/soil, where they pose environmental pollution and health concerns while emitting huge amounts of greenhouse gases (GHG) (Panda et al., 2016).

Managing this group of solid organic waste is a huge problem for waste management and government officials as they are linked with the spread of diseases,

bad smells in communities, contamination of water sources, and rodent infestation. Asides from affecting the environment and health, as the population increases, the real cost of managing this waste will begin to impact the standard of living of the people in the communities where they are generated (Chatterjee & Mazumder, 2020). Sustainable waste management practices are necessary to manage the useful organic fraction to successfully mitigate the existing problems they cause, from GHG emissions to soil and water pollution (Surendra et al., 2014). For these reasons, there is a strong need to find environmentally friendly, and cost-effective uses for these waste materials. If successfully done, it has positive repercussions economically and to the environment.

The suggestion that around 1% of microbiota can be cultured under controlled laboratory conditions has fuelled microbiologists to look into other approaches that greatly differ from traditional culture methods to increase this data. Some of these methods are high throughput extinction culturing, high throughput single-cell encapsulation, diffusion chambers, use of various gelling agents, antioxidants, or signaling molecules, increase in the incubation times along with reducing nutrient concentrations or simple alterations in the media preparation, soil printing or biological laser printing (BioLP). Using metagenomics approaches has allowed a probing of select environments which has giving new and clearer understanding of a plethora of ecological systems and diversity that exists in these environments (Jaswal et al., 2019). Metagenomic approaches are used to gather information on the unculturable fraction of environmental ecosystems, as the metagenomic DNA (mDNA) is taken directly from the environmental sample and then high throughput sequencing technologies are used to analyse the mDNA obtained and to identify potential enzymes and other useful functions (Jackson et al., 2022). It has been established that culture-independent techniques need to be employed to boost culture-dependent techniques, which are

limited in output, to garner more information on microbial communities and their possible function in the environment (Riesenfeld et al., 2004).

2.2 Lignocellulose Biomass

Lignocellulosic biomass is viewed as dry plant matter from agricultural, forestry and industrial activities. Biomass from agriculture and forest related sources are richly available and most promising (Cai et al., 2017).

Lignocellulosic biomass is affordable, superabundant, and green materials, that are vital in reaching the goal of clean and alternative energy production and minimizing our reliance on fossils (Ge et al., 2018; Isikgor & Becer, 2015; Qian, 2013; Zhou et al., 2011). They include kitchen residue, sewage sludge, farm residue, environmental refuse, livestock waste, organic fraction of municipal solid residues, vegetable market wastes, agricultural and food wastes, animal manure, cow dung, mango pulp, leaves, rice husks, and peels (Fajobi et al., 2022). Other lignocellulose biomass examples are miscanthus, wheat bran, hazelnut shell, rice husk, and poplar wood (Kok & Ozgur, 2017). Table 2.1 below shows the various compositions of lignin, cellulose and hemicellulose in selected biomass (Baruah et al., 2018)

Table 2.1 Lignin, cellulose, and hemicellulose composition in various lignocellulosic biomass

Lignocellulosic Biomass	Percentage (%)			References
	Cellulose	Hemicellulose	Lignin	
Bamboo	45	24	20	(X. Li et al., 2015)
Barley straw	38	35	16	(Sun et al., 2005)
Coastal Bermuda Grass	30	29	23	(Lee et al., 2009)
Corn Strover/cob	38-41	23-31	12-20	(S. Chen et al., 2010; Wan & Li, 2010)
Elephant Grass	36	24	28	(Scholl et al., 2015)
Napier Grass	47	31	22	(Reddy et al., 2018)
Pine	42	21	30	(Sannigrahi et al., 2010)
Poplar	44	20	29	(Y. Kim et al., 2009)

Rice straw /Husk	37-38	29-32	12-24	(Kalita et al., 2015; Lu & Hsieh, 2012)
Soybean straw	34	16	22	(Wan et al., 2011)
Sugarcane bagasse	40-45	30-35	20-30	(Cardona et al., 2010)
Sweet sorghum bagasse	45	27	21	(M. Kim & Day, 2011)
Wheat straw	33-40	20-25	15-20	(Talebnia et al., 2010)

2.2.1 Lignocellulose structure

Lignocellulosic biomass is made up mainly of three polymers: cellulose, hemicellulose, and lignin alongside pectin, protein, extractives, and ash in smaller amounts. The association of these polymers with one another varies depending on the type, species, and source of the biomass (Bajpai, 2016). The cell wall's framework is dependent on the arrangement of cellulose molecules which is usually regular in bundles. The bonding between cellulose, hemicellulose, and lignin, which are usually present in a ratio of 4:3:3, is usually different. There is an extra chemical bonding between hemicellulose and lignin, which results in lignin, isolated from natural lignocelluloses to always contain a small amount of carbohydrates (Hongzhang Chen, 2014).

2.2.2 Composition of lignocellulosic feedstock

Lignocellulosic substances content in feed stocks such as animal manure and crop residues is known to be higher than that in fruit and vegetable wastes (FVW) (Paudel et al., 2017). FVW are rich in cellulose, hemicellulose, pectin, and minerals but have very low concentrations of lignin compared to other crop residues. FVW is characterised by low lipid content but a high cellulose content. They also have higher pH and Carbon- nitrogen (C/N) ratio than other food wastes, but their lignocellulosic materials are more resistant to microbial attacks and impede acidification (Bong et al., 2018). FVW are known to be low in total solids (TS) and high in volatile solids (VS) (Shen et al., 2013). Fruit waste has a higher TS, VS, and C/N ratio than vegetable wastes. The TS for fruits is usually around 7.5–23% (wet weight basis), while TS for vegetables is between 3–11%. VS of fruits at 5–12% exceeds that of vegetables at 2–9%. Then C/N ratio of fruit wastes is 19–53% higher than that of vegetables at 10–21% (Esparza et al., 2020).

FVW residue after processing contains pulp, peel, seeds, and stem. The main components of this waste is pectin that accounts for 1-13%, 7-44% cellulose, 4-34% hemicellulose, 15-69% lignin, and trace amounts of gums (Szymańska-Chargot et al., 2017). These components affect the value that any particular FVW can produce. They are the major factors that make up the cell wall saccharification potential of a lignocellulosic biomass feedstock which drives its suitability in the biorefinery process. This, in turn, affects the extent and efficiency of hydrolysis and other steps in the degradation process (Szymańska-Chargot et al., 2017).

FVW have high moisture content, which alongside high VS (>90%), makes it a very suitable choice for anaerobic digestion (AD) to produce organic products (B. Chatterjee & Mazumder, 2020).

2.2.3 Pretreatment

Native lignocellulosic biomass in its natural form has complex structural and compositional factors that make it recalcitrant and difficult to be hydrolyzed by enzymes (Tomás-Pejó et al., 2011). Thus, pretreatment is needed to overcome these drawbacks and is a vital tool in the breakdown of lignocellulose biomass to useful products (Mosier et al., 2005). Pretreatment could either be physical, chemical biological or a combination of methods. It is dependent on the feedstocks, enzymes and organisms involved and how compatible they are together (Hongyan Chen et al., 2017). Table 2.2 shows the various types of pretreatment processes grouped under physical, chemical, physico-chemical and biological.

Table 2.2 Summary of various pre-treatment methods

Pre Treatment Process	Physical	Chemical	Biological	Physico-chemical
	Milling	Alkaline hydrolysis	Enzymatic pre-treatment	Steam explosion
	Microwave	Acid hydrolysis	Whole cell pre-treatment	Ammonia fiber explosion (AFEX)
	Extrusion	Ionic liquids		Carbon dioxide explosion
	Ultrasonication	Organosolv process		Liquid hot water
		Deep eutectic solvents		

Biological pretreatment is done with microorganisms that secrete specific enzymes with lignocellulolytic ability. It is also possible to do it with the enzymes that these microorganisms have pre-produced (Vasco-Correa et al., 2016). Pretreatment is to break down the lignin structure and disrupt the crystalline structure of cellulose to increase enzyme accessibility. It is done to change the structure in the biomass as depicted in Figure 2.1. It makes the cellulosic component more accessible to enzymatic

hydrolysis into fermentable sugars (Mosier et al., 2005). In addition to making the biomass more digestible, biological pretreatment has other uses in biorefinery. The enzymes it produces can be extracted for use in other things. Other products and derivatives can be used as chemicals and industrial compounds. Microorganisms are biocatalysts for the biorefinery process, as fungi or bacteria can handle microbial breakdown of lignocellulose (Vasco-Correa et al., 2016).

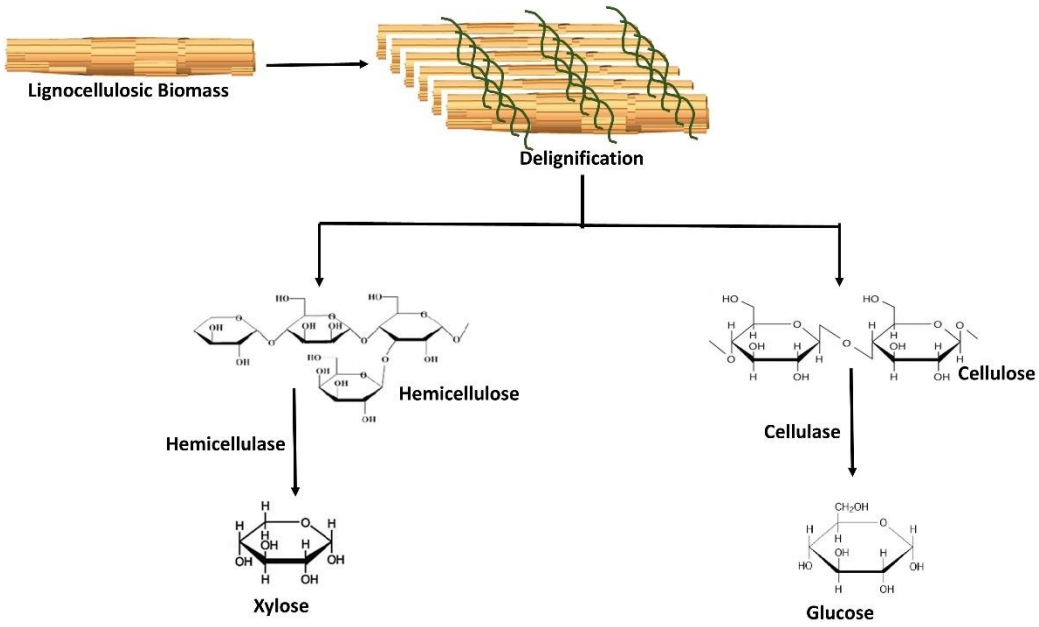

Figure 2.1 Breakdown of lignocellulose into sugars (Chukwuma et al., 2020)

2.3 Lignocellulolytic Bacteria

In recent times, research focus is turning to bacteria for lignocellulose conversion to useful products as a result of their ability and versatility (López-Mondéjar et al., 2019). Bacteria are among the first and simplest life forms on earth. They are ubiquitous, vital in nutrient cycling, and in maintaining the earth's balance through involvement in various natural processes (Ho et al., 2020). In the process of breaking down lignocellulose, bacterial cellulases and hemicellulases are known to have several

advantages including ease to culture, possibility of speeding up production and boosting expression (Taha, Kadali, et al., 2015). Bacterial strains generally have short generation times which means they can be grown with ease for further use in biofuel production. They can withstand environmental stress better as they are biochemically versatile with ability to adapt to changes in temperature, salinity, pH, oxygen availability (Daniel, 1998; Muaaz-Us-Salam et al., 2020).

Bacterial growth at the latter stage of lignocellulose breakdown can increase, which is especially useful as this stage is known to have materials usually difficult to breakdown. Bacterial enzymes are known to be effective between several pH ranges and their high growth rates leads to high rates of enzymes being produced. Bacterial lignocellulases form multienzymatic complexes that are more suited for the complex degradation of biomass (López-Mondéjar et al., 2019).

Studies have shown that lignocellulosic biomass can be broken down by contributions from several microorganisms (Kumar et al., 2008) with manifold fungal and bacterial genera giving rise to cellulolytic and hemicellulolytic enzymes (Chukwuma et al., 2020) under aerobic and anaerobic surroundings to achieve this (Wongwilaiwalin et al., 2010). The use of enzymes from microbial sources is being preferred to others because they are not difficult to nurture and manipulate for desired yields when compared to other sources (Prakash et al., 2013). Bacteria and fungi have the ability to breakdown both manmade and natural polymers (Pathak & Navneet, 2017).

Bacteria and the enzymes they produce have shown that they can adapt better to pH and temperature changes as opposed to fungi (Jones et al., 2018). Recent analysis of a synthetic microbial community showed that bacteria in the overall structure are more significant to lignocellulolytic enzyme activity than fungi (Hu et al., 2017).

Bacterial Glucuronoyl esterases (GEs) which arise from carbohydrate esterase family 15 were biochemically characterized and structurally determined on model substrates deepen the knowledge on their biological roles and functions and the results revealed few enzymes with higher catalytic efficiencies than previously characterized fungal GEs (Arnling Bååth et al., 2018). A halotolerant lignocellulose degrading microbial consortia was produced from salt marsh soil microbiome using wheat straw as carbon and energy source. The consortium showed bacteria possess a unique role in the breakdown of recalcitrant lignocellulose under saline conditions, as opposed to fungi. The final consortia showed greater lignocellulolytic haloenzymes than the initial one with the results affirming bacteria's more central role in lignocellulose degradation in saline environment, as compared to fungi (Cortes-Tolalpa et al., 2018).

To achieve success in degradation of lignocellulose biomass in the production of biofuels, it is necessary to have novel and efficient enzyme mixtures, consortia of microorganisms and appropriate use of bioengineering. Bioengineering can improve promising strains and create microbial communities that can use synergistic relationship with each other to breakdown the biomass. It is interesting that in nature bacterial abundance increases when simple carbon sources are diminished leaving their complex counterparts and higher lignin levels. These reasons, as well as, a functional diversity, a wide range of terminal electron acceptors and the lignin degrading abilities are responsible for the increased bacterial interest in future biotechnological strategies (Rosnow et al., 2017). A lot of research on lignin degrading bacteria has come from guts of insects. Alphaproteobacteria, Gammaproteobacteria and Actinomycetes are some of the recognized species for breaking down lignin from the gut. Discovering novel bacteria with lignin degrading abilities is vital to industrial production of next-generation biofuels. This is largely due to bacteria's potential for use as engineered

organisms involved in biofuel generation, their flexible oxygen demands and ability and range in extreme environmental conditions (DeAngelis et al., 2011). Recent work involving bacteria is shown in Table 2.3. Time's effect on the synergism that exists among lignocellulolytic enzymes involved in hydrolysis showed aerobic bacteria are one of the groups with the prevalent mechanism for the breakdown of lignocellulosic biomass via the free enzyme system. Anaerobic bacteria instead use an alternative lignocellulolytic system that makes use of complex protein structures like cellulosomes and xylanosomes which are supporting enzymes when biomass is hydrolyzed (Malgas et al., 2017). Bacteria also possess a handful of characteristics that make more advantageous in the production of hydrolytic enzymes, which are vital to the degradation of lignocellulosic biomass (Daniel, 1998). Research findings from several researchers shows the bacteria-driven breakdown of lignocellulosic biomass was facilitated by *Acetovibrio, Bacillus, Bacteroides, Cellulomanas, Clostridium, Erwinia, Microbispora, Ruminococcus, Streptomyces* and *Thermomonospora* amongst others (Vijayalaxmi et al., 2013).

Table 2.3 Recent bacterial studies using lignocellulose biomass in biorefinery of various products (Chukwuma et al., 2021b).

S/N	Bacteria Strain	Substrate (Biomass)	Degradation % (Yield)	Degradation time	Method of Analysis	Product	References
1	*Pantoea ananatis Sd-1*	Pesticide carbaryl, rice straw	45	24 hours	Enzyme assays	Reducing sugars	(Yao et al., 2020)
2	*Firmicutes, Proteobacteria*	Wheat, rice, sugarcane, and pea ball-milled straws	-	84 hours	Biolog (MT2) microplate system	Cellulase and xylanase	(Taha, Kadali, et al., 2015)
3	*Aeromonas*	Wheat, rice, sugarcane, and pea straw	100	72 hours	Enzyme assays &	Strawase,	(Taha, Shahsavari, et

	hydrophila, Pseudomonas poae, Streptomyces thermoviolaceus, Klebsiella oxytoca, Bacillus amyloliquefaciens				genomics	cellulase	al., 2015)
4	*Streptomyces, Bacillus and Paenibacillus*	Saw dust	40-100	7-10 days	Enzyme assays & genomics	Cellulase	(Buraimoh, et al., 2015)
5	*Opitutus terrae, Spirosoma linguale, Solibacter usitatus*	Lignite, molasses	NA	5 days	Biogas and organic acid	Fermentation	(Arnling Bååth et al., 2018)
6	*phosphate solubilizing (PSB) and potassium solubi*	Bagasse	NA	7 days 28 days	Carbon dioxide	Randomized blocks design and assays	(Setiawati et al., 2019)

	lizing (KSB) bacteria						
7	Ochrobactrum sp.	Palm oil mill effluent	71	6 days	Aerobic treatment	CMCase and xylanase	(Neoh et al., 2016)
8	Pantoea ananatis Sd-1	Rice straw	46	3 days	Enzyme assays, Fenton chemistry	Biofuels	(Jiangshan Ma et al., 2016)
9	*Bacillus, Streptomyces, Burkholderia*	Beech wood	NA	7 days	Enzyme assays.	Xylanase	(Gong et al., 2017)
10	*Dickeya sp. WS52*	Sweet Pepper and Tomato Stalk	NA	4 days	Enzymatic Hydrolysis and genomics	CMCase and pectinase	(Y. J. Yang et al., 2019)
11	*Clostridium sp., Petrimonas sp., Methanosarcina sp. and Methanospirillum sp.*	Palm oil mill effluent (POME)	NA	18 hours	PCR-DGGE and fermentation	Methane	(Nuntongkaew et al., 2020)
12	*Streptomyces lividans*	Sunflower stalks and rape straw	N/A	6 days	Fatty acid profiling	Triacylglycerol	(Dulermo et al., 2016)
13	*Bacillus, Enter*	Rice straw	N/A	4 days	Enzyme activity assay	Cellulase	(Hu et al., 2017)

	ococcus, Lactococcus, Afipia, Alkaliphilus, Burkholderia, Erwinia, Geobacillus, Ralstonia, Rhodanobacter, Sediminibacterium, and Streptococcus.						
14	*Firmicutes, Actinobacteria, Proteobacteria and Bacteroidetes*	Molasses	NA	124 days	Organic acids and other compounds	Fermentation	(Detman et al., 2018)
15	*Proteobacteria, Firmicutes and Bacte*	Corn stover				Enzymes	(N. Zhu et al., 2016)

	roidetes						
16	*Pseudomonas sp. GO2.*	Corn stover	99.8	131 hours	Fermentation	Bioflocculant	(H. Guo, Hong, et al., 2017)
17	*Geobacillus sp. strain WSU CF1*	Prairie cordgrass	100	120 hours	Single pot bioconversion	Biohydrogen	(Bibra et al., 2018)
18	*Streptomyces sp. MDS*	Rice waste biomass, wood straw, local grass powder, sugar cane barboja and sugar cane bagasse	6	6 days	Solid state fermentation	Endoglucanase, exoglucanase, cellobiases, filter paperase, amylase, and xylanase	(Saratale et al., 2017)
19	*Bacillus sp. BS-5*	Corn cob	NA	72 hours	Enzymes assays	Xylanase, endoglucanase	(J. Xu et al., 2017)
20	*Paenibacillus, Streptomyces*	Switch grass	NA	10 days	Solid-state and submerged-state cultivation	Biofuel	(Jain et al., 2017)
21	Actinobacteria	Olive pomace	NA	6 days	Submerged fermentation	Laccase, xylanase	(Medouni-Haroune et al., 2017)
22	*Bacillus sp. K1*	Wheat Bran	44	24 hours	Lipid extraction and enzyme assays	Lipid, biodiesel	(H. Guo, Chen, et al., 2017)
23	*Bacillus sp. G0*	Miscanthus	88	100 hours	Pre-treatment	Xylanase	(H. Guo, Wu, et