EFFECTS OF PRETREATMENTS ON CHEMICAL AND PHYSICAL ALTERATIONS OF OIL PALM (*Elaeis guineensis*) EMPTY FRUIT BUNCH FOR ENHANCING ENZYMATIC SACCHARIFICATION YIELD

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by

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LIST OF SYMBOLS

α	Alpha
~	Approximately
β	Beta
cm	Centimetre
°	Degree
°C	Degree Celsius
°C/min	Degree celsius per minute
$	Dollar
eV	Electronvolt
γ	Gamma
g	Gram
g/L	Gram per litre
g/mol	Gram per mole
kHz	Kilohertz
kV	Kilovolt
λ	Lambda
<	Less than
L	Litre
MHz	Megahertz
MPa	Megapascal
m	Metre
µ	Micro
µL	Microlitre
µm	Micrometre
µmol	Micromole

mA	Milliampere
mg/g	Milligram per gram
mL	Millilitre
ml/min	Millilitre per minute
mm	Millimetre
M	Molarity
mol dm^{-3}	Moles per cubic decimeter
>	More than
nm	Nanometer
ln	Natural logarithm
N	Normality
%	Percent
±	Plus minus
psi	Pounds per square inch
rpm	Revolutions per minute
K	Scherrer constant
θ	Theta
V	Voltage
v/v	Volume per volume
W	Watt
w/v	Weight per volume
w/w	Weight per weight

LIST OF ABBREVIATIONS

AFEX	Ammonia fibre expansion
ANOVA	Analysis of variance
ATR	Attenuated Total Reflectance
AH	Autohydrolysis
BRF	Brown rot fungi
CBH	Cellobiohydrolase
CPS	Chemical-physical sequence
R^2	Coefficient of determination
CS	Crystallite size
CrI	Crystallinity index
DES	Deep eutectic solvent
DNS	Dinitrosalicylic assay
EG	Endoglucanase
ES	Enzymatic saccharification
FPU	Filter paper unit
E.g.	For example
FTIR	Fourier-Transform Infrared Spectroscopy
FFB	Fresh fruit bunch
FWHM	Full width at half maximum
HPLC	High Performance Liquid Chromatography
HMF	Hydroxymethylfurfural
LOI	Lateral order index
LHW	Liquid hot water
OPEFB	Oil palm empty fruit bunch
OD	Oven dry
O/C	Oxygen: carbon ratio
PCS	Physical-chemical sequence
KHP	Potassium hydrogen phthalate
Py-GCMS	Pyrolysis-Gas Chromatography Mass Spectrometry
RID	Refractive Index Detector
SEM	Scanning Electron Microscopy

$NaClO_2$	Sodium chlorite
NaOH	Sodium hydroxide
SRF	Soft rot fungi
SE	Steam explosion
H_2SO_4	Sulphuric acid
SLC	Surface lignin coverage
S/G	Syringyl: guaiacyl ratio
TAPPI	Technical Association of the Pulp and Paper Industry
TGC	Theoretical Glucose Concentration
TGY	Total Glucose Yield
H_2O	Water
WRF	White rot fungi
XRD	X-ray Diffraction
XPS	X-ray Photoelectron Spectroscopy
BG	β-glucosidase

LIST OF APPENDICES

KESAN PRA-RAWATAN KE ATAS PERUBAHAN KIMIA DAN FIZIKAL TANDAN KOSONG KELAPA SAWIT (*Elaeis guineensis*) UNTUK MENINGKATKAN HASIL SAKARIFIKASI ENZIMATIK

ABSTRAK

Kesan langsung perubahan komposisi kimia dan sifat fizikokimia dalam biojisim yang telah dipra-rawat terhadap keberkesanan hasil sakarifikasi enzimatik belum dijelaskan secara menyeluruh. Oleh itu, kajian ini menyiasat kesan komposisi kimia lignoselulosa dan sifat fizikokimia gentian tandan kosong kelapa sawit (OPEFB) yang telah dipra-rawat terhadap sakarifikasi enzimatik (jumlah hasil glukosa, TGY). Tiga pra-rawatan akueus ringkas (autohidrolisis, pra-rawatan asid, dan pra-rawatan alkali) serta penghalusan mekanikal telah diaplikasikan pada gentian tandan buah kosong kelapa sawit (*Elaeis guineensis*) (OPEFB) untuk meningkatkan keberkesanan hidrolisis enzimatik oleh enzim selulolitik. Pra-rawatan akueus dijalankan pada suhu yang tertentu selama 45 minit dengan nisbah pepejal: cecair 1:7. Gentian OPEFB yang tidak dipra-rawat dan yang telah dipra-rawat telah disakarifikasikan secara enzimatik pada keadaan tetap 50°C dan pH 4.8 selama tempoh 48 jam dengan kepekatan substrat 25 g/L dan dos enzim 70 FPU/g substrat Celluclast 1.5 L. Hasil kajian mendapati bahawa menjalankan pra-rawatan akueus pada gentian OPEFB yang belum dihaluskan menyebabkan degradasi dan larutan lignoselulosa yang lebih rendah berbanding dengan gentian yang telah dihaluskan terlebih dahulu, menyebabkan kehilangan selulosa yang minimum dan TGY yang lebih tinggi. Berdasarkan analisis komposisi kimia gentian OPEFB dan hasil sakarifikasi enzimatik/ jumlah hasil glukosa (TGY) melalui HPLC, gentian OPEFB yang telah dipra-rawat secara autohidrolisis mencapai TGY maksimum (> 99.9%), disebabkan oleh penyingkiran hemiselulosa sebanyak 77% dan lignin sebanyak 24%, dengan hanya kehilangan selulosa sebanyak 16% semasa pra-rawatan. Selain itu, perubahan kimia dan fizikal gentian OPEFB yang telah dipra-rawat dinilai oleh pelbagai analisis instrumental—SEM, FTIR, XRD, Py-GCMS dan XPS, dikaitkan dengan TGY berdasarkan kepekatan glukosa teoretikal (TGC). Di antara semua analisis instrumental, hanya indeks kekristalan (CrI) menunjukkan korelasi yang signifikan dengan TGY berdasarkan kepekatan glukosa teoretikal (TGC), manakala morfologi, kumpulan berfungsi, saiz kristalit, nisbah S/G dan nisbah O/C tidak relevan seperti yang ditunjukkan oleh tren rawak. Tambahan pula, gentian OPEFB dengan CrI dalam julat 30 hingga 40 telah dikenal pasti boleh mencapai TGY berdasarkan TGC menghampiri 100%. Kajian ini mengesahkan bahawa kebanyakan analisis fizikokimia yang biasa dicadangkan bagi gentian OPEFB mempunyai hubungan yang tidak signifikan dengan keberkesanan sakarifikasi enzimatik kecuali indeks kekristalan.

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ABSTRACT

The direct impacts of chemical composition and physicochemical properties alterations in pretreated biomass on the effectiveness of enzymatic saccharification yield have not been comprehensively elucidated. Therefore, this study investigates the effects of lignocellulose chemical composition and physicochemical properties of pretreated oil palm empty fruit bunch (OPEFB) fibre on enzymatic saccharification (total glucose yield, TGY). Three simple aqueous pretreatments (autohydrolysis, acid pretreatment and alkaline pretreatment) and mechanical refining were applied on oil palm empty fruit bunch (*Elaeis guineensis*) (OPEFB) fibres to enhance its enzymatic hydrolysability by the cellulolytic enzyme. The aqueous pretreatments were conducted at appropriate temperatures for 45 minutes with a solid: liquid ratio of 1:7. The refined untreated and pretreated OPEFB fibres were enzymatically saccharified at a fixed condition of 50°C and pH 4.8 for a duration of 48 hours with a substrate concentration of 25 g/L and an enzyme dosage of 70 FPU/g substrate of Celluclast 1.5 L. The results found that conducting aqueous pretreatment on unrefined OPEFB fibres caused lower lignocellulose degradation and dissolution than the pre-refined one, resulted in minimum cellulose loss and higher TGY. Based on the chemical composition analysis of OPEFB fibres and their enzymatic saccharification/ total glucose yield (TGY) by HPLC, autohydrolysed OPEFB fibre achieved the maximum (> 99.9%) TGY, attributed to 77% hemicellulose and 24% lignin removals, with only 16% cellulose loss during pretreatment. Moreover, the chemical and physical alterations of pretreated OPEFB fibres evaluated by various instrumental analyses—SEM, FTIR, XRD, Py-GCMS and XPS, were correlated with TGY based on theoretical glucose concentration (TGC). Among all the instrumental analyses, only the crystallinity index (CrI) showed a significant correlation with the TGY based on theoretical glucose concentration (TGC), whilst morphology, functional groups, crystallite size, S/G ratio and O/C ratio were irrelevant as shown by random trends. Additionally, OPEFB fibre with CrI in a range of 30 to 40 was identified could achieve TGY based on TGC approaching 100%. This study ascertains most of the commonly proposed physicochemical analyses of OPEFB fibre possess insignificant relationships to the effectiveness of enzymatic saccharification except crystallinity index.

CHAPTER 1

INTRODUCTION

1.1 General Background

By 2055, the world population is anticipated to grow from 7.4 billion to 10 billion, which might cause natural resources to deplete, especially the petroleum-based resources. Therefore, it is expected that by 2030, human being consumption of biomass materials annually would have enlarged to 100 gigatons from its present level of about 72 gigatons (Ashokkumar et al., 2022). As a result, there is a budding pursuit in evolving novel biorefinery technologies for the generation of bioenergy and biopolymers from renewable resources. Hereby, circular bioeconomy theories emphasis on preserving the long-term utilisation of renewable biomass resources and biotechnology, limiting pollution on both the end-product and the environment besides ensuring food security while creating jobs for humanity (Tong et al., 2022).

Considering this, global interest in lignocellulosic biomass as a viable substitute for fossil carbon resources to manufacture second-generation biofuels and other bio-based chemicals without jeopardising the world food security is still cultivating (Zoghlami & Paës, 2019). The idea of "Sugar is the new oil" acknowledges the possibility of efficient conversion of recalcitrant plant materials known as lignocellulosic biomass into sugars, for further generating multiple valuable products. Ultimately, this transformation is a key segment of a process termed lignocellulose biorefinery. If successful, this change could significantly modify the technique we acquire essential products such as chemicals, materials and biofuels from plant sources in an environmentally friendly manner, making it more sustainable for our society and its growth (Chandel et al., 2018).

Global biofuel production has surged to above 192 billion litres annually and is forecasted to keep growing progressively whereby, the United States topped with over 38% and followed by Brazil at 21.4% in year 2022. The primary biofuels produced are bioethanol and biodiesel, the former serving as an additive in or substitute to gasoline and the latter as an alternative for diesel, respectively. In 2022, bioethanol continued to dominate production, achieving 129.4 billion litres, with expectations to escalate over 147 billion litres by 2030, a 17 billion increase from 2022. The United States is leading the bioethanol production with 15.4 billion gallons (58.3 billion litres) and followed by Brazil with 7.5 billion gallons (28.4 billion litres) in year 2022. The first-generation bioethanol, an alcohol derived from starch or sugar-based feedstocks such as sugarcane, sugar beet, coarse grain, maize and wheat which varies regionally, is chiefly blended with gasoline at ratios ranging from 5% (E5) to 25% (E25) for regular gasoline-powered whereas blends such as E85 (85%) and E100 demand specially fitted engines (Statista, 2024).

In spite of this, the development of first-generation biofuels arisen a conflict amongst the energy and food industries for the control of raw resources, and thus driving up food costs. A workable solution to the "food or fuel" debate could be the development of second-generation biofuels derived from lignocellulosic biomass. Advanced biofuels developed from lignocellulosic biomass possess the potential to help accomplish three Sustainable Development Goals (SDGs): Climate Action (SDG 13), Industry, Innovation, and Infrastructure (SDG 9), and Sustainable Consumption and Production (SDG 12) (Sharma et al., 2023; Valladares-Diestra et al., 2022). However, the recalcitrant nature of biomass hinders the ability to convert it into fuel ethanol. In order to achieve maximum fuel conversion while also making biorefinery applications cost-effective, a systematic biomass pretreatment for improving enzymatic hydrolysis/ saccharification is essential (Moyer et al., 2018).

In fact, biomass recalcitrance has influenced structure-related factors such as cellulose specific surface area, cellulose crystallinity, degree of polymerisation of cellulose chain, pore size and volume and chemical-related factors that can be distinguished from each composition and content in cellulose, lignin and hemicellulose (Zoghlami & Paës, 2019). In light of this, the rise of the biorefinery platform demands the availability of economical sugar streams for downstream biological and/or chemical conversion whereby, biomass pretreatment followed by enzymatic saccharification has been viewed as a promising technique to obtain sugars from biomass. However, two areas still require improvement in order to enhance the sugar-based biorefinery operations. Firstly, an eco-friendly pretreatment process which is vital to overcome the recalcitrance of lignocellulose should be successfully developed (Luo et al., 2019). Secondly, it is nevertheless important to unveil the effect (physical and chemical alterations) of different types of biomass pretreatment on the enzymatic accessibility and saccharification efficiency.

Since lignocellulosic biomass feedstocks are diverse, it is challenging to devise a universal pretreatment method for all of them. Several pretreatment approaches comprising physical pretreatments, chemical pretreatments, hydrothermal pretreatments, biological pretreatments or combination of pretreatments have been proposed throughout the years in biomass conversion research area (Arce & Kratky, 2022; Ávila-Lara et al., 2015; Awoyale & Lokhat, 2021; Baral & Shah, 2017; Buratti et al., 2018; Gundupalli et al., 2022; Gundupalli et al., 2022; Jeong et al., 2022; Loow et al., 2016; Luo et al., 2014; Luo et al., 2021; Yang et al., 2023). The pretreatment method used for pretreating the biomass has a substantial impact on the yield of cellulosic sugar and subsequently, bioethanol production (Awoyale & Lokhat, 2021). Thus, an effective pretreatment method should minimise the polysaccharides (cellulose) loss and the formation of inhibitors for subsequent enzymatic saccharification and microbial fermentation.

Among existing aqueous pretreatment methods, dilute acid (H_2SO_4), alkaline (NaOH), hydrothermal pretreatment methods such as autohydrolysis and steam explosion are among the most widely investigated biomass pretreatments in various feedstocks, such as agricultural wastes, grasses, hardwoods etc. (Ávila-Lara et al., 2015; Lee et al., 2015; Valladares-Diestra et al., 2022). On the other side, multiple researchers have proposed to employ instrumental analyses in order to characterise the physicochemical alterations or changes in biomasses after undergoing pretreatments as evaluating chemical composition solely may not be sufficient to determine the outcome of various pretreatments to correlate and verify its effect on enzymatic saccharification efficiency.

Biomass analysis using several analytical methods can provide extensive data regarding its properties for enhancing yield and recovery whereby, characterising biomass both before and after pretreatments is crucial to understand the conversion process, its mechanism and design aspect. Nonetheless, identifying the exact mechanisms or components of biomass that are relevant to the enzymatic yield remains a significant obstacle to studying the impact of biomass pretreatments and quantifying the influencing factors for subsequent process (Ha et al., 2020; Hoang et al., 2021; Kumar et al., 2023; Yiin et al., 2018).

1.2 Justification/ Problem Statements

Globally, approximately 11.9 billion tons of biomass (on a dry basis) are generated yearly, with 7.26 billion tons (equivalent to 61%) originating from agricultural waste sources (Suhartini et al., 2022). Only from oil palm industry, almost 200 million metric tons of biomass are generated annually (Tye et al., 2016b) and more than 87 million metric tons are contributed by oil palm empty fruit bunch (OPEFB) fibre in 2022 (US Department of Agriculture & Foreign Agricultural Service, 2022). Thus, research on utilising OPEFB fibre for various types of products such as papermaking pulp (Sunday Noah, 2022), dissolving pulps (Chong et al., 2013; Harsono et al., 2016; Leh et al., 2008), cellulose derivative (Rama Rao & Ramakrishna, 2022) and fibrous filler for composites production (Almeida-Naranjo et al., 2022; Hassan et al., 2018; Rama Rao & Ramakrishna, 2022) as well as for the production of biofuel (Kaniapan et al., 2021) have been reported. In order to enhance the effectiveness of converting cellulose from OPEFB fibre into fermentable sugar, studies of the effects of various pretreatments and their optimization on augmenting the enzymatic hydrolysability of OPEFB fibre have been carried out extensively (Darus et al., 2022; Fatriasari et al., 2021; Nurdin et al., 2021; Tye et al., 2017).

The research found that the types of pretreatments employed will have significant impacts on the chemical composition of OPEFB fibre and the efficiency of enzymatic saccharification particularly, total glucose yield based on untreated biomass. Total glucose yield based on untreated biomass will provide a clear picture on the percentage of cellulose that will be converted from the cellulose of the original, raw biomass, which has not been reported in previous studies by other researchers such as Gundupalli et al. (2022); Valladares-Diestra et al. (2022); Yang et al. (2023). Therefore, this approach guides in evaluating the percentage of cellulose that had been degraded and lost during the preceding pretreatment processes (cellulose loss) and failed to be converted into sugar.

Furthermore, biomass size influenced by pretreatment sequences also plays a key role in influencing the efficiency of chemical pretreatments. It has been reported that variations in biomass sizes impact the effectiveness of pretreatment in enhancing the subsequent enzymatic saccharification process. This phenomenon is mainly attributed to differences related to chemical penetration into the biomass during pretreatment, leading to varying degrees of alteration in the chemical and/or physical properties of the treated biomass. Additionally, in biomass treatment for papermaking pulp, hybrid pulping, which involves chemical treatment followed by mechanical refining, exhibits improved energy efficiency. Therefore, it is vital to compare the pretreatment effect on both unrefined and pre-refined biomass properties, as well as their respective impact on the saccharification yield (Liu et al., 2013).

Although the close relationship between the chemical composition of the OPEFB fibre and the total glucose yield has been comprehensively stated, there was less discussion on other fundamental alterations in OPEFB fibre examined by using analytical instruments that occurred during pretreatment, which have direct effects on the enzymatic saccharification as well as glucose yield based on untreated biomass.

Many previous research findings proposed that structural or physical destruction of biomass (examined by Scanning Electron Microscopy, SEM) caused by pretreatments have remarkable effects on increased enzymatic accessibility and hydrolysability (Abraham et al., 2013; Hernández-Mendoza et al., 2021; Sunkar & Bhukya, 2022). Moreover, the alteration of biomass’s crystallinity property (determined by X-ray Diffraction, XRD) and chemical properties (analysed by

Fourier-Transform Infrared Spectroscopy, FTIR; Pyrolysis-Gas Chromatography Mass Spectrometry, Py-GCMS; X-ray Photoelectron Spectroscopy, XPS) also possess notable direct or indirect correlation to the biomass hydrolysability (Gundupalli, et al., 2022; Huang et al., 2020; Li et al., 2023; Liu et al., 2017; Ridwan et al., 2023; Shi et al., 2020; Sorn et al., 2019; Sunkar & Bhukya, 2022; Wu et al., 2021; Xing et al., 2013; Xu et al., 2019; Xu et al., 2012; Zhang et al., 2011).

Therefore, in this research, besides reconfirming the relationship between the chemical composition of various pretreated OPEFB and their total glucose yield, the effect of different order of physical and chemical pretreatment sequences on the chemical composition alteration of biomass as well as physicochemical alterations induced by pretreatments that influences the effectiveness of glucose conversion will be investigated. Also, to fill up the research gap, various instrumental analyses namely, SEM, XRD, FTIR, Py-GCMS and XPS as commonly proposed by previous studies (Abraham et al., 2013; Gundupalli, et al., 2022; Hernández-Mendoza et al., 2021; Huang et al., 2020; Li et al., 2023; Liu et al., 2017; Ridwan et al., 2023; Shi et al., 2020; Sorn et al., 2019; Sunkar & Bhukya, 2022; Wu et al., 2021; Xing et al., 2013; Xu et al., 2019; Xu et al., 2012; Zhang et al., 2011), will be applied to analyse the alterations occurred on the pretreated OPEFB and then comprehend their correlation to enzymatic glucose yields. Hence, the current study focused on verifying the instrumental analyses (chemical or physical) appropriateness based on OPEFB fibre as a single raw material by correlating the results with glucose yield besides identifying the most suitable instrumental analysis to determine the effectiveness of pretreatments on biomass (OPEFB fibre).

1.3 Objectives

1. To compare the impact of different physical-chemical sequences used for pretreatment of oil palm empty fruit bunch (OPEFB) fibres on cellulose loss.

2. To study the effect of various pretreatments on the chemical composition of oil palm empty fruit bunch (OPEFB) fibres and enzymatic saccharification particularly, total glucose yield based on untreated biomass.

3. To determine and correlate the physical and chemical alterations (instrumental analyses results) to enzymatic saccharification/ total glucose yield based on theoretical glucose concentration.

CHAPTER 2

LITERATURE REVIEW

2.1 Lignocellulose

Lignocellulose is the primary structural component of all plant biomass, which is composed of three fundamental constituents—cellulose, hemicellulose, and lignin as illustrated in Figure 2.1. These three components are typically distributed in three major layers which are middle lamella; a layer rich in lignin, the primary cell wall, consists of randomly oriented cellulose microfibril interlocked by hemicellulose and the secondary cell wall, which is the biggest layer and built by organised cellulose microfibril and linked with hemicellulose and lignin (Ansari et al., 2023; Mathew et al., 2016; Pendse et al., 2023). Table 2.1 summarises the basic unit and characteristics of cellulose, hemicellulose and lignin. The percentage of each component varies among different biomasses in terms of sources, species, ages, location of biomass in a plant and etc. (Ali et al., 2017; Chen et al., 2023; Mankar et al., 2021; Mathew et al., 2016).

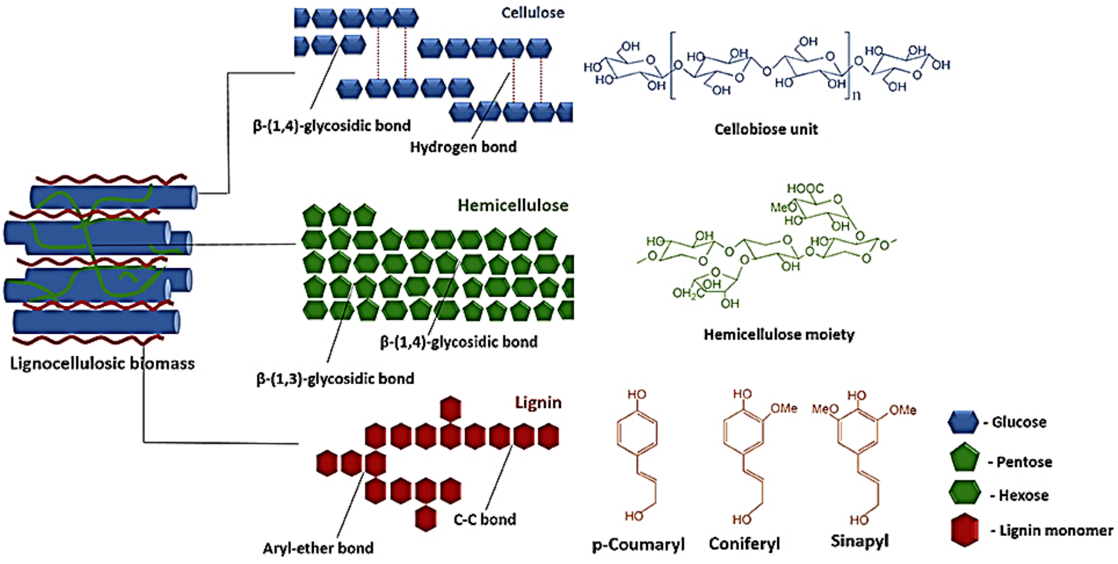

Figure 2.1 Schematic structure of lignocellulose (Baruah et al., 2018)

Table 2.1 Features of lignocellulose

Components	Basic unit	Characteristics	References
Cellulose (40 – 60%)	Most abundant natural, linear polymer (equal distribution of hydroxyl groups on both sides of the polymer chains) on Earth made up of monomer units and anhydro glucopyranose monomer units. Two monomers form cellobiose units linked by β-1,4-D glycosidic bonds. Degree of polymerisation: 5000 to 15,000 glucose molecules	Cellulose chains arranged regularly and align with each other by hydrogen bonds to form large crystalline regions and some small cellulose chains disperse into amorphous segments. The crystalline structure packs together to form bundles or microfibrils linked by hydrogen bonds between hydroxyl groups, thus strengthening its resistance to destruction and chemical stability. The amorphous cellulose is less compact and easily hydrolysed, while the dissolution of crystalline cellulose is limited (resistant).	Deng et al., 2023; Mankar et al., 2021; Pendse et al., 2023; Wang et al., 2021; Zhang et al., 2021
Hemicellulose (25 – 35%)	Biopolymer made up of heterogenous group of polysaccharides, which comprises of monosaccharides including hexoses (glucose, mannose, and galactose), pentoses (xylose and arabinose), with a tracer amount of other saccharides (rhamnose and fucose) with some sugar acids (α-D-glucuronic, α-D-galacturonic acid, and α-D-4-O-methylgalacturonic) and a small amount of acetyl groups and uronic acids. Degree of polymerisation: 50 to 300	Hydrophilic, amorphous, lower chemical and thermal stability than cellulose, being more soluble and susceptible to acidic hydrolysis. Can be classified into 4 major groups: xylan, xyloglucan, mannan and mixed-linkage β-glucans, where xylan (composed of β-1, 4-linked D-xylopyranose units) is abundant in herbaceous plants and hardwoods while mannan (containing β-1, 4-linked D-mannopyranose D- glucopyranose) is dominant in softwood. Closely linked with cellulose via extensive hydrogen bonds and with lignin mainly through covalent bonds while providing mechanical strength to plants.	Chen et al., 2022; Deng et al., 2023; Mankar et al., 2021; Pendse et al., 2023
Lignin (10 – 25%)	Second most abundant biopolymer in Earth, non-carbohydrate aromatic polymer made up of complex methoxylated phenylpropanoid monomers: - (p-hydroxyphenyl (H), guaiacyl (G) and syringyl (S) units lignin) derived from monolignols (p-coumaryl, coniferyl, and sinapyl alcohols) and crosslinked with	A 3-dimensional, amorphous macromolecule which is recalcitrant, hydrophobic and unable to dissolve in water but dissolve in alkaline aqueous solutions. It strongly binds with cellulose and hemicellulose polymers by ether bonds, and specifically to hemicellulose by ester bonds while supplies rigidity and resistance to the plant cell wall.	Arce & Kratky, 2022; Das et al., 2024; Deng et al., 2023; Khadem et al., 2024; Mankar et al., 2021; Okonkwo et al., 2023; Pendse et al., 2023; Zhang et al.,

	each other via C-O-C as well as the C–C linkages. These building blocks of lignin are connected via various chemical bonds, such as β-O-4- aryl ether linkages, α-O-4-aryl ether, 5-O-4-diphenyl ether, β-5-phenylcoumaran, 5-5-, α-1-biphenyl, β-1-(1,2-diarylpropane) and β-β-(resinol).		2021; Zhang et al., 2015

2.1.1 Lignocellulosic Biomass as Cellulosic Sugar Feedstock for Bioethanol Production

Among renewable energy sources, lignocellulosic biomass-based biofuels from agricultural waste materials, including wood, straw, bagasse and etc., emerged as promising options within the bioenergy sector due to no threat to world food security (Baruah et al., 2018; Zoghlami & Paës, 2019). Considering the swift growth of the oil palm industry in progressing countries of South-East Asia such as Thailand, Malaysia and Indonesia, the oil palm tree (*Elaeis guineensis*) is one of the leading sources of biofuel among the agricultural residues (Pangsang et al., 2019; Sutikno & Kismurtono, 2018). Globally, palm oil production was approximately 79.2 million metric tonnes (MT) in 2022 according to the United States Department of Agriculture (USDA), where Indonesia and Malaysia contributed nearly 46.5 million MT and 19.8 million MT, respectively. Therefore, Malaysia held about 25% of the world's palm oil production, falling behind Indonesia which accounted for 59% of the global palm oil generation, thus appearing as the second-biggest crude palm oil producer, after Indonesia (US Department of Agriculture & Foreign Agricultural Service, 2022).

Every tonne of palm oil originated from fresh fruit bunches (FFB) produces large amounts of residual biomass, including an estimated 1.0 ton of oil palm empty fruit bunches (OPEFB), 0.7 tons of oil palm fibres, 0.3 tons of oil palm shells and 0.3 tons of oil palm kernels, totalling 2.3 tons of palm biomass (Chang, 2014). OPEFB specifically refers to the remaining stalk and spikelets (Figure 2.2) after palm fruits are separated from fresh fruit bunch (FFB) (Chang, 2014; Mohammad et al., 2012; Obada et al., 2023). Over 23% OPEFB fibre is generated from FFB weight, along with 14% of palm mesocarp fibres and 7% of palm kernel shells, implying OPEFB fibre as the most abundant biomass from oil palm processing (Masran et al., 2020; Obada et al., 2023). In the past, OPEFB fibre was commonly utilised as a fuel in boilers to create steam for the sole purpose of generating power. However, the government had imposed a restriction on the usage of OPEFB fibre due to the controversial issue of its excessive smoke emission (James Rubinsin et al., 2020). In light of this, OPEFB fibre is now recognised as a highly promising resource for the synthesis of numerous bioproducts (Tang et al., 2020) particularly, composites (Latip et al., 2019), chemicals (Francis Prashanth et al., 2020) and liquid biofuels (Intasit et al., 2020).

Considering that approximately 1.1 metric tonnes of OPEFB fibre are generated for every metric tonne of palm oil produced (global palm oil production: 79.2 million MT), applying the conversion factor of 1.1 as proposed by Ma Ah Ngan (1999) results in a global generation of approximately 87 million metric tonnes of OPEFB fibre. This figure denoted that OPEFB fibre can be aptly recognised as one of the most economically viable and abundantly available plant fibres (Loh, 2017). Moreover, as OPEFB fibre contains more than 80% of carbohydrates content (Tye et al., 2017), it possesses superior characteristics and chemical compositions for bioethanol production (Loh, 2017). In the production of bioethanol from biomass including OPEFB fibre, pretreatment is a crucial step to maximize enzymatic saccharification by enhancing cellulose accessibility via altering the lignocellulosic structure (Jose et al., 2023) or chemical composition, or both (Jeong et al., 2021; Tye et al., 2017).

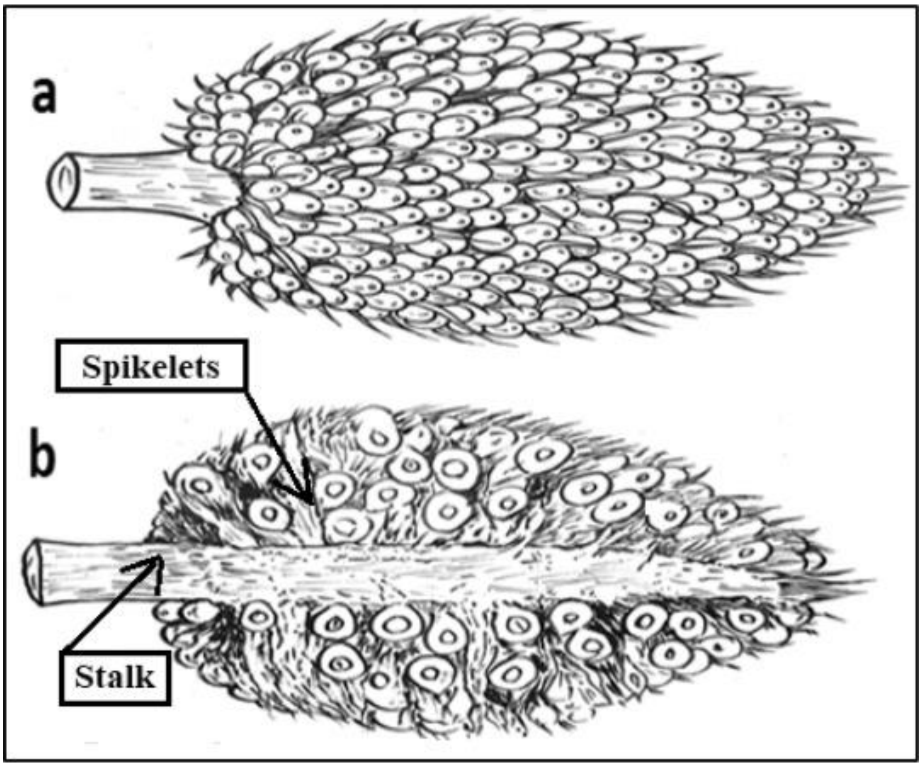

Figure 2.2 a) Oil palm fruit bunch b) Cross-section of oil palm empty fruit bunch (OPEFB) displaying fibre arrangements (Rama Rao & Ramakrishna, 2022)

2.2 Pretreatments of Lignocellulose for Enzymatic Saccharification

Various pretreatment methods, including physical, chemical, biological as well as combination of physico-chemical, and thermo-chemical approaches (Figure 2.3), have been extensively practised to enhance the effectiveness of converting polysaccharides of lignocellulosic materials into fermentable sugars by enzymatic saccharification. Table 2.2 demonstrates various type of pretreatments and their conditions that were applied on different types of biomasses (Ashokkumar et al., 2022; Dharmaraja et al., 2023; du Pasquier et al., 2023; Mankar et al., 2021).

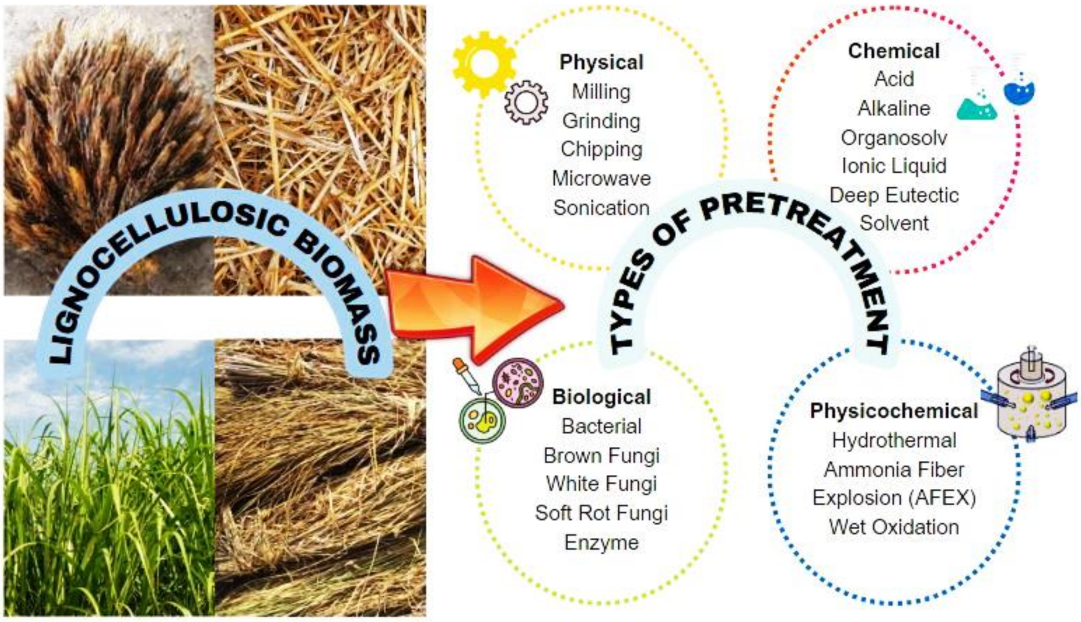

Figure 2.3 Schematic diagram of different types of pretreatment

Table 2.2 Summary of different types of pretreatments with their corresponding findings

Pretreatment methods	**Lignocellulosic biomass**	**Pretreatment conditions**	**Components removal**	**Enzymatic saccharification conditions**	**TGY/ reducing sugar based on pretreated (%)**	**TGY/ reducing sugar based on untreated (%)**	**References**
Sulphuric acid	Soybean hull	1% w/w H_2SO_4, 1:20 solid: liquid ratio, 120°C, 60 minutes	40.5% solid yield, 73.2% hemicellulose removal, 26.7% lignin removal	20 g/L, 50°C, 72 hours, pH 4.8, 160 rpm	86.90	35.19	Qing et al., 2017
Sodium hydroxide		1% w/w NaOH, 1:20 solid: liquid ratio, 100°C, 60 minutes	45.5% solid yield, 47.9% hemicellulose removal, 37.9% lignin removal	20 g/L, 50°C, 72 hours, pH 4.8, 160 rpm	75.60	34.40	Qing et al., 2017
Citric acid	Sugarcane bagasse	6% w/w citric acid, 1:25 solid: liquid ratio, 100°C, 102.4 min	84.99% solid yield,	20 g/L, 8.5 FPU/g 50°C, 72 hours, pH 4.8, 150 rpm	71.63	60.88	Gomes et al., 2020
Triethylbenzyl ammonium chloride/lactic acid (TEBAC/LA) deep eutectic solvent (DES)		30.0 g TEBAC/LA DES, 1:15 solid: liquid ratio, 120°C, 4 hours, 200 rpm, recycle number of 5 times	53.23% solid yield, 73.93% xylan removal, 88.72% lignin removal	80 g/L, 35 FPU/g, 50°C, 72 hours, pH 4.8, 200 rpm	88.23	47.00	Liu et al., 2021
Sodium hydroxide	Sweet sorghum	2% NaOH, 121°C, 50 minutes, 15 psi, 1 mm particle size	48.74% solid yield, 54.03% hemicellulose removal, 62.5% lignin removal	20 g/L, 20 FPU/g, 50°C, 72 hours, pH 5.0, 150 rpm	62.40	30.41	Punia & Singh, 2024

Dilute maleic acid	Oil palm empty fruit bunch	1% w/w maleic acid, 1:13 solid: liquid ratio, 200°C, 45 minutes	–	10 g/L, 10 FPU/g, 50°C, 72 hours, pH 5.0, 120 rpm	89.80	–	Risanto et al., 2023
Hydrothermal		1:10 solid: liquid ratio, 230°C, 20 minutes, severity factor (Log R0) of 5.13	62.6% solid yield, 100% hemicellulose removal, 0% lignin removal	60 g/L, 10 FPU/g, 50°C, 72 hours, pH 5.0	99.99	62.60	Zakaria et al., 2015
Dilute sulphuric acid	Oil palm trunk	1:5 solid: liquid ratio, 3% w/w H_2SO_4, 180°C, 40 minutes, severity factor 3.47	46.8% solid yield, 100% hemicellulose removal, 0% lignin removal,	60 g/L, 15 FPU/g, 50°C, 72 hours, pH 4.8, 200 rpm	50.80	23.77	Noparat et al., 2015
Sodium hydroxide	Sweet sorghum	2% NaOH, 121°C, 50 minutes, 15 psi, 1 mm particle size	48.74% solid yield, 54.03% hemicellulose removal, 62.5% lignin removal	20 g/L, 20 FPU/g, 50°C, 72 hours, pH 5.0, 150 rpm	62.40	30.41	Punia & Singh, 2024
Hydrothermal	Sunflower straw	1:15 solid: water ratio, 180°C, 120 minutes, severity factor (Log R0) of 4.1	58.8% xylan removal, 33.5% lignin removal	100 g/L, 15 FPU/g, 50°C, 72 hours, pH 4.8, 180 rpm	61.80	–	Yang et al., 2023
Soaking in aqueous ammonia	Corn stover	1:12 solid: liquid ratio, 29.5% NH_4OH, room temperature, 60 days	13% hemicellulose removal, 73.5% lignin removal	10 g/L, 15 FPU/g, 50°C, 72 hours, pH 4.8, 150 rpm	97.00	–	Kim & Lee, 2005
Microwave assisted 1-Butyl-3-methylimidazolium chloride ([Bmim]Cl)	Rice straw (RS)	1:20 RS:[Bmim]Cl 130°C (oil bath), 45 minutes	81.9% solid yield, 4.36% hemicellulose removal, 57.02% lignin removal	10 g/L, 50 FPU/g, 50°C, 72 hours, pH 4.8, 150 rpm	61.14	50.07	Sorn et al., 2019

It can be clearly seen from Table 2.2 that most of the lignocellulosic biomass that are being widely studied are from agricultural wastes rather than woody biomass. This is mainly because lignocellulose-derived biomass from agricultural residues is abundantly available globally, which is accounted to more than 7.26 billion tons per year (Suhartini et al., 2022; Tye et al., 2016b), sustainable and does not contend with edible food feedstocks or crops as it does not need a specific land area quality, water supply and fertilizer to cultivate (Ali et al., 2017; Chen et al., 2023).

Besides that, most of the pretreatments employed or studied come from chemical pretreatment or hydrothermal methods due to its stand in the industrial commercialisation sector. Each of these pretreatment methods offers distinct advantages and possesses its own set of limitations, which have been thoroughly elucidated within the scientific literature over the years (du Pasquier et al., 2023). While a multitude of pretreatment techniques have been discussed in the literature, only a few of them have demonstrated the potential near to industrial-scale implementation notably, hydrothermal pretreatment methods such as autohydrolysis and steam explosion, as well as specific alkaline pretreatments, and dilute acid pretreatment (Valladares-Diestra et al., 2022), which will be thoroughly discussed in this review.

2.2.1 Physical Pretreatments

Physical pretreatment techniques employ external mechanical and electrical forces to modify lignocellulosic biomass physical structures, with the aim to enhance polymeric chain porosity and surface area without involving chemical modifications within the biomass chains using external chemicals (Zhang et al., 2023). Methods like mechanical, microwave and ultrasound technologies demonstrate this approach.

2.2.1(a) Mechanical

Various techniques of physical pretreatments including milling, grinding, refining, shredding, and chipping reduces biomass particle size mechanically. In the literature, the terms "milling", “refining” and "grinding" are often used interchangeably. During the treatment, the material experiences intense mechanical stress, inducing a series of physical changes in the raw biomass, ultimately enhancing its enzymatic reactivity through the reduction in cellulose crystallinity, improved surface accessibility for enzymatic saccharification, decreased cellulose polymerisation, and enhanced mass transfer due to particle size reduction and total surface area increment (Gallego-García et al., 2023; Mankar et al., 2021; Sai Bharadwaj et al., 2023).

In general, the final particle size is dependent on the employed method; for instance, grinding or milling yields particles of 0.2 – 2 mm, while chipping results in 10 – 30 mm particles (Mahmood et al., 2019; Malik et al., 2022; Veluchamy et al., 2018). Nevertheless, as reported by some researchers (Malik et al., 2022; Tayyab et al., 2018; Veluchamy et al., 2018), a further reduction of biomass to a size smaller than 0.4 mm by physical pretreatment does not give significant impact to the hydrolysis rates and bioethanol yields.

Mechanical pretreatment is occasionally criticized for its high-energy consumption and low effectiveness in enhancing biomass enzymatic saccharification. However, it does not yield toxic or inhibitory compounds that hamper enzymatic saccharification due to abstaining from the external chemicals consumption and avoiding any chemical alterations within the biomass chains, making it suitable for both ethanol and methane production. The motive to search for a procedure without chemical consumption and reduced waste generation has renewed interest in mechanical pretreatment. Hence, this resurgence is driven by the environmental friendliness and simplicity of physical pretreatment, as it avoids the production of chemical liquid waste (spent liquor) (Amin et al., 2017; Zhang et al., 2021).

Although the mechanical pretreated biomass exhibits enhanced microbial and enzyme accessibility in biorefinery processes, the energy-intensive size reduction process makes it economically unviable compared to other pretreatment methods. Optimizing and designing biomass size-reduction equipment requires a deep understanding of mechanical properties, material feed rates, initial particle sizes, machine variables, and moisture content. Fine grinding, particularly, demands substantial energy input, necessitating a delicate balance between efficiency enhancement and cost concerns. Therefore, research should focus on determining the optimal particle size requirements for milled biomass. Given the high energy demand and rising energy costs, coupled with limitations in lignin removal and low cellulose enzyme access, mechanical pretreatment may still not stand alone as an economically viable selection (Amin et al., 2017; Gallego-García et al., 2023; Khan et al., 2021; Khan et al., 2022; Malik et al., 2022).

On the other hand, since the mechanical pretreatment is only limited to the physical changes of biomass, thus it is frequently combined with other pretreatment methods, especially chemical pretreatment for better effect in processing and disrupting the raw lignocellulosic materials structure, both in laboratory and industrial settings (Gallego-García et al., 2023; Sai Bharadwaj et al., 2023).

2.2.1(b) Microwave

Microwave pretreatment, falling within the 1 mm to 1 m wavelength range and 300 – 300,000 MHz on the electromagnetic spectrum, are nonionizing radiation that selectively transfers energy to substances (Hassan et al., 2018). In microwave pretreatment, the primary mechanism involves the breakdown of lignocellulosic structures through molecular collisions due to dielectric polarization during heating that resulted in rapid temperature increases and reduced processing times. Therefore, microwave irradiation contributes to the decomposition of polymers, disintegration of cellulose crystalline structures, and the breakdown of lignin and hemicellulose (Fia & Amorim, 2023; Hoang et al., 2021; Mohan et al., 2018).

Microwave-induced thermal effects aided to depolymerise cellulose by disrupting the hydrogen bonds and reducing cellulose crystallinity (Chen et al., 2011; Hoang et al., 2021) which leads to reduced particle size and increased surface area (Hoang et al., 2021). The disruption of biomass cell walls during microwave pretreatment occurs primarily due to rising pressure and temperature (Mohan et al., 2018). Hence, microwave heating facilitates enzymatic saccharification by promoting fibre swelling and fragmentation through uniform and rapid internal heating of large biomass particles (Diaz et al., 2015). An efficient destruction of hydrogen bonds and cell-wall components plays a role to improve the subsequent enzymatic saccharification efficiency (Ma et al., 2022).

Despite its advantages, like other physical pretreatments, microwave pretreatment is hampered by high energy consumption and equipment costs, partially offsetting its efficiency gains as well as producing heat-induced inhibitors such as phenolic and furan compounds (Li et al., 2016; Ma et al., 2022). Hence, microwave-assisted pretreatment, commonly combined with other methods, tackles issues like low thermal efficiency and uneven heating found in pretreatment techniques (Li et al., 2022) by catalysts providing ions and polar molecules to accelerate physical and chemical reactions (Akhlisah et al., 2021). Combining microwave heating with low sodium hydroxide (NaOH) or other chemicals has been promising in enhancing biomass deconstruction, potentially requiring lower NaOH amounts than chemical treatment alone (Bichot et al., 2021). Studies by Chen et al. (2011) reported that increased surface area and decreased particle size in the microwave pretreatment of sugarcane bagasse with rising temperatures and time.

2.2.1(c) Ultrasound

On the other hand, ultrasound pretreatment, operating in the frequency range of approximately 10 kHz to 20 MHz, imparts substantial energy to the reaction medium through cavitation and associated physical and chemical effects (de Farias Silva et al., 2020; Luo et al., 2014). Ultrasonic irradiation generates mechanoacoustic effects through cavitation of microbubbles, which can grow up to around 4 – 300 μm in diameter, expanding and contracting periodically during acoustic cycles. This radial oscillation produces turbulence and disintegrates biomass surface structures, enhancing mass transfer and accessibility to enzymatic saccharification (Bussemaker & Zhang, 2013; Luo et al., 2014).

In the reviewed physical pretreatment methods, it is apparent that this approach proposes benefits like reduced chemical usage, saving costs and time for chemical disposal, and eliminating concerns about managing solvent reuse and recycling, as well as minimising the generation of toxic by-products. Although physical methods enhance surface area accessibility and porosity for enzymatic action without any chemical usage, they face challenges in breaking down the recalcitrant components in biomass whereby, non-productive adsorption of enzyme on lignin can negatively affect enzymatic saccharification as well as elevate the cost of enzyme usage.

To tackle these issues and boost enzymatic yield, physical methods are commonly combined with other pretreatment methods. A combined ultrasound and

NaOH pretreatment approach exhibited notable results, including approximately 63% delignification, 560 mg/g of wheat waste biomass for reducing sugar production, improved hydrolysis of approximately 77.0%, and yields of 86% glucose and 62% xylose. These outcomes surpassed those achieved with individual alkaline or ultrasound pretreatments (Saratale et al., 2020).

2.2.2 Chemical Pretreatments

Chemical pretreatment emerged as the most extensively investigated method among the various pretreatment techniques (Smullen et al., 2017; Zhang et al., 2021). This approach employs a diverse range of chemicals including acids, alkalis, organic solvents, ionic liquids, deep eutectic solvents, and renewable solvents like ethyl lactate to facilitate the extraction of biomolecules from lignocellulosic biomass (Mohammad Rahmani et al., 2022; Sai Bharadwaj et al., 2023; Zhang et al., 2021).

Over time, chemical pretreatment has evolved into one of the most efficient technologies to enhance the effectiveness of the subsequent enzymatic saccharification through alteration of the crystalline structure of cellulose (degree of crystallinity), reducing the degree of polymerisation, and eliminating recalcitrant hemicellulose and lignin parts from lignocellulosic biomass by employing various chemical agents (Akhlisah et al., 2021; Behera et al., 2014; Mohammad Rahmani et al., 2022; Sai Bharadwaj et al., 2023).

During the chemical pretreatment process, a large quantity of chemicals and water is typically essential, necessitating the disposal of waste solutions, recycling of chemicals apart from occasionally elevated temperatures which then can lead to elevated facility investment, high treatment expenses, and potential environmental implications (Behera et al., 2014). Despite all the aforementioned criteria, when compared to physical and biological pretreatment methods, chemical pretreatment methods still offer the advantages of high efficiency and ease of operation (Tang et al., 2023). Nonetheless, the effectiveness of any type of chemical pretreatment is largely dependent on the properties of the reagents and the severity of the pretreatment (Ma et al., 2022). Hereby, the most common, simple aqueous chemical pretreatment approaches are acid, hot water and alkali pretreatments (Akhlisah et al., 2021; Huang, Jiang, et al., 2022; Zhang et al., 2021).

Techno-economic analysis of various pretreatment methods for bioethanol generation revealed that the dilute acid and hot water pretreatments are the most practical techniques to commercialise bioethanol in the short term where hot water demonstrated the best values of $1.78/L for minimum ethanol selling price (MESP) (Huang, Jiang, et al., 2022; Zhai et al., 2022). Alkaline pretreatment, inspired by the tremendous delignification ability in pulping technology and paper making industry has been commonly applied to disrupt the lignocellulosic materials, especially for agricultural wastes. Therefore, this review will particularly emphasise acid, hot water (autohydrolysis) and alkaline pretreatment within the area of chemical pretreatment.

2.2.2(a) Acidic Hydrolysis

Acidic pretreatment is a highly efficient chemical method employed for disrupting the lignocellulosic matrix by cleaving the glycosidic bonds via hydrolysis, leading to the solubilisation of the hemicellulosic portion and a fraction of lignin components. Therefore, this treatment makes cellulose more accessible to successive enzymatic hydrolysability (Lorenci Woiciechowski et al., 2020; Solarte-Toro et al., 2019).

Acidic pretreatment can be carried out by applying diluted mineral acid (commonly used is sulphuric acid) (du Pasquier et al., 2023; Kang et al., 2018), namely as acid-catalysed pretreatment or simply known as acid prehydrolysis or