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A Review on 1st and 2nd Generation Bioethanol Production-Recent Progress

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Abstract

Today's society is based on the use of fossil resources for transportation fuels. The result of unlimited consumption of fossil fuels is a severe depletion of the natural reserves and damage to the environment. Depleting fossil reserves and increasing demand for energy together with environmental concerns have motivated researchers towards the development of alternative fuels which are eco-friendly, renewable and economical. Bioethanol is one such dominant global renewable transport biofuel which can readily substitute fossil fuels. Conventionally, bioethanol has been produced from sucrose and starch rich feedstocks (edible agricultural crops and products) known as 1st generation bioethanol; however this substrate conflicts with food and feed production. As an alternative to 1st generation bioethanol, currently there is much focus on advancing a cellulosic bioethanol concept that utilizes lignocellulosic residues from agricultural crops and residues (such as bagasse, straw, stover, stems, leaves and deoiled seed residues). Efficient conversion of lignocellulosic biomass into bioethanol remains an area of active research in terms of pretreatment of the biomass to fractionate its constituents (cellulose, hemicellulose and lignin), breakdown of cellulose and hemicellulose into hexose and pentose sugars and co-fermentation of the sugars to ethanol. The present review discusses research progress in bioethanol production from sucrose, starch and cellulosic feedstocks. Development of efficient technology to convert lignocellulosic biomass into fermentable sugars and optimization of enzymatic hydrolysis using on-site/in-house enzyme preparation are the key areas of development in lignocellulosic bioethanol production. Moreover, finding efficient fermenting microorganisms which can utilize pentose and hexose sugars in their metabolism to produce ethanol together with minimum foam and glycerol formation is also an important parameter in fermentation. Research has been focusing on the application of genetically modified strains, thermoanaerobes and mixed cultures of different strains in bioethanol production from sucrose, starch and lignocellulosic feedstocks.

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1. Introduction

Energy sources and their utilization determine the economic status and growth of developing countries all over the world [1]. The Statistical Review of World Energy estimated that in 2013 the primary sources of energy consisted of petroleum 32.9%, coal 30.0%, and natural gas 23.7%, amounting to an 87.0% share for fossil fuels in primary energy consumption in the world. In the year 2003 the world consumed 9943.8 million tonnes oil equivalent primary as energy; this value increased by 7.8%, 20.2% and 28.0% in 2005, 2010 and 2013, respectively.

Today's society is based on the use of fossil resources for transportation fuels and petrochemicals. World energy consumption by fuel type is given in **Figure 1**. It is evident that the consumption of oil, coal and natural gas greatly exceeds the consumption of renewable energy and hydroelectricity. The result of unlimited consumption of fossil energy, due to its low cost and ready availability is a severe depletion of the natural reserves. However, the use of fossil fuels also leads to environmental damage. The burning of every tonne of fossil-fuel adds 180 kg of sulphur oxides to the atmosphere, causing irritation to the respiratory system and adding to the formation of acid rain.

In addition, the burning of fossil fuel produces around 21.3 giga tonnes of carbon dioxide (CO_2) per year, but it is estimated that natural processes can only absorb about half of that amount, so there is a net increase of 10.7 billion tonnes of atmospheric carbon dioxide per year (one tonne of atmospheric carbon is equivalent to 44/12 or 3.7 tonnes of carbon dioxide) (http://www.eia.gov/oiaf/1605/ggccebro/chapter1.html).

Depleting fossil reserves and increasing demand for energy together with environmental concerns have led to focused research on the development of alternative fuels which are eco-friendly, bio-degradable and economical. The use of renewable resources to produce liquid biofuels offer attractive solutions to reducing greenhouse gas emissions, decreasing reliance on foreign oils, addressing energy security concerns, strengthening rural and agricultural economies and increasing the sustainability of the world transportation system [2]. Currently only

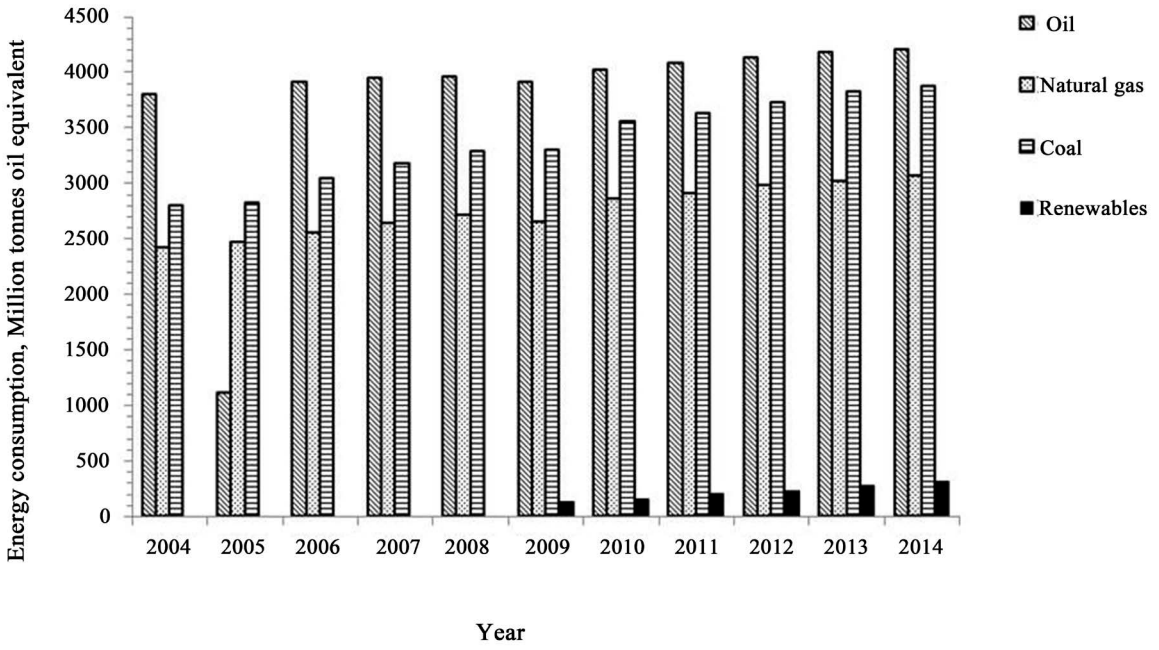

Figure 1. World energy consumption by fuel type in million tonnes oil equivalent (data collected from statistical review of world energy. http://www.bp.com/en/global/corporate/energy-economics/statistical-review-of-world-energy.html).

3.0% of global energy consumption is supplied from renewable sources. Yet in 2050, potentially around 20% - 80% of the world's primary energy demand could be provided by sustainable renewable resources [Statistical Review of World Energy. http://www.bp.com/en/global/corporate/energy-economics/statistical-review-of-world-energy.html].

Bioethanol is the dominant global renewable transport biofuel and offers greenhouse gas savings of up to 80% over conventional fossil fuels depending on the feedstock. Other types of biofuels include biodiesel, biomethanol, biogas, bio-syngas, bio-oil and bio-hydrogen [3] produced from a wide range of agricultural or waste sources.

2. 1st Generation versus 2nd Generation Biofuel Production

The raw materials for bioethanol production can broadly be classified as (i) sucrose-containing feedstock (sugarcane, sugar beet and sweet sorghum), (ii) starch-containing feedstock (wheat, corn and cassava) and (iii) cellulosic feedstock (straw, grasses, wood, stovers, agricultural wastes, paper, etc.). However, bioethanol is currently produced chiefly from traditional food crops such as corn (USA), sugar cane (Brazil), wheat (France, England, Germany, and Spain), cassava (Thailand, Nigeria) and sorghum (India), the feedstock depending on location and dominant agricultural product [4]. Most current bioethanol production processes utilize more readily degradable biomass feedstock such as cereals (corn or grain) and sugar cane juice. However, the utilization of edible agricultural crops exclusively for biofuel production conflict with food and feed production [5]. The bioethanol produced from these sucrose-and starch-containing feedstock is classified as 1st generation bioethanol (ethanol from corn and sugarcane) and those produced utilizing cellulosic feedstock is 2nd generation bioethanol (ethanol from corn stover, rice straw, palm empty fruit bunches and other lignocellulosic biomass) [https://www.iea.org/publications/freepublications/publication/2nd_Biofuel_Gen.pdf].

2.1. Sucrose-Containing Feedstock for Bioethanol Production

Sugarcane, sugar beet and sweet sorghum are the main sucrose-containing feedstocks for bioethanol production with feedstock yields of 62 - 74 tonnes·ha^{-1} [6], 54 - 111 tonnes·ha^{-1} [7] and 50 - 62 tonnes·ha^{-1} [6], respectively, and are mostly exploited in Brazil, India, France and Germany. Black strap/sugarcane molasses from sugarcane processing, aqueous juice expelled from sugar beets and sweet sorghum stalks were employed as raw material in bioethanol production. The proximate composition of sucrose-containing feedstock [8]-[10] and starch-containing feedstocks [11]-[13] for bioethanol production are given in **Table 1**. Sugarcane molasses is composed of sucrose (31%) and inverted sugar (15%) [8]. Therefore, sucrose concentration in sugarcane molasses must be diluted (to 14% - 18%) before fermentation to facilitate the optimum growth of fermenting microorganism. The juice extracted from sugar beet is composed of 16.5% sucrose [9] and in sweet sorghum, stalks are the main store of sugar and are mechanically pressed to recover a sugar juice of 12% - 22% concentration [10] which can be directly fermented by *Saccharomyces cerevisiae* (yeast).

Table 1. Proximate composition of starch-containing and sucrose-containing feedstock.

Sugarcane molasses [8]		Sugar beet juice [9]		Sweet sorghum stalks [10]		Corn grain [11]	Wheat grain [12]	Cassava [13]
Component	% w	Component	% w	Component	% w	Component	% w	% w	% w
Water	18.9	Water	65.6	Cellulose	8.7	Starch	72.0	53.0 - 57.0	77.0 - 94.0
Sucrose	31.8	Solids	17.3	Hemicellulose	6.3	Fiber	9.5	9.9 - 11.8	1.5 - 3.7
Invert sugar	15.4	Sucrose	16.5	Lignin	0.6	Sugars	2.6	0.0	0.0
Ash	13.8	Sugars	0.2	Sucrose	67.4	Protein	9.5	12.5 - 15.1	1.7 - 3.8
Others	20.1	Impurities	0.3	Glucose	3.7	Oil	4.3	2.1 - 2.6	0.2 - 1.4
				Ash	0.2	Minerals/Ash	1.4	0.0	1.8 - 2.5
				Others	13.1	Water	0.0	12.0	59.0 - 70.0
						Others	0.7	4.9 - 5.8	0.0

Although bioethanol production using sucrose-containing feedstock has been well reported, research is still ongoing, including the testing of different yeast species available in the market and also newly isolated species to achieve high ethanol yields and to reduce the formation of foam and glycerol during fermentation. Foaming and glycerol formation are the major parameters which can have a significant impact on ethanol production costs. A summary of the latest research reports [14]-[23] on ethanol production from sucrose-containing feedstocks together with feedstock availability is presented in **Table 2**. Conventionally, bioethanol production has been carried out by anaerobic fermentation using yeast. However, Jayus and co-workers [14] reported the effect of aeration during fermentation of sugarcane molasses using commercially available New Aule Alcohol yeast and New Aule Baker's yeast on ethanol production. Among the two species tested Baker's yeast (0.7 $g \cdot g^{-1}$) showed higher

Table 2. Bioethanol production from sucrose-containing feedstock- recent research.

Feedstock	Yield, tonnes ha^{-1}	Sugar content, % w/w	Fermentation organism/conditions	Ethanol	Reference
Sugarcane molasses	62 - 74 (Sugarcane) [6]	31% sucrose and 15% invert sugars	New Aule Alcohol yeast and New Aule Baker's yeast; fermentation at room temperature, pH 4.3 for 72 h, inoculum 1% w/v	Alcohol yeast-74.8 $g \cdot L^{-1}$, $Y_{p/s}$ 0.4 $g \cdot g^{-1}$ and Baker's yeast-102.9 $g \cdot L^{-1}$, $Y_{p/s}$ 0.7 $g \cdot g^{-1}$ from 300 $g \cdot L^{-1}$ sugar concentration	[14]
Sugarcane molasses			*Saccharomyces species* isolated from molasses; fermentation at 30˚C for 144 h, inoculum 0.5 $g \cdot L^{-1}$	128.7 $g \cdot L^{-1}$, $Y_{p/s}$ 0.6 $g \cdot g^{-1}$ from 250 $g \cdot L^{-1}$ sugar concentration	[15]
Sugar beet molasses and thick juice	54 - 111 [7]	Total sugars in Sugar beet molasses: 53.0% and in thick juice: 60.0%	Immobilized yeast; fermentation at 30˚C, pH 5.5, 144 h, inoculum 1 $g \cdot L^{-1}$	From molasses: $Y_{p/s}$ 0.5 $g \cdot g^{-1}$, 96.8%, 83.2 $g \cdot L^{-1}$ and from thick juice: $Y_{p/s}$ 0.4 $g \cdot g^{-1}$, 90.6%, 132.4 $g \cdot L^{-1}$ from 300 $g \cdot L^{-1}$ sugar concentration	[16]
Sugar beet raw, thin and thick juice and molasses		In raw juice:13.4% Thin juice:13.0% Thick juice: 58.3% Molasses: 50.1%	Commercial yeast strain; fermentation at 30˚C, 60 h, inoculum 3 $g \cdot L^{-1}$	From raw juice: 0.08 v/v Thin juice: 0.08 v/v Thick juice: 0.08 v/v Molasses: 0.07 v/v from an initial sugar concentration of 130 $g \cdot kg^{-1}$ media	[17]
Sweet sorghum stalk juice	50 - 62 [6]	Sucrose 12% - 22%	Immobilized *S. cerevisiae* in bioreactor 5L; fermentation at 37˚C, pH 5, 12 h, inoculums 10^8 cells$\cdot mL^{-1}$	33 $mg \cdot mL^{-1}$, yield 98.0%	[19]
Sweet sorghum stalk juice			Immobilized *S. cerevisiae* in fluidized bed fermenter; fermentation at 32˚C, pH 4, 9 h, inoculum 10^8 cells$\cdot mL^{-1}$	Ethanol content 6.2% v/v; yield 91.6%	[20]
Sweet sorghum juice			*S. cerevisiae;* fermentation at 30˚C, 48 h, inoculums 5^*10^8 cells$\cdot mL^{-1}$	Ethanol 133.5 $g \cdot L^{-1}$, 87.6% of the theoretical yield	[22]
Sweet sorghum juice			*S. cerevisiae* strain BY4741; fermentation at 30˚C, pH 5.2, 48 h, inoculum 5^*10^8 cells$\cdot mL^{-1}$	Ethanol 115.2 $g \cdot L^{-1}$, 87.1% of the theoretical ethanol yield, $Y_{p/s}$ 0.4 $g \cdot g^{-1}$	[21]
Sweet sorghum juice from three varieties: GK-coba; Mn-4508; SS-301		GK-cba: 16.9%, Mn-4508: 17.4%, SS-301: 19.1%	*Zymomonas mobilis* and *S. cerevisiae* mixed culture (1:1); fermentation at 30˚C, 4 days, inoculum 5 mL of 48 h old liquid seed cultures	45.2 $mL \cdot L^{-1}$; 1075.4 $L \cdot ha^{-1}$ 46.9 $mL \cdot L^{-1}$; 1318.2 $L \cdot ha^{-1}$ 50.2 $mL \cdot L^{-1}$; 1232.6 $L \cdot ha^{-1}$	[23]

ethanol formation per unit of substrate consumed ($Y_{p/s}$, $g \cdot g^{-1}$) than alcohol yeast (0.4 $g \cdot g^{-1}$). Muruaga *et al*. [15] reported ethanol production from sugarcane molasses using *Saccharomyces* species isolated from molasses and grapes. They achieved ethanol formation of 128.7 $g \cdot L^{-1}$ which corresponds to an $Y_{p/s}$ of 0.6 $g \cdot g^{-1}$ from molasses with initial sugar concentration of 250 $g \cdot L^{-1}$.

Sugar beet molasses and thick juice are the other promising raw sources for ethanol production due to their high sugar content *i.e*. 53.0% and 60.0%, respectively. Razmovski *et al*. [16] studied the very high gravity (VHG) fermentation of sugar beet molasses and thick juice using *S. cerevisiae* (strain DTN) in free and immobilized form. During VHG fermentation by the immobilized yeast, the maximum ethanol concentrations achieved were 83.2 $g \cdot L^{-1}$ and 132.4 $g \cdot L^{-1}$ from sugar beet molasses and thick juice, respectively, with an initial sugar concentration of 300 $g \cdot L^{-1}$. The intermediate products (raw juice, thin juice and thick juice) obtained during sugar beet processing were also employed as feedstock for bioethanol production along with beet molasses. It was reported that little difference was observed in the amount of ethanol formed (v/v) but a significant difference was reported in terms of fermentation duration. The optimal fermentation duration of intermediates was 36 h whereas that of molasses was 50 h [17]. Moreover, it was reported that 0.07 kg ethanol can be obtained from aqueous sugars extracted from 1 kg sugar beet [18]. Employment of immobilized yeast in different modes of fermentations *i.e.* batch fermentation in a bioreactor [19] and continuous fermentation in a fluidized bed reactor [20] were other aspects which improved ethanol yields. Concentration of sweet sorghum raw juice before being subjected to fermentation was reported to have a positive effect on improving the ethanol titer. Sasaki and co-workers [21] reported improved ethanol titer of 115.2 $g \cdot L^{-1}$ corresponding to a $Y_{p/s}$ of 0.4 $g \cdot g^{-1}$ after concentrating the sweet sorghum raw juice from an initial concentration of 125 $g \cdot L^{-1}$ to 278.6 $g \cdot L^{-1}$ using nanofiltration. Apart from these process improvements, supply of a nitrogen source (87.6% ethanol yield) [22], inorganic carbon source (91.6% ethanol yield) [20] and application of mixed culture of fermenting organisms during fermentation were reported to have a significant effect on the fermentation process for bioethanol production. Khalil *et al*. [23] employed a mixed culture of *Zymomonas mobilis* and *S. cerevisiae* as fermenting agents on juice extracted from different varieties of sweet sorghum (GK-coba, Mn-4508 and SS-301) and reported that sweet sorghum SS-301 variety gave maximum ethanol yield of 1233 $L \cdot ha^{-1}$ among the different varieties of sweet sorghum tested.

2.2. Starch-Containing Feedstock for Bioethanol Production

Corn, wheat and cassava are the most employed starch-containing feedstocks in bioethanol production in North America, Europe and tropical countries. Starch is a polymer of glucose which can be broken into glucose monomers by the action of α-amylase and gluco-amylase enzymes. The proximate chemical composition of the starch-containing feedstock is provided in **Table 1**. The conversion of starch-containing feedstock to obtain fermentable sugars is mainly comprised of three operations which are: (i) milling, (ii) liquefaction and (iii) saccharification using enzymes. Commercially, corn grain is converted to ethanol by two methods, wet milling and dry milling. In wet milling corn grain is soaked in water to fractionate the grain into starch, fiber and germ involving separate processing of each fractionated component. Dry milling involves processing of whole grain and the residual components are separated at the end of the process.

In corn grain based ethanol production, corn grain variety and quality contribute to the final ethanol yield. Research carried out on 258 corn varieties for bioethanol production confirmed that the corn samples with higher starch content have lower efficiency of starch saccharification [24]. Corn quality in terms of kernel composition, endosperm hardness, planting location and the presence of mycotoxins affected ethanol yield, with differences in ethanol yield ranging between 3% - 23% due to grain quality [25]. Moreover, the high free sugar content in corn kernels has the potential to decrease enzyme consumption during saccharification resulting in higher ethanol yields. A brief description of the latest research on bioethanol production from starch-containing feedstock [4] [12] [26]-[33] is given in **Table 3**.

Ethanol production from corn of the high sugary corn genotype, HSG and its parent field corn lines PFC confirmed that the enzyme requirement for HSG corn was 1.5 $kg \cdot tonne^{-1}$ of dry corn whereas PFC corn consumed 2 $kg \cdot tonne^{-1}$ [26]. Therefore, it is evident that starch content in corn grains is not the only factor which determines ethanol productivity.

Wheat is another main cereal feedstock for grain distilleries and ethanol production and it replaced barley 30 years ago. Dry milling of wheat to separate bran from grain improves the starch content in flour resulting in a high ethanol titer. Sosulki *et al*. [12] reported ethanol production using wheat flour from dry milling with a

Table 3. Bioethanol production from starch-containing feedstock.

Feedstock	Feedstock availability	Starch content, % w/w	Liquefaction and Saccharification	Fermentation organism/conditions	Ethanol formed	Reference
Corn HSG and PFC varieties	7.2 tonnes·ha^{-1}	HSG: Starch 67.3%, total sugar 7.4% and PFC: starch 73.6%, total sugar 1.2%	SSF; Saccharification using STARGEN 002 at 2 kg·tonne^{-1} of corn with 300 g·L^{-1} initial solid concentration	*S. cerevisiae ATCC* 96581; fermentation at 30˚C, pH 4.2, 96 h and inoculum 2 mL per 100 mL media	From HSG: 0.4 g·ethanolg^{-1} dry corn, 141.5 g·L^{-1} From PFC: 0.4 g·ethanolg^{-1} dry corn, 130.5 g·L^{-1}	[26]
Corn meal		Starch 70.8%	Two-step enzymatic treatment using commercially available α-amylase and glucoamylase	*S. cerevisiae*; fermentation at 32˚C, pH 5.0, 48 h and inoculum 1.3% w/w	80% of the theoretical ethanol yield	[4]
Wheat	3.6 tonnes·ha^{-1} annum^{-1}	Total carbohydrates 69.7% and crude fiber 1%		*S. cerevisiae*; fermentation conditions not available	423 L·tonne^{-1}; 1560 L·ha^{-1}·annum^{-1}	[29]
Wheat			Mash obtained under VHG conditions at 38% w/v solid concentration	Active dry yeast; fermentation at 20˚C and inoculums 10^6 cells per 1 g wheat mash	23.80% v/v after 200 h at 20˚C	[27]
Wheat		Starch 53% - 57%	Liquefaction at 95˚C for 2 h and 45 min using α-amylase and saccharification at 55˚C for 2 h using glucoamylase at grain/flour to water weight ratio of 1:2.7	*S. cerevisiae;* fermentation 30˚C, 72 h and inoculum $1.6^{*}10^7$ cells·mL^{-1}	From grain: 10% - 11% w/v, 12.7% - 13.8% v/v, 377 - 399 L·tonne^{-1}; from flour: 12% - 12.7% w/v, 15.0% - 15.9% v/v and 344 - 367 L·tonne^{-1}	[12]
Cassava	36.3 tonnes·ha^{-1} annum^{-1} [29]	Starch 76% - 81%	Liquefaction using Spezyme enzyme for 30 min at 90˚C followed by SSF using Stargen enzyme at 1:100 w/w ratio of Stargen to starch and 10% w/v solid concentration	Dry bake's Yeast; fermentation at 30˚C, 48 h, pH 5.5, and inoculum 10 mL yeast suspension having O.D 3.8 - 4.0 at 450 nm	558 g ethanol·kg^{-1} cassava starch, fermentation efficiency 98.4%	[30]
Cassava flour			Liquefaction at 80˚C for 90 min using Spezyme followed by SSF using alpha amylase, beta glucanase and two glucoamylases	*S. cerevisiae*; fermentation at 30˚C, pH 5.5, 72 h and inoculum $1.5^{*}10^7$ cells·mL^{-1}	At lab scale: 17.2% v/v, 86.1% of the theoretical ethanol yield; At pilot scale: 16.5% v/v, 83.6% of the theoretical ethanol yield	[31]
Wild cassava			Liquefaction and saccharification using α-amylase and β-glucanase	*Caloramator boliviensis* (Thermoanaerobe); fermentation at 60˚C, pH 7, 48 h and inoculum 50 mL overnight culture per 240 mL media	33.0 g·L^{-1}, 1.7 mol·mol^{-1}, 85% of the theoretical ethanol yield	[33]

maximum ethanol concentration of 15% - 15.89% (v/v) at 344 - 367 L·tonne^{-1} of wheat flour whereas under very high gravity conditions a maximum ethanol titer of 23.8% (v/v) was reported by [27] Thomas and co-workers. Moreover, a recent study proposed by [28] Belboom *et al*. reported that the consumption of 1 MJ bioethanol produced from wheat instead of 1 MJ gasoline can reduce greenhouse gas emissions by 42.5% - 61.2%.

Cassava is a promising feedstock for bioethanol production due to the high starch yield per hectare and availability of raw material all year round (36.3 tonnes·ha^{-1}·annum^{-1}) [29]. Although several workers have reported bioethanol production from cassava, research is still focused on the evaluation of optimum slurry concentration, enzyme load and fermentation conditions to obtain high ethanol titer and maximum ethanol yield [30].

Shanavas *et al*. reported Spezyme (a highly powerful α-amylase) liquefying enzyme treatment followed by saccharification and fermentation of cassava starch (10% w/v slurry concentration) was the best process strategy

to obtain 558 g ethanol per kg cassava starch within 48.5 h of duration using Stargen enzyme (granular starch hydrolyzing enzyme) at 1:100 w/w ratio of the enzyme to cassava starch and dried baker's yeast as fermenting organism at 30˚C. In another approach simultaneous saccharification and fermentation (SSF) under very high gravity (VHG) conditions employing 315 $g \cdot L^{-1}$ slurry concentration was reported by Nguyen *et al*. [31] for bioethanol production from cassava flour at lab and pilot scale level. Liquefied cassava flour at 80˚C for 90 min using α-amylase and β-glucanase was subjected to SSF at 30˚C with simultaneous addition of glucoamylase and active dry yeast. The ethanol content achieved at lab and pilot scale were 17.2% (v/v) and 16.5% (v/v) corresponding to 86.1% and 83.6% of the theoretical ethanol yield, respectively. VHG technology has some disadvantages due to the high viscosity of starch after liquefaction, which leads to solid-liquid separation problems, incomplete hydrolysis of starch and lower fermentation efficiency. In order to overcome these drawbacks of VHG technology the feedstock can be pretreated using cell-wall degrading enzymes (cellulase and pectinase) and viscosity reduction enzymes (xylanase) which will give low viscosity starch paste from VHG operation [32]. Currently, the application of thermoanaerobes during fermentation has been gaining attention in bioethanol production. The high growth temperatures of thermoanaerobes promote higher rates of starch/cellulose conversion to sugars and reduce cooling costs in fermentation. Moshi *et al.* [33] reported an ethanol titer of 33 $g \cdot L^{-1}$ corresponding to 85% of the theoretical ethanol yield from α-amylase and β-glucanase treated cassava subjected to fed-batch fermentation under high hydrogen pressure using a thermoanaerobe, *Caloramator boliviensis* at 60˚C.

Globally, among the countries which produce bioethanol from sugar and starch containing feedstock the United States produces 40 billion liters of bioethanol from corn/wheat while Brazil accounts for 25 billion liters from sugar cane. Apart from these two major bioethanol producing countries, China (3 billion liters from corn/cassava/rice), Canada (2 billion liters from corn/wheat), India (1 billion liters from sugarcane/molasses), France (1 billion liters from wheat/sugarcane/sugar beet), Germany (750 million liters from wheat/sugarcane/sugar beet) and Australia (500 million liters from sugar cane) are the remaining countries producing significant bioethanol [http://biofuel.org.uk/major-producers-by-region.html]. To preserve the sustainability of the bioethanol production from sugar and starch-containing (1st generation) feedstock and to improve energy economics of the process it is necessary to recover intermediate products and to integrate pulp/bagasse fermentation with the process.

1st generation bioethanol production from food crops have several limitations including the fact that it has a direct impact on food production in terms of food price and quality and soil usage for crop growth while providing only limited greenhouse gas emission reduction benefits [34]. Currently there is much focus on advancing a cellulosic bioethanol concept (2nd generation) that utilizes lignocellulosic biomass. 2nd generation bioethanol produced from lignocellulosic biomass, non-food crops, industrial and municipal wastes results in greater greenhouse gas reductions and does not compete for agricultural land with food crops.

2.3. Cellulosic Feedstock for Bioethanol Production

Lignocellulosic biomass represents a promising resource for bioethanol production which is renewable in nature. Lignocellulosic biomass is defined as "the biodegradable fraction of products, waste and residues from biological origin from agriculture (including vegetable and animal substances), forestry and related industry" [http://ec.europa.eu/agriculture/bioenergy/potential/index_en.htm]. Not only an energy source, biomass is also a promising raw material for the production of chemicals [35]. As biomass represents a renewable energy source it can potentially be utilized without depleting reserves. However, the structural features of lignocellulosic biomass pose challenges to conversion technologies. An effective conversion technology must be developed to enable the processing of lignocellulosic biomass that has a very complex and resistant structure and allow the efficient exploitation of every part of the biomass. The relative portions of the different parts of lignocellulosic biomass vary greatly depending on the source. There is no current technology for conversion of the biomass to bioethanol available. What is now required is to develop techno-economic routes for the production of bio-based compounds to make the bio-industry competitive in the market.

Lignocellulosic biomass is composed of carbohydrate polymers (cellulose and hemicellulose), lignin and a small remaining fraction of extractive acid, salts and minerals. **Figure 2** depicts the structural components of lignocellulosic biomass.

Cellulose is a homo-polymer of glucose subunits (cellobiose) with a crystalline structure; hemicellulose is a heteropolymer of pentose sugars with an amorphous structure, whereas lignin is a highly crystalline and rigid component of biomass. Cellulose and hemicellulose typically comprise two-thirds of the dry mass and varies with the type of biomass feedstock. The cellulose, hemicellulose and lignin composition of different renewable

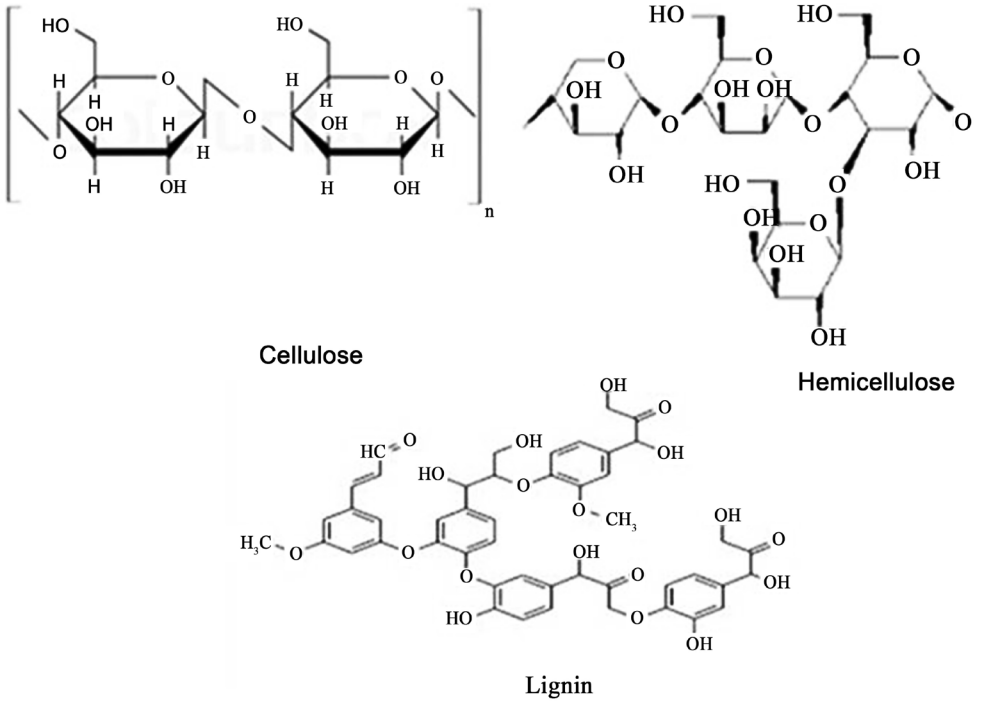

Figure 2. Lignocellulosic biomass structural components (cellulose, hemicellulose and lignin).

feedstocks [36]-[38] is presented in **Table 4**. These three components of biomass can be converted to various value added products through different pathways. There are a number of recent reviews reporting the state of the art in biofuel and biochemical production and the use of different feedstock for this developing bioindustry (e.g. [39]).

Bioethanol production from lignocellulosic biomass feedstock typically comprises the following steps:

- Pre-treatment: process where the structural carbohydrates that compose the biomass are made more accessible for the subsequent steps;
- Enzymatic hydrolysis: breakdown of the polymeric carbohydrates into simple sugars that can be fermented by the microorganisms into ethanol;
- Fermentation: conversion of the carbohydrates into ethanol by the selected microorganism or culture;
- Downstream processing: recovery of the ethanol from the fermentation broth (typically by distillation) and management of the remaining streams.

The economic feasibility of biofuel production from lignocellulosic feedstock largely depends on (i) the type of biomass and (ii) the pretreatment process before fermentation. Availability, cost, transportation to the processing facility and physical state of the biomass are major factors affecting the selection of feedstock for bioethanol production. Agricultural residues and pulp/bagasse generated from 1st generation bioethanol process represent a promising feedstock for 2nd generation bioethanol production.

A list of different processes for 2nd generation bioethanol production from corn stover [40]-[43], Japanese ceder [44], wheat straw [45] [46], cassava residues [47]-[49], sugarcane bagasse [50]-[55], sugar beet pulp [56] [57], sweet sorghum bagasse [54] [58]-[61], sweet sorghum stover [62], rice straw [63]-[65] and palm empty fruit bunches [66] are presented in **Table 5** together with feedstock availability, chemical composition and ethanol yield from the process.

The need for a pre-treatment step is the major distinction between a 1st and a 2nd generation bioethanol process. Existing ethanol production processes have (i) separate hydrolysis and fermentation steps (SHF) [67]; (ii) simultaneous saccharification and fermentation (SSF) [68] refers to saccharification and fermentation of hexose sugars taking place within the same bioreactor; (iii) simultaneous saccharification and co-fermentation (SSCF) refers to the saccharification and co-fermentation of both pentose and hexose sugars in a single step and (iv) consolidated bioprocessing step (CBP) (**Figure 3**). In CBP a single organism is used to produce the enzymes required and to perform both cellulose hydrolysis and fermentation [69]. CBP is considered potentially the most

Table 4. Cellulose, hemicellulose and lignin composition of lignocellulosic biomass feedstocks [36]-[38].

Lignocellulosic biomass	% of total dry weight		
	Cellulose	Hemicellulose	Lignin
Bamboo	49 - 50	18 - 20	23
Corn cobs	45	35	15
Corn stover	35 - 40	21 - 25	11 - 19
Grasses	25 - 40	35 - 50	10 - 30
Hardwood stems	40 - 50	24 - 40	18 - 25
Nut shells	25 - 30	25 - 30	30 - 40
Rice straw	29 - 35	23 - 26	17 - 19
Softwood stems	45 - 50	25 - 35	25 - 35
Sugar cane bagasse	25 - 45	28 - 32	15 - 25
Switch grass	30 - 50	10 - 40	5 - 20
Wheat straw	33 - 40	20 - 25	15 - 20

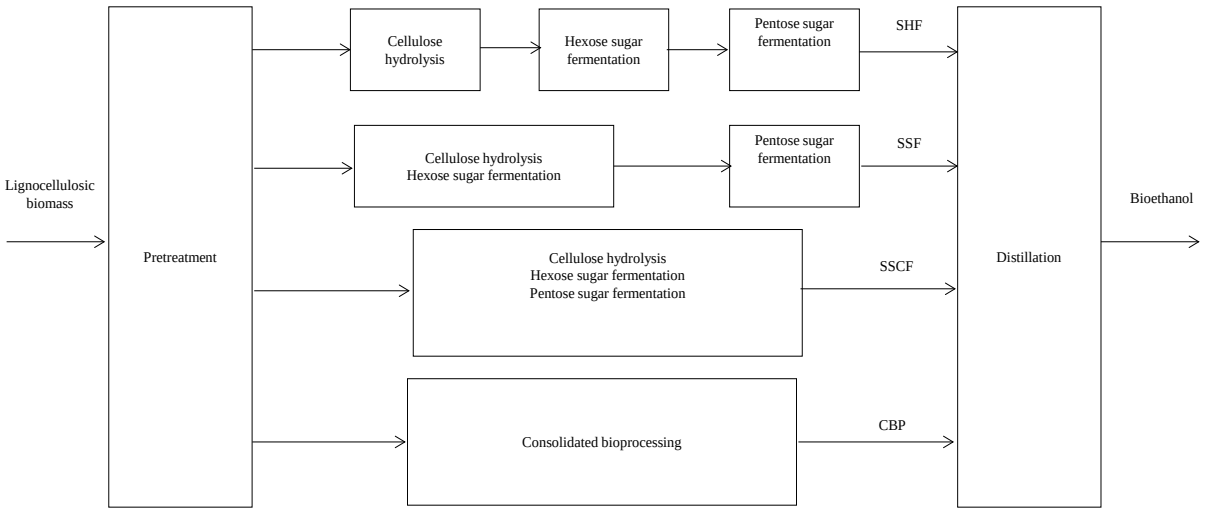

Figure 3. Process steps in lignocellulosic ethanol production reproduced from [71].

cost-effective process as the processes, namely enzyme production, hydrolysis and fermentation are taking place within the same bioreactor making the capital cost lower [70].

Lignocellulosic biomass represents a promising but challenging substrate for ethanol production. Hydrolysis of lignocellulosic substrates results in the formation of both hexose and pentose sugars from cellulose and hemicellulose, respectively. Ethanol is produced primarily by the fermentation of glucose liberated from cellulosic feedstock using fermentative microorganisms, principally yeasts, *S. cerevisiae* [72]. The most common microbe used has been *S. cerevisiae* which, as Lin and Tanaka [73] reported, can produce ethanol at concentrations as high as 18% in the fermentation broth. It is a relatively easy microbe to handle as it is generally recognized as safe. *Z. mobilis*, a Gram-negative bacterium, can also be used in fermentation of glucose into ethanol [74]. Biomass formed during fermentation using *S. cerevisiae* and *Z. mobilis* are recognized as safe for fodder, making these organisms suitable for metabolic engineering for application in co-fermentation of both pentose and hexose sugars. Recent reports suggest that some white rot fungi [75], namely *Agaricus bisporus*, *Bjerkandera adusta* and *Iprex lacteus*, are able to produce ethanol from glucose under semi-aerobic conditions. Jung and

Table 5. 2nd generation bioethanol production from agricultural residues and residues from 1st generation bioethanol process.

Feedstock Feedstock availability	Composition, % w/w	Pretreatment	Hydrolysis and fermentation	Ethanol	Reference
Corn stover (agricultural by-product 80 - 100 million dry tonnes annum^{-1} [40]	Glucan-41.0% Xylan-25.3% Arabinan-6.1% Galactan-3.0% Lignin-21.0%	Steam pretreatment of SO_2 impregnated corn stover at 200˚C for 5 min	SSF at 10% WIS using cellulase and β-glucosidase mixture and *S. cerevisiae* (Baker's yeast); fermentation at 30˚C, pH 5, 72 h and inoculum 2 g yeast·L^{-1}	74% of the theoretical ethanol yield, ethanol 25 g·L^{-1}	[41]
Corn stover	Glucan-42.2% Xylan-19.6% Arabinan-2.9% Galactan-1.1% Lignin-20.8%	Steam pretreatment as described in [41]	Pre enzymatic hydrolysis using Thermo-active enzyme mixture followed by SSF using *S. cerevisiae* (Baker's yeast) at 11.50% WIS; fermentation at 35˚C, pH 5, 96 h and inoculum 1.8 g yeast 100 mL^{-1}	Ethanol 33.8 g·L^{-1}, 80.2% overall ethanol yield	[42]
Corn stover		Steam pretreatment as described in [41] Ohgren *et al*., 2006	8% WIS and 10 FPU/g WIS SSF Vs. SHF; *S. cerevisiae* in fermentation at 35˚C, pH 5, 144 h and inoculum 1 g dry yeast L^{-1}	Theoretical ethanol yield in SSF-72.4 % and in SHF-59.1 %	[43]
Japanese cedar (*Cryptomeria japonica*) 7.6 million tonnes annum^{-1} forest residues		Crushed to 20 micron of particle size using Cogwheel mill	SSF using commercial cellulase and *S. cerevisiae* after 24 h. Fermentation conditions not available	270 L ethanol tonne^{-1} of Japanese cedar, 0.2 g ethanol g^{-1} of Japanese cedar	[44]
Wheat straw 84.5 million dry tonnes of wheat straw annum^{-1} at residue to wheat grain ratio of 1.3 - 1.7:1 [45]	Cellulose 33.5%, Hemicellulose 22.4%, Klason lignin 16.4%, Ash 5.8%, Residual 21.8%	A three step pretreatment: Presoaking at 80˚C for 20 min followed by thermal treatment at 170˚C - 180˚C for 7.5 - 15 min then steam treatment at 195˚C for 3 min	Enzymatic hydrolysis of the solid residue using Cellubrix L enzyme	203 - 205 kg ethanol tonne^{-1} of straw from cellulose fraction; 350.5 kg tonne^{-1} from both cellulose and hemicelluloses fractions (calculated based on sugars obtained in hydrolysis step)	[46]
Cassava stems and peelings Cassava stems and peelings: 403 tonnes·ha^{-1}	Cellulose 28.9%, 9.7% Hemicellulose 21.1%, 32.3% Klason lignin 30.6%, 16.9% Proteins 1.4%, 3.7% Ash 7.3%, 11.3% Lipids 0.7%, 1.7% others 9.9%, 24.2% in stems, peelings, respectively	Thermohydrolysis at 225˚C for 50 min	Enzymatic hydrolysis using Cellulase followed by fermentation using *S. cerevisiae* or *Rhyzopus spp.* at 1 g dry biomass inoculum per 100 mL hydrolyzate and other fermentation conditions not available	Stems: 5.2 g ethanol 100 g^{-1} stems Peelings: 2.6 g ethanol 100 g^{-1} peelings	[47]
Cassava cellulosic wastes from starch processing Liquid waste (1% total solids): 8.9 - 10.6 tonnes and Wet cassava bagasse: 0.9 - 1.1 tonnes from 1 tonne of dry cassava processed [48]	Carbohydrate 76.6% Starch 60.8% Fibre 15.8% Protein 0.8%	Hydrolysis using α-amylase for 1 h at 97˚C - 100˚C followed by Dilute HCl hydrolysis	Saccharification of hydrolyzed starch using amyloglucosidase at 50˚C - 60˚C followed by fermentation using *S. cerevisiae* at 40˚C - 50˚C, pH 4.6 - 5.5, 8 h and inoculum 0.2 g dry biomass per 100 mL hydrolyzate	2.7 g ethanol 15 g^{-1} cassava cellulosic waste, 32.4% w/w ethanol concentration	[49]
Sugar cane bagasse 276 kg bagasse·tonne^{-1} of sugarcane; Sugarcane harvest in South Central Brazil 516 million tonnes in 2011/2012 [50]	Cellulose 52% Hemicellulose 20% Lignin 24% [51]	Delignification using NaOH (1N) at reflux temperature for 2 h	SSF using cellulase and *S. cerevisiae* using 1 g de-lignified bagasse per 20 mL medium; fermentation 5 days and other conditions not available	11.8 g ethanol·L^{-1}	[52]

Continued

Egyptian sugarcane bagasse	Cellulose 41.3% Hemicellulose 27.4% Lignin 12.1%	Chipped, ground bagasse autoclaved at 121˚C for 20 min	Separate hydrolysis and fermentation: (i) Biological hydrolysis using *Trichoderma viride* and fermentation using *Candida tropicalis*; (ii) Biological hydrolysis using *Aspergillus terreus* and fermentation using *S. cerevisiae* Fermentation at 30˚C, 48 h and inoculum 10% v/v	(i) 249 kg of ethanol tonne^{-1} bagasse; (ii) 204 kg of ethanol tonne^{-1} bagasse	[53]
Sugar cane bagasse	Cellulose 42% Hemicellulose 25% Lignin 20% [54]	Dilute sulphuric acid (1% v/v) treatment at 121˚C for 45 min with a solid to liquid ratio of 1:2 followed by NaOH (4% w/v) treatment at 121˚C for 30 min with a solid to liquid ratio of 1:20	Simultaneous Saccharification using enzyme prepared using *Pencillium funiculosum* ATCC 11797 and pretreated sugarcane bagasse substrate followed by fermentation using *S. cerevisiae* at 37˚C, pH 5, 144 h and inoculum 15 g·L^{-1}	Ethanol 100 g·L^{-1}, 121.2 L of ethanol tonne^{-1} of sugarcane bagasse	[55]
Sugar beet pulp 4717360 tonnes annum^{-1} from US, Europe and Asia [56] and 17 million tonnes sugar beet annum^{-1}	Total carbohydrate 80%: Rhamnose 2.4% Arabinose 23.0% Galactose 6.2% Glucose 25.9% Mannose 1.0% Xylose 1.7% Galacturonic acid 14.4%	Steam pretreatment at 152˚C - 175.5˚C and 4 - 8 bar(g) pressure	Enzymatic hydrolysis using commercially available cellulase at 50˚C for 24 h followed by fermentation using *S. cerevisiae* at 30˚C for 24 h and other conditions not available	0.5 g ethanol per g of glucose from sugar beet pulp	[57]
Sweet sorghum bagasse (SSB) 36.0 - 45.4 tons/ha [58]	Carbohydrates 58.3% Lignin 18.6% Ash 1.9% Extractives 21.2%	Steam pretreatment of 2.5% SO_2 impregnated bagasse at 200˚C for 7.5 min	Enzymatic hydrolysis using commercial cellulase and β-glucosidase at 50˚C for 72 h followed by fermentation using *S. cerevisiae* at 30˚C, pH 6, 48 h and inoculum 3 - 5 g·L^{-1}	15.3 g ethanol 100 g^{-1} SSB, 72.7% conversion of hexose sugars to ethanol	[59]
Sweet sorghum bagasse (SSB)	Cellulose 34% - 45% Hemicellulose 25% - 27% Lignin 18% - 21 % [54]	SSB in dilute NaOH solution (2% w/v) autoclaving at 121˚C for 60 min and H_2O_2 immersing	Enzymatic hydrolysis at 50˚C using Celluclast supplemented with β-glucosidase followed by fermentation using Active dry yeast at 30˚C and inoculum at 1:10 volume ratio of yeast medium to fermentation broth	Total sugar yield of 90.9 g sugar 100 g^{-1} dry SSB, Ethanol 6.1 g·L^{-1}	[60]
Sweet sorghum bagasse (SSB)		NaOH pretreatment	Enzymatic hydrolysis using commercial cellulase and xylanase from Novozyme followed by fermentation using *Zymomonas mobilis*at 32˚C, pH 6, 30 h and inoculum *Z. mobilis* culture at 10% v/v	61.8% of the theoretical ethanol yield	[61]
Sweet sorghum stover		Dilute sulphuric acid (0.37% v/v) treatment in a high pressure reactor at 150˚C for 15 min	Enzymatic hydrolysis using commercial cellulase (Zytex) at 50˚C for 48 h followed by fermentation using *S. cerevisiae* at 30˚C, 48 h and inoculum 0.27 g·L^{-1}	91.9 g ethanol·kg^{-1} native sorghum	[62]
Rice straw 34.3 million tonnes annum^{-1} from Thailand	Cellulose 32% - 47 % Hemicellulose 13% - 27 %	Aqueous ammonia (27% w/w) treatment at 1:12 solid: liquid ratio at room temperature (25˚C ± 3˚C) for 14 days followed by washing	SSF using cellulase (Cellic Ctec2) and xylanase (Cellic Htec2) and *S. cerevisiae* and *Candida tropicalis* at 37˚C for 72 h and inoculum 1.2 g yeast in 10 mL YP medium	25.1 g·L^{-1}, $Y_{p/s}$ 0.4 g·g^{-1}	[63]

Continued

Rice straw	Glucan 44.8% Xylan 20.8% Lignin 18.3%	0.5 M Na_2CO_3 pretreatment at 100˚C for 3 h	SSF using cellulase (Celluclast 1.5 L), β-glucosidase (Novozyme 188) and *Mucor hiemalis* as fermenting organism at 37˚C, pH 5.5, 72 h and inoculum 1 g dry biomass L^{-1}	12.8 $g{\cdot}L^{-1}$, 154 g ethanol $k{\cdot}g^{-1}$ rice straw, 83% ethanol yield	[64]
Rice straw		Two step pretreatment Step1: Dilute sulphuric acid (1% w/w) treatment at 100˚C for 2 h at 10% w/v ratio of rice straw; Step2: Sulphomethylation treatment at 160˚C for 5 h at 15% w/v of acid treated rice straw	SSF using cellulase (Onozuka R-10) and β-glucosidase and *S. cerevisiae* at 40˚C, 72 h and inoculum 5 mL yeast culture of $1.5^{*}10^{8}$ cells$\cdot mL^{-1}$	Ethanol concentration 40.6 $g{\cdot}L^{-1}$; Ethanol yield 86.4%	[65]
Palm empty fruit bunch 6.7 million tonnes $annum^{-1}$	Cellulose 50.3% Hemicellulose 23.3% Lignin 23.5% Ash 3.0%	Dilute H_2SO_4 (1% v/v) treatment at 125˚C for 90 min followed by NaOH (1% w/v) treatment at 100˚C, for 60 min	Enzymatic hydrolysis using Cellulase (Novozymes) at 50˚C for 72 h followed by fermentation using *S. cerevisiae* at 30˚C, pH 4.0, 72 h and inoculum 10% v/v	Ethanol 12.1 $g{\cdot}L^{-1}$; 89.1% of the theoretical ethanol yield	[66]

co-workers [76] reported the use of *Kluyveromyces marxianus* for ethanol fermentation from empty palm fruit bunches. Much research continues in this field in search of efficient fermentative microorganisms for application in the simultaneous fermentation of pentose and hexose sugars. *S. cerevisiae* can readily ferment hexose sugars but it is not able to use pentose sugars in its metabolism to produce ethanol. Therefore, the co-fermentation of hexose and pentose sugars is expected to improve ethanol yields from lignocellulosics which can be possible by applying engineered/recombinant yeast strains in the fermentation of ethanol, an area of active research at the present [77].

3. Lignocellulosic Biomass Pretreatment Techniques

The main aim of lignocellulosic biomass pretreatment is to separate the biomass components *i.e*. cellulose, hemicellulose and lignin and eventually to remove lignin without losing hemicellulose while decreasing the crystallinity of cellulose and increasing the porosity of the biomass material. A number of techniques are available for the pretreatment of biomass; these include hot water treatment, steam explosion, ammonia fiber explosion, alkali treatment, organic solvent treatment and enzymatic hydrolysis. A brief description of the pretreatment methods is presented here.

3.1. Hot Water Treatment [78]

This type of pretreatment is also termed aqua-solve, aqueous fractionation, hydrothermolysis, and uncatalyzed solvolysis. In hot water treatment, biomass is treated with liquid hot water at elevated temperature and the treatment uses pressure to maintain the water in the liquid state. Water at high temperatures acts as an acid in the fractionation of the biomass rigid structure. The main component of the operating cost for this method is the energy required to feed the water as a saturated liquid. The treatment time for this process is 15 - 20 minutes at temperatures in the range of 200˚C - 230˚C. Approximately 40% - 60% of the total biomass is dissolved in this process.

3.2. Steam Explosion [79]

Steam explosion is the most commonly used method for the pretreatment of biomass. In this method, biomass is treated with high-pressure saturated steam, and then the pressure is suddenly reduced, which makes the materials undergo an explosive decompression.

Steam explosion is initiated at a temperature of 160˚C - 260˚C for several seconds to a few minutes before the material is exposed to lower pressure. The process causes hemicellulose degradation and lignin transformation

due to high temperature, thus improving cellulose hydrolysis. Addition of acid ≤3% (w/w) in steam explosion can decrease time and temperature, effectively improving hydrolysis, and leads to the complete removal of hemicellulose.

3.3. Ammonia Fiber Explosion [80]

Ammonia fiber explosion is a physicochemical pretreatment process in which lignocellulosic biomass is exposed to liquid ammonia at high temperature and pressure for a period of time, and then the pressure is suddenly reduced. The process is very similar to steam explosion. During pretreatment only a small amount of the material is solubilized. The structure of the material is changed, resulting in increased water holding capacity and higher digestibility in subsequent processing. Ammonia fiber explosion has been reported to be ineffective for biomass with higher lignin content (~25%).

3.4. Carbon Dioxide Explosion [81]

In the carbon dioxide explosion method biomass is treated with supercritical carbon dioxide at comparatively lower temperatures than steam explosion. It is hypothesized that CO_2 forms carbonic acid when dissolved in water, increasing the hydrolysis rate. Increased rate of penetration of CO_2 molecules into the crystalline structure of biomass is facilitated by an increase in pressure. Carbon dioxide hydrolyzes hemicellulose as well as cellulose. Moreover, the low temperature treatment helps in preventing the decomposition of monomer sugars formed during the treatment. However the yields are relatively low compared to those of other pretreatment methods. A comparative study on the pretreatment of sugar cane bagasse and recycled paper and its re-pulping waste using different treatment methods including CO_2 explosion, steam explosion and ammonia fiber explosion concluded that CO_2 explosion is more cost-effective than other methods.

3.5. Organosolvation [82]

In the organosolvation process biomass is treated with a mixture of organic/aqueous organic solvents and acid catalysts (inorganic and organic). The most commonly used solvents are methanol, ethanol, acetone, ethylene glycol, triethylene glycol and tetrahydrofurfuryl alcohol. The process facilitates simultaneous hydrolysis and delignification of lignocellulosic biomass. Lignin can be recovered as a fine precipitate by flash exposure of the liquor to atmospheric pressure, followed by rapid dilution with water. Other products such as sugars and sugar degradation products can be recovered from the water soluble stream. Solvents from the process can be recycled to reduce the cost.

3.6. Alkaline Hydrolysis [83]

Alkaline hydrolysis processes use lower temperature and pressures than other pretreatment methods. The most commonly employed alkaline pretreatment agents are sodium hydroxide, potassium hydroxide, calcium and ammonium hydroxides. Alkali pretreatments carried out under mild conditions require long pretreatment times, in the order of hours to days. However, treatment at mild temperatures (25˚C - 55˚C) selectively removes lignin and hemicellulose while cellulose is unaffected. Lignin removal increases enzyme effectiveness by increasing access to cellulose and hemicellulose and by eliminating non-productive adsorption sites. The effect of alkaline pretreatment of different biomass feedstocks depends on the lignin content of the materials.

4. Hydrolysis of Lignocellulosic Biomass

In bioethanol production from lignocellulosic biomass the pretreated feedstock must be hydrolyzed to convert cellulose and hemicellulose fractions to simple sugars. Therefore, a pre-hydrolysis of the feedstock is needed to improve the conversion of cellulose and hemicellulose to free sugars for application in further bioethanol production. Hydrolysis of lignocellulosic biomass for sugars synthesis can be carried out using either acid or enzyme treatment and a brief discussion is presented in the following sections.

4.1. Acid Hydrolysis of Lignocellulosic Biomass

Acid hydrolysis is a process in which biomass is treated with water in the presence of acid to give sugars. The

treatment process converts the cellulose and hemicellulose to sugars. Acid hydrolysis is the most common methodology for biomass conversion to fermentable sugars, where virtually any acid (H_2SO_4, HCl, H_3PO_4) can be used. Hydrolysis of biomass for the release of sugars takes place through either a dilute acid treatment or concentrated acid treatment. Existing acid hydrolysis processes consists of two stage acid hydrolysis [84], using double acids and heterogeneous acids. Important parameters such as reaction temperature, acid concentration, reaction time and particle size determine the conversion and yield of sugars obtained. Dilute acid hydrolysis can be carried out at lower temperature with longer reaction times and at higher temperatures with shorter reaction times. Longer reaction time results in the degradation of monomers released from hemicellulose; this observation was reported by Cruz and coworkers with barley husks [85].

Different biomass feedstocks such as bark rich saw mill waste, rice straw, grass, silage press cakes, sugar maple wood extract, oil palm empty fruit bunch [86], wood shavings, sweet sorghum bagasse [87] and nitrogen rich dairy manure [88] have been processed using dilute acids for sugar release from the feedstock. Reports on the dilute acid hydrolysis processes, carried out in two steps with different acid concentrations at each stage [84] [89] [90], varying from 0.05% to 2.5% state that yields reached around 80% - 85% of the sugars available in the biomass. For example, a pre-extraction step with water at low/high temperature [91] followed by acid hydrolysis of maple wood resulted in around 160 g sugar·L^{-1} concentrated wood extract [92].

A range of acids have been employed for the breakdown of the crystalline structure of biomass constituents; these include sulfuric acid, hydrochloric acid, phosphoric acid and H-USY zeolite treated with oxalic acid [93]. The specific interest in the use of H_3PO_4 in acid hydrolysis is that after neutralization with sodium hydroxide, it will yield sodium phosphate which will remain in the hydrolyzate and subsequently be used as a nutrient by microorganisms in the fermentation for ethanol production negating the requirement for filtration [94]. However, hydrolysis with H_3PO_4 does require higher temperatures and increased acid loading compared to hydrolysis with sulphuric acid.

The use of concentrated acid hydrolysis represents a promising process for the hydrolysis of biomass for both biofuel and bio-refinery applications, with high sugar yields, lower levels of fermentation inhibitors, good fermentability and a general robustness towards changes in raw material quality. The treatment of cellulose with concentrated sulphuric acid solution (50% - 60%) at room temperature [95] resulted in good solubility and the recovered cellulose had an amorphized structure characterized by high enzymatic digestibility. This regenerated cellulose had reduced crystallinity (25% - 30%), and a lower degree of polymerization (40% - 50%). A two stage concentrated acid hydrolysis [96] of soft wood biomass resulted in good sugar yields and a low concentration of fermentation inhibitors. However, concentrated acid hydrolysis has some major drawbacks, namely:

- Consumption of large quantities of concentrated acids.
- High costs of neutralization.
- Gypsum disposal problems.

Concentrated acid hydrolysis requires expensive materials for process equipment construction and to make the process economically feasible acid recovery is needed which itself represents an energy consuming step. Therefore, dilute acid hydrolysis is a more suitable option compared to concentrated acid hydrolysis.

Currently, biomass treatment technologies are energy intensive due to the large amount of water usage and the requirement for heating the process material to pretreatment temperatures of 100˚C - 200˚C [97]; in addition, the conversion process results in the accumulation of salts and inhibitors that are toxic to subsequent bio-refinery processes. Therefore, conversion of lignocellulosic biomass to biofuels requires efficient pretreatment technology, achieved through optimization of pre-hydrolysis in terms of both maximizing the sugar yield and minimizing the energy requirement.

4.2. Enzymatic Hydrolysis of Lignocellulosic Biomass

The use of enzymes in biomass conversion processes can often eliminate the requirement for high temperatures, chemicals and extremes of pH, while at the same time offering increased reaction specificity, product purity and reduced environmental impact. Enzymatic hydrolysis of cellulose and hemicellulose components of lignocellulosic biomass is carried out by cellulase and hemicellulase enzymes which are highly specific. Cellulases are mainly a mixture of endoglucanases, exoglucanases, and β-glucosidases and catalyze the hydrolysis of cellulose to simple sugars. Xylanases and β-xylosidases are the enzymes that attack the backbone of hemicellulose resulting in the production of xylose monomers. Pretreatment of lignocellulosic biomass is a prerequisite to achieve

better conversion in the enzymatic hydrolysis of biomass. The role of pretreatment is that it usually breaks down the lignin structure, as shown in **Figure 4** [36] [98], thereby facilitating the hydrolysis of cellulose and hemicellulose, resulting in the production of hexose and pentose sugars. Lignin acts as physical barrier limiting the accessibility of enzymes to cellulose and hemicellulose substrates. The available techniques for the pretreatment of biomass have been discussed in the previous section [71]. Biological pretreatment can represent an ecofriendly and a low cost alternative to physico-chemical and chemical pretreatments of lignocellulosic biomass. However, biological pretreatment requires an appropriate microorganism-biomass combination, as for example it is reported that fungal treatment can cause carbohydrate loss [75]. Pretreatment results in increased porosity in the biomass substrate due to the removal of the lignin, disruption of hemicellulose, size reduction of the particles and reduction in the crystallinity of cellulose depending on the specific pretreatment technology. Enzymatic delignification can also be achieved using laccase and lignin peroxidase enzymes but the technique is limited by long residence times. Improvements in enzymatic hydrolysis for the production of bioethanol from sustainable biomass are necessary in order to reduce enzyme requirements and the overall processing times.

The other major limiting factor in the enzymatic conversion of biomass to biofuels is the cost of cellulase enzymes for use in the hydrolysis of pretreated biomass [99]. Techno-economic analysis of lignocellulosic bioethanol production costs report that the enzymes cost about $ 132 per cubic meter of ethanol when the enzymes are supplied by commercial enzyme manufacturers, such as Novozymes [100]. However in the case of on-site enzyme production the overall cost of enzymes was reported to be $ 90 per cubic meter of ethanol, significantly lower than Novozymes. Therefore, to achieve cost effective biomass conversion for biofuel production an on-site/in house enzyme production for the continuous supply of cellulases to the process appears as one of the most economically attractive options.

Much information is available on the preparation of cellulase enzymes using different substrates and a variety of cellulolytic microorganisms for application in lignocellulosic bioethanol production have been reported. Both bacteria (e.g. *Bacillus*, *Bacteriodes*, *Cellulomonas*, *Clostridium*, *Streptomyces*) and fungi (e.g. *Phanerochaete chrysosporium* [101], *Tricoderma reesei*, *Aspergillus niger* [102], *Gracibacillus* species [103], *Penicillium oxalicum* [104]) can produce cellulases. A variety of substrates have been employed in cellulase production; for instance Humbird *et al.* [105] reported cellulase preparation using corn syrup substrate and *T. reesei*; Jing *et al.* [106] used hydrolyzed sugarcane bagasse residue as substrate for cellobiohydrolase production using *P. oxalicum*; Vijayaraghavan & co-workers [107] reported carboxymethyl cellulase production from cow dung by *Bacillus halodurans* ID 18. The use of cheap lignocellulosic biomass substrates for enzyme production can significantly reduce the production cost of cellulases. Wheat bran has been reported to be an effective substrate for the preparation of cellulases using *T. reesei* and *A. niger* [102]. Other potential woody and herbaceous substrates used in cellulase production by white rot fungi and brown rot fungi via solid state fermentation include eucalyptus wood chips, pine wood chips, beech leaves, wheat straw, wheat bran, corn fiber, corn stover, reed grass, bean

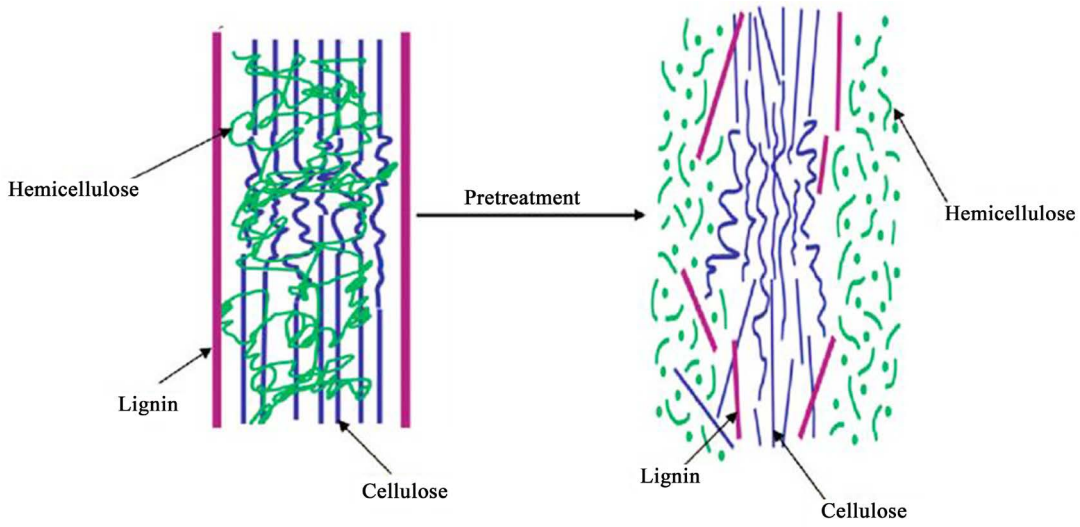

Figure 4. Pretreatment for the breakdown of the rigid structure of biomass [36] [98].

stalk and sago waste. Solid state fermentation for enzyme production is the most adopted technology as it requires less infrastructure and less skilled manpower to operate and has lower operational costs.

5. Conclusion

Depleting fossil reserves and deleterious effects of fossil fuel burning on the environment led to the search for alternate fossil fuels which must be ecofriendly and renewable. Bioethanol is a promising renewable biofuel produced from agricultural crops (sugarcane, sugar beet, corn, wheat) and cellulosic feedstock. Conventional bioethanol (1st generation) production based on edible agricultural products conflicts with food supply and causes food price increase. As an alternative to edible agricultural feedstock, lignocellulosic biomass (2nd generation) has been gaining attention as a sustainable feedstock (pulp, stover, stalk, stems and leaves) for bioethanol production. Ligncellulosic biomass based bioethanol requires a multi-step complex conversion technology due to its rigid structure, comprised of milling (size reduction), pretreatment, hydrolysis and fermentation. Optimization of the pretreatment strategy aimed at reducing the formation of degradation products and optimization of enzyme mixtures for efficient conversion of pretreated biomass together with improving fermentation efficiency using genetically modified strains, mixed cultures and the application of thermoanerobes which can ferment hexose and pentose sugars to improve ethanol yield are key areas of future research. On-site/in-house enzyme preparation in solid state fermentation is also gaining significant research attention in the 2nd generation bioethanol process as commercial enzymes are expensive, representing a significant barrier to the commercialization of this technology.

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