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REVIEW

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Microbial conversion of pyrolytic products to biofuels: a novel and sustainable approach toward second-generation biofuels

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Abstract This review highlights the potential of the pyrolysis-based biofuels production, bio-ethanol in particular, and lipid in general as an alternative and sustainable solution for the rising environmental concerns and rapidly depleting natural fuel resources. Levoglucosan (1,6-anhydrous-β-D-glucopyranose) is the major anhydrosugar compound resulting from the degradation of cellulose during the fast pyrolysis process of biomass and thus the most attractive fermentation substrate in the bio-oil. The challenges for pyrolysis-based biorefineries are the inefficient detoxification strategies, and the lack of naturally available efficient and suitable fermentation organisms that could ferment the levoglucosan directly into bio-ethanol. In case of indirect fermentation, acid hydrolysis is used to convert levoglucosan into glucose and subsequently to ethanol and lipids via fermentation biocatalysts, however the presence of fermentation inhibitors poses a big hurdle to successful fermentation relative to pure glucose. Among the detoxification strategies studied so far, over-liming, extraction with solvents like (*n*-butanol, ethyl acetate), and activated carbon seem very promising, but still further research is required for the optimization of existing detoxification strategies as well as developing new ones. In order to make the pyrolysis-based biofuel production a more efficient as well as cost-effective process, direct fermentation of pyrolysis oil-associated fermentable sugars, especially levoglucosan is highlly desirable. This can be achieved either by expanding the search to identify naturally available direct levoglusoan utilizers or modify the existing fermentation biocatalysts (yeasts and bacteria) with direct levoglucosan pathway coupled with tolerance engineering could significantly improve the overall performance of these microorganisms.

Keywords Pyrolysis oil · Pyrolytic sugars · Levoglucosan · Bio-ethanol · Levoglucosan kinase

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Introduction

The growing environmental threat posed by air pollution, and the increasing oil demand have forced the world in general and China in particular to find alternative and sustainable solution to fossil fuel. Air pollution is a serious issue for most of the developing countries, killing more people than malaria, tuberculosis, AIDS, and breast cancer [127, 134, 135, 193]. The observed air pollution contributes to an estimated 1.6 million deaths every year in China (0.7–2.2 million people per year at 95 % statistical confidence), or about 4400 people a day and around 17 % of the total deaths in China [159]. The ever increasing gap between domestic oil supply and demand is another reason driving China to seek alternative fuels, especially biofuels in transportation sector [20]. The quantity of domestic crude oil produced in China is supposed to remain constant around 200 million tons by 2020 [180] and even by 2050 [107]. Meanwhile, the projected oil demand for domestic purposes will increase to 600–700 million tons by 2030 and 700–800 million tons by 2050 [27]. According to the research of the International Energy Agency (IEA), biofuels could contribute about 27 % of the total transportation fuel by 2050. The projected use of biofuels has the potential to avoid CO_2 emission (2.1 gigatonnes/year) into the atmosphere if produced in a sustainable manner [74].

Table 1 World fuel ethanol production by country or region (million gallons)

Countries	2007	2008	2009	2010	2011	2012	2013	2014
USA	6.521	9.309	10.938	13.298	13.948	13.300	13.300	14.300
Brazil	5.019	6.472	6.578	6.922	5.573	5.577	6.267	6.190
European Union	570	734	1.040	1.209	1.168	1.179	1.371	1.445
China	486	502	542	542	555	555	696	635
Canada	211	237,7	291	357	462	449	523	510
Rest of World	315	389	914	985	698	752	1.272	1.490
World	13.123	17.644	20.303	23.311	24.404	21.812	23.429	24.570

Source: F.O. Licht, cited in renewable fuels association, ethanol industry outlook 2007–2014 reports. Available at http://www.afdc.energy.gov/data/10331

Bio-ethanol is a biofuel produced from sugar, starch, or cellulose-based feedstocks. Production of bio-ethanol from sugar-based crops, such as sugar cane, sugar beet, sweet sorghum, and fruits is the most efficient and easiest process since yeast and bacteria can readily ferment the sugar. However, yeast and bacteria cannot directly ferment the starch-based feedstocks, such as corn, wheat, milo, barley, potatoes, and cassava, hence enzymes are used to convert the complex polysaccharides into monomeric sugar units before fermentation [30]. Currently more than 90 % of the world's bio-ethanol is produced from edible feedstock (sugars and starch-based), which is also known as the "first generation" bio-ethanol feedstock [117], and the bio-refineries based on edible food are known as "first generation" bio-refineries, while the fuel is identified as "first generation" biofuel [29, 52, 123]. In 2014, United States was the world's leading bio-ethanol producer with 14.3 billion gallons (bg) followed by Brazil with 6.2 bg, together accounting more than 84 % of the world bio-ethanol production [57]. Asian countries and especially China are now on the way for bio-ethanol production but still lag behind USA and other developed countries, as shown in Table 1.

The advantages of using first-generation feedstock for bio-ethanol production are the high sugar content of the feedstock material, relatively simple conversion processes, and the available well-developed technology. But unfortunately, first-generation bio-ethanol feedstock is also used for food and feed purposes, which means that its usage would compete with food and feed industries for feedstock, fertile agricultural land, and fresh water [160, 178, 203]. Therefore, the first-generation biofuel becomes controversial due to food vs fuel debate, ethical, and environmental reasons.

To overcome the limitations of first-generation biofuels, the concept of second-generation biofuel based on nonedible lignocellulosic biomass, such as forestry wood, agricultural residues (sugarcane bagasse, grasses, straw etc.), and municipal solid wastes was introduced [22, 54, 80]. The biomass feedstock used to produce second-generation biofuels contain high amount of sugars in the form of polysaccharides, which can be converted to second-generation biofuels [52, 112]. Lignocellulosic biomass has numerous advantages over the first-generation feedstock such as, it is widely distributed, inexpensive, as well as abundantly available, further it does not compete with food, freshwater, and agriculture land [23, 112]. However, converting lignocellulosic feedstock is much more difficult task than sugar and starch-based biomass due to the recalcitrance nature of lignocellulose materials. Cellulosic feedstock is converted into bio-ethanol in three steps: pretreatment, hydrolysis, and fermentation. The pretreatment step releases the cellulose and hemicelluloses by breaking down the complex structure of lignocelluloses, hydrolysis is applied to convert the cellulosic and hemicellulosic polysaccharides into simple sugars, and finally the sugars are fermented by yeast or bacteria to produce bio-ethanol [55]. The traditional conversion methods aimed to release sugars from lignocellulosic biomass involve either the use of acids or enzymes in bulk quantities, which is no way easy and economical [47, 158]. The other drawback is that during the pretreatment and hydrolysis, along with the release of fermentable sugars, a variety of unwanted by-products are also produced from the lignocellulosic biomass. These by-products have been reported to be highly toxic to the enzymatic hydrolysis step as well as microbial fermentation.

In recent times, pyrolysis process has attracted attention as an efficient and faster method for the depolymerization of lignocellulosic biomass compared to acid hydrolysis or enzymatic hydrolysis. Pyrolysis oil, also commonly called bio-oil, is a product of biomass processing in a process called fast pyrolysis. Depending on the type of lignocellulosic material and the operating conditions used during pyrolysis, bio-oil can contain up to 33 wt.% of levoglucosan, an anhydrosugar that can be readily hydrolyzed to glucose [96]. Although several technologies are available that can be used to convert sugars into advanced generation biofuels (butanol, hydrocarbons, etc.), fermentation of sugars to produce ethanol is still the dominant technology in practice [31, 190].The concept of converting pyrolytic sugars into bio-ethanol was initially proposed by Shafizadeh and Stevenson [168], since then several researchers studied the feasibility of producing bio-ethanol from pyrolysis oil

[10, 96, 197]. Microbial conversion of pyrolytic products into bio-ethanol is a relatively unexplored area of biological research and information on how to separate, hydrolyze, neutralize, detoxify, and ferment the levoglucosan from pyrolysis oil for the production of bio-ethanol fuel is still very limited. This review highlights bio-ethanol production from pyrolytic sugars mainly levoglucosan, the challenges, possible solutions, and the possibility of microbial engineering for direct utilization of pyrolytic sugars.

Products of pyrolysis

Pyrolysis is a thermo-chemical decomposition of biomass material at elevated temperatures in the absence of oxygen or other oxidizing agents. It involves the simultaneous changes of chemical composition and physical phase, which are irreversible. Typically three different types of products are produced from pyrolysis, a liquid products known as bio-oil (~17 MJ kg^{-1}), a solid fraction known as biochar (~18 MJ kg^{-1}), and a gaseous fraction known as syngas (~6 MJ kg^{-1}). The average yields of liquid, char, and gaseous products are 60, 25, and 15 %, respectively, and the yields mainly depend on the type of biomass and process conditions [41, 116]. During the pyrolysis, the organic material is thermally decomposed at temperatures above 400 °C in the absence of oxygen into vapors, leaving behind solid residual (biochar). The polar and high molecular weight compounds condense into a liquid bio-oil, whereas the relatively low molecular weight compounds remain in the gaseous phase. The physical and chemical reactions that take place during the pyrolysis are very complex depending on the conditions of the reactor and type of biomass [5, 40]. Biomass pyrolysis is an environmental friendly biotechnology process as it generates zero waste, the liquid fraction (bio-oil) and the solid part (biochar) are commercially important, while the gaseous fraction (syngas) can be used for internal energy requirements [6, 10].

Though this article focuses more on pyrolysis oil and its biological conversion to biofuels, but here we will also briefly mention about other products of pyrolysis: biochar and syngas, which have attracted attention in recent times. Biochar is inexpensive, sustainable, and easily produced, but still many of its applications are in their infancy, further research is required to explore its commercial applications. Biochar can be used in many applications with remarkable effects. As a precursor material, biochar can be used to produce catalyst for syngas cleaning, syngas conversion into liquid hydrocarbons, and solid acid catalyst for biodiesel production [152]. Biochar can also be applied as soil amendment to increase soil quality, reduce greenhouse gas emission, and as a sorbent to remove organic and inorganic contaminants from soil and water [152]. Biochar-based activated carbon has shown promising results as a gas adsorbent to capture and store carbon dioxide [58] and hydrogen [94]. Biochar also has the potential to replace coal in direct carbon fuel cell systems (DCFC) [49, 79]. Recently, many researchers used biochar as a raw material for fabricating supercapacitor [8, 50, 59, 103].

Another useful product of pyrolysis is syngas that mainly contains carbon monoxide and hydrogen, with small amounts of carbon dioxide, methane, and water. Syngas can be used for power generation and heating purposes [77, 89]. Many microorganisms can use syngas as a substrate to produce chemicals, such as ethanol, butanol, methane, acetate, and biopolymers [77, 120]. *Clostridium ljungdahlii* is a rod-shape, anaerobic bacterium that was discovered in 1987, and is considered as a model syngas-consuming microorganism due to its remarkable capability to convert CO and H_2 into ethanol and acetate [135, 176]. Several other microorganisms including *Acetobacterium woodii* [169], *Clostridium aceticum* [171], and *Clostridium carboxidivorans* [102] have also been studies for syngas fermentation. All these microorganisms are mesophilic and produce ethanol through the reductive acetyl-CoA pathway which functional only under anaerobic conditions [33]. The most challenging issue regarding syngas fermentation is to establish culture conditions which would provide optimum gas–liquid mass transfer in such a way that the syngas is readily dissolved and available for microbial fermentation [21].

Pyrolysis oil, also a product of fast pyrolysis, commonly known as bio-oil or pyrolysis liquid, is dark brown or dark green in appearance, free-flowing in nature, and chemically very complex, comprising more than 400 compounds [15, 72, 75, 111, 116]. Chemically bio-oil is a complex mixture of water, anhydrosugars, acids, aldehydes, furans, and phenols, with yields up to 500 l of bio-oil per dry ton of biomass [17]. Table 2 shows the chemicals found in bio-oils that are derived from the major groups, such as sugars, ketones, carboxylic acids, aldehydes, alcohols, and phenolic compounds. The physical and chemical properties of bio-oil are determined by the type of biomass material and the operating conditions applied [37]. The water content of bio-oil is 15–30 wt.%, which is much higher than the petroleum oil, the high water content contributes to phase separation and low heating value of bio-oil [116]. Pyrolysis oil also has a much higher oxygen content compared to petroleum oil, which is partly due to the high water content as well as the high concentrations of sugars, aldehydes, carboxylic acids, ketones, and phenolics, in overall contributing to

Table 2 Potential oxygenated chemicals in pyrolysis oil

Compounds	Minimum (wt.%)	Maximum (wt.%)	References
Levoglucosan	0.1	30.5	[12, 37]
Cellobiosan	0.4	3.3	[37]
1,6-Anhydrogluco-furanose	0.7	3.2	[37]
Fructose	0.7	2.9	[37, 113]
Glycolaldehyde	0.9	17.5	[37, 113]
Acetic acid	0.5	17.0	[12, 37]
Formic acid	0.3	9.1	[113]
Propionic acid	0.1	2.0	[2, 113]
Acetaldehyde	0.1	8.5	[12, 37]
Ethanedial	0.9	4.6	[113]
Methyl glyoxal	0.6	4.0	[37]
Formaldehyde	0.1	3.3	[113]
Furfural	1.5	3.0	[37]
Glyoxal	0.6	2.8	[37]
Methanol	0.4	8.2	[12, 113]
Furfuryl alcohol	0.1	5.5	[37, 113]
Ethanol	0.5	3.5	[37]
Ethylene glycol	0.7	2.0	[113]
Hydroquinone	0.3	1.9	[37]
Acetol	0.2	7.4	[37, 113]
1-hydroxy-2-butanone	0.3	1.3	[37]
Isoeugenol	0.1	7.2	[113]
Catechol	0.5	5.0	[37]
Syringol	0.7	4.8	[113]
Phenol	0.1	3.8	[113]
Guaiacol	2.8	2.8	[12, 37]
Cresol	1.03	2.5	[37]
4-Methyl-2,6-dimethoxyphenol	0.5	2.3	[37]
Eugenol	0.1	2.3	[75]
Syringaldehyde	0.1	1.5	[37, 75]
3-Ethylphenol	0.2	1.3	[37]
Acetone	0.4	2.8	[37, 75]
2-Cyclopenten-1-one	0.3	1.5	[37]
2-Furanone	0.1	1.1	[75]
Methyl formate	0.2	1.9	[37]

Source: http://alexandria.tue.nl/extra2/738958.pdf

the instability, immiscibility, and low heating value of pyrolysis oil. For these reasons, the viscosity of pyrolysis oil increase with the passage of time due to the evaporation of volatile components, and the chemical reactions that take place between oxygenated compounds to achieve equilibrium [43].

Pyrolysis oil: a viable source for platform chemicals

The rich chemical composition of pyrolysis oil makes it a viable source for the thermo-chemical-based biorefinery, where both platform chemicals and conventional biofuels can be produced [183]. The three important bio-based chemicals in future are glycolaldehyde, acetic acid, and acetol, that are present in pyrolysis oil in considerable quantities; wood-derived pyrolysis oils contain about 5–13 wt.% glycolaldehyde, 0.7–7.4 % acetol [45], and 3–12 wt.% acetic acid [14, 172]. Glycolaldehyde is used as a food browning agent as well as a substrate for making renewable ethylene glycol [36]. Acetic acid is used as a common solvent and a feedstock for producing cellulose-derived biopolymers and vinyl acetate [7, 24]. Acetol is applied as an intermediate chemical to synthesize glycol, acetone, acrolein, propylene, propionaldehyde, and furan derivatives [34]. Acetol is also used in food industry to provide flavor to milk and food [115]. The recovery of these bio-based platform chemicals is difficult due to the complex chemical composition of pyrolysis oil and also the dilute concentration of these chemicals in pyrolysis oil [44]. Distillation is not a good choice due to the chemical and thermal instability of pyrolysis oil, and also the minor differences in boiling points of the various chemicals present in the oils. Therefore, solvent extraction is considered a good option [153]. In order to reduce the chemical complexity of pyrolysis oil, water is used to separate it into distinct polar and non-polar fractions [183].

Glycolaldehyde and acetic acid can be extracted from the aqueous polar fraction either by physical or reactive extraction [154, 184, 185]. Physical extraction is straightforward and back-extraction can also be performed by simple addition of water; however, physical extraction of glycolaldehyde with medium polar organic solvents gives low yield and selectivity. Research shows that 9 % of glycolaldehyde could be extracted from the aqueous fraction of pyrolysis oil derived from a forest residue in a single-step extraction with a solvent-to-feed ration of 0.5. Besides 6 % of acetol and 15 % of acetic acid were also co-extracted. Glycolaldehyde up to 85 % could be recovered in a multi-stage cross-current back-extraction with water [184]. Physical extraction using organic solvents has also been widely studied to extract acetic acid from the aqueous fraction [56, 60, 85, 170]. However, physical extraction is thought to be rather ineffective, because the distribution coefficients are remarkably low and almost temperature independent [68, 81].

Therefore, reactive extraction with tertiary amines has been widely investigated to extract acetic acid from diluted solutions of waste water streams [157], fermentation broth

[67, 68, 73] and aqueous fraction of pyrolysis oil [108, 154]. Reactive extraction with tri-*n*-octylamine (TOA) [108] and sodium bisulfite [182] was used to extract acetic acid and glycolaldehyde directly from pyrolysis oil. However, both of these methods did not prove a good choice due to significant TOA losses [108], further the stability of glycolaldehyde-bisulfite complicates the product recovery [182]. Several possible integrated process designs were assessed to recover glycolaldehyde and acetic acid from pyrolysis oil via water extraction in Aspen Plus®. These process designs were evaluated for their yields, energy requirements, and projected total annual costs [35]. One such process configuration makes use of 40 wt.% TOA/2-ethyl-1-hexanol to recover glycolaldehyde and acetic acid simultaneously. This process can extract acetic acid up to 89.4 %, and glycolaldehyde up to 99.8 %, besides it also consumes 2.5 times less energy compared to the separate glycolaldehyde and acetic acid extraction [35]. In a more recent study, a conceptual process was designed for the integrated extraction of bio-based glycolaldehyde, acetic acid, and acetol form forest residue- and pine-derived pyrolysis oils. This process can extract more than 99 % of the acetic acid and glycolaldehyde as well as about two-third of the acetol present in pyrolysis oils [186].

Pyrolysis oil-associated sugar: levoglucosan

The anhydrosugar, levoglucosan (LG, 1,6-anhydro-β-D-glucopyranose) is the most abundant sugar in bio-oil and the most attractive fermentation substrate. The thermochemical degradation of cellulose during fast pyrolysis leads to the formation of levoglucosan, and hence the chemical composition of levoglucosan ($C_6H_{10}O_5$) is the same as cellulose. Bio-ethanol and other chemicals can be produced from levoglucosan if the intermolecular glycosidic bond is broken down to give glucose [192]. LG can be obtained in large quantities under appropriate pyrolysis conditions using cellulosic materials [18, 151]. Pyrolysis of untreated biomass can produce bio-oil that contains up to 12 % levoglucosan [143, 144], and pretreatment of the biomass to remove cations can result in bio-oil that contains up to 30 % levoglucosan [148]. For the purpose of bio-ethanol production, levoglucosan is the compound of interest and is found in relatively high amount as a source of glucose [195].

The first studies regarding the production of levoglucosan via the process of pyrolysis were conducted by Pictet and Sarisin [146]. Their study is considered as a first attempt to explore the production of glucose and ethanol by the process of cellulose pyrolysis. They obtained two phases, the aqueous phase (32 wt.%) containing levoglucosan, and a yellow paste of (44 wt.%) during vacuum pyrolysis. Although fast pyrolysis of woody biomass material (lignocellulosic) produces crude bio-oil (75 wt.%), however, only 10–20 % of cellulose is converted into levoglucosan, while the remaining is converted into small molecules and charcoal with less economic importance. Levoglucosan yields of up to 60 % have been reported from vacuum or fast pyrolysis of cellulose [98, 118, 166–168]. Researchers have found that the addition of small amounts of sulfuric acid to lignocellulosic biomass causes a twofold increase in levoglucosan yield [168]. However, in most situations the levoglucosan yield is low. The reasons behind low levoglucosan yield from the fast pyrolysis of lignocellulosic biomass are still not clear, however many researchers have conducted studies to investigate the causes of low levoglucosan yield [4, 39, 42, 93, 114, 177]. It has been reported that even small amounts of alkaline metals, such as potassium and sodium, naturally found in biomass can decrease the production of pyrolytic sugars due to initiation of fragmentation reactions [93]. The alkaline metals naturally found in biomass cause passivation due to the formation of stable salts, decreasing the overall yield of levoglucosan [88]. This effect of alkaline metals can be reduced by washing the biomass with acids [137]. The undesirable reactions taking place between cellulose and other components of lignocellulosic biomass during the process of fast pyrolysis also cause a reduction in the yield of levoglucosan. In a more recent study, Zhou et al. [201] reported that the yield of levoglucosan can further be increased by removing minerals and applying a mild acid impregnation. Although a combination of acid wash and acid impregnation have been proved to be a promising approach for increasing the yield of levoglucosan, however to achieve theoretically possible yield of levoglucosan from lignocellulosic biomass by the process of fast pyrolysis is still a challenge for the scientific research. Table 3 shows the different pretreatments methods used during biomass pyrolysis in order to increase the yield of levoglucosan.

Extraction and hydrolysis of levoglucosan

In order to reduce the chemical complexity of pyrolysis oil, firstly it is separated into distinct fractions using water. The aqueous extract containing the polar compounds mainly the anhydrosugars is then separated from the non-polar fraction [183]. Due to the hydrophilic nature of levoglucosan, water is used as it is easily available and also inexpensive [129]. Bio-oils have high water content which depends on the moisture content of the biomass materials as well as the process conditions. The water content for wood bio-oils [16, 128] is 15–30 wt.%, for hay and straw bio-oils [130] is 39–51 wt.%, and for rice husk bio-oils [198] is 28 wt.%. Pyrolysis oils contain lignin-derived hydrophilic

Table 3 Important chemical pretreatments of cellulose and biomass to increase pyrolytic sugars

Pretreatment methods	Biomass; pyrolysis	LG yield (mass %)	Explanation provided	References
Sulfuric acid wash (5 %) 90 °C 5.5 h followed by water wash to pH 6.6	Cellulose (Avicel); fluidized bed (500 °C)	26.9 w/o acid 38.41 w/acid	Hydrolysis of cellulose breaks glycosidic bonds; ash removal	[149]
Sulfuric acid wash (5 %) 90 °C 5.5 h followed by water wash to pH 6.6	Poplar wood (50 % cellulose); fluidized bed (500 °C)	3.04 w/o acid 30.42 w/acid	Hydrolysis of cellulose breaks glycosidic bonds; ash removal	[149]
Organic extraction (8 h toluene-ethanol) followed by hydrolysis (0.1 M H_2SO_4, >100 °C), rinse w/H_2O, then 0.1 % (feedstock) H_2SO_4 impregnation	Douglas fir (46 % cellulose); tube furnace (400 °C)	12 w/o acid 26 w/acid 56 w/acid (on cellulose basis)	Extractives, hemicellulose, ash removal; suppressed lignin–cellulose reactions	
Acid wash and H_2O rinse, 0.1 % (feedstock) H_2SO_4 impregnation	Douglas fir (46 % cellulose); tube furnace (400 °C)	9 w/o acid 19 w/acid 41.3 w/acid (on cellulose basis)	Ash removal	[168]
Water wash, 0.1 % (feedstock) H_2SO_4 impregnation	Douglas fir (46 % cellulose); tube furnace (400 °C)	7 w/o acid 14 w/acid 30 w/acid (on cellulose basis)	Suppressed lignin–cellulose reactions and inorganics	[168]
Acid wash, 0.1 % H_2SO_4 impregnation	Cellulose; tube furnace (400 °C)	36 w/o 35 w/		[168]
10 % acetic acid, 3.75 % acetone, 3.75 %, 1.5 % propionic acid, 1.5 % guaiacol; rinse with DI water	Pine; fluidized bed (530 °C)	3.4 w/o 18 wash + rinse (39 on cellulose basis) 11.1 wash, no rinse (24 on cellulose basis)	Mineral removal	[137]
0.3 % H_2SO_4, 2L/600 g biomass followed by drying	Douglas fir (45 % cellulose); augur reactor (500 °C)	5.5 w/o (12 on cellulose basis) 12 w/ (26 on cellulose basis)	Alkaline inorganics suppression	[201]
0.2 mmol/g various acids, 15 g Aq. w/5 g biomass, dry	Switchgrass (33.3 % cellulose); Py-GC/MS (500 °C)	2 w/o acid (6 on cellulose basis) 1.5 acetic/formic acid (4.5 on cellulose basis) 3 nitric acid (9 on cellulose basis) 5 HCl (15 on cellulose basis) 12 H_3PO_4 (36 on cellulose basis) 16 sulfuric acid (48 on cellulose basis)	$H_2SO_4 > H_3PO_4 > HCl > HNO_3$ chlorides, phosphates, and sulfates form thermally stable salts (inorganic suppression)	[88]
0.05 % H_2SO_4, 2L/600 g biomass followed by drying	Douglas fir (45 % cellulose); fluidized bed reactor (500 °C)	3 w/o (6.7 on cellulose basis) 5.5 w/ (12 on cellulose basis)	Alkaline inorganics suppression	[201]

Source: http://www.pyne.co.uk/?_id=148

compounds that are soluble in water. Bio-oils separate into two distinct phases if the water content exceeds a certain limit which typically ranges from 30 to 45 wt.% [181]. The phase separation results in the formation of an aqueous fraction rich in sugars along with other hydrophilic compounds, and a non-aqueous fraction mainly containing the lignin-derived less polar compounds [116]. Many researchers have worked on the optimization of parameters applied to separate bio-oils into aqueous fractions that would contain the highest levoglucosan concentration. Bennet et al. [11] studied the optimization of the extraction conditions, such as temperature, volume of water, and the contact time in order to recover maximum levoglucosan into the aqueous phase from pyrolysis oil of scots pine material. In their work they obtained an aqueous fraction of bio-oil containing levoglucosan up to 87 g/l (7.8 wt.% of bio-oil) by the addition of water to pyrolysis oil until it reached 62 mass % at 34 °C for 22 min. Chan and Duff [19] applied an optimal ratio of water to bio-oil (100 wt.%), that resulted in levoglucosan yield of 4.98 wt.% (g levoglucosan/g bio-oil). Whereas, Wang et al. [187] achieved complete phase separation of bio-oil produced from loblolly pine particles using 1:1 (v/v) ratio of water to bio-oil, mixed for some time, and then let the mixture to settle down for 48 h at ambient temperature. Li et al. [98] reported that levoglucosan concentration up to 12.7 wt.% could be extracted from bio-oil obtained from loblolly pine wood particles using a water to bio-oil ratio of 1.3:1 at 25 °C and 20 min shaking in a water bath shaker. In a more recent study by Zheng et al. [199] recovered an aqueous fraction containing 4.1 wt.% of levoglucosan with the addition of water to bio-oil in a 1:1 ratio at 50 °C and 20 min contact time. These findings suggest that phase separation behavior of bio-oil depends on the type of biomass material used for the production of bio-oil as well as the ratio of water to bio-oil, temperature, and contact time.

For bio-ethanol production from lignocellulosic material, hydrolysis is used to release the monomeric sugar units from the relatively complex lignocellulosic feedstock. In the case of indirect ethanol fermentation from pyrolysis oil, levoglucosan and cellobiosan are first hydrolyzed into glucose which are later fermented into ethanol. Hydrolysis can be carried out by different methods such as dilute acid, concentrated acid, and enzymes, but acid hydrolysis is more favored compared to enzymatic hydrolysis due to its low cost. The disadvantage of using acid hydrolysis is that it introduces additional inhibitors to the system [10, 122, 175]. For two reasons acid hydrolysis is favored over enzymatic hydrolysis for the breakdown of pyrolytic sugars into glucose; first is the chemical composition of bio-oil is very complex, and the second is that cellulases are specific for glycosidic bonds found in cellulose only and not the 1,6-anhydro bonds in levoglucosan. Hydrolysis of bio-oil with sulfuric acid is considered to be effective as well as inexpensive option [10]. Yu and Zhang [195] used concentrated sulfuric acid (0.2 M) to hydrolyze a fourfold diluted cellulosic pyrolysate of waste cotton, autoclaved it for 20 min at 121 °C and obtained glucose yield up to 17.35 %. They also suggested that glucose concentration increased after hydrolysis due to the hydrolysis of other carbohydrate oligomers. It was later reported by Helle et al. [63] that glucose concentration exceeds the 100 % theoretical yield of glucose (based on the original concentration of levoglucosan) due to the hydrolysis of cellobiosan. The cellobiosan is hydrolyzed into levoglucosan and cellobiose and finally glucose. Bennet et al. [11] reported a maximum glucose concentration of 216 % (when based on original levoglucosan) by autoclaving bio-oil with 0.5 M sulfuric acid at 121 °C for 44 min. The resulting high glucose concentration was supposed to be due to the presence of some other glucose precursors in the bio-oil.

Fermentable substrates in pyrolysis oil

Pyrolysis oil is a good source of fermentable substrates, such as sugars (levoglucosan and cellobiosan), carboxylic acids (mainly acetic acid), glycolaldehyde, and hydroxyacetone, that could be converted into lipids, ethanol, and other chemicals via biological conversion. Levoglucosan is not very abundant in nature, however it can be found in large quantities in places where biomass burning or forest fires incidents have occurred [151]. Microbial utilization of levoglucosan can occur indirectly by hydrolyzing bio-oil containing levoglucosan to glucose with mild acid treatment in solution [63] or solid acid catalysts [132]. Also many microorganisms can directly metabolize levoglucosan via their direct LG utilization pathways [82, 124, 125, 202].

Indirect fermentation of pyrolytic sugars

Several studies have been accomplished in the past regarding the indirect fermentation of pyrolysis oil to ethanol involving separation of the anhydrosugars first using water, hydrolyzing the sugars (mainly levoglucosan) to glucose applying acids, and then the subsequent fermentation of glucose to ethanol via fermentation microorganisms (Table 4). Studies by Prosen et al. [151] evaluated a variety of fungi and yeast strains for their potential to ferment bio-oil rich in levoglucosan into ethanol. They noticed that a variety of fungal and yeast strains could consume both activated charcoal-treated liquid and acid hydrolysate of the bio-oil. However, ethanol yield for the acid hydrolysate was much higher compared to the activated charcoal-treated liquid, suggesting that acid hydrolysis might be converting levoglucosan to glucose, which is a preferred substrate for ethanol fermentation. Since then several scientific studies aiming to produce biofuels, bio-ethanol in particular, and lipids in general via indirect fermentation of bio-oil hydrolysate containing

Table 4 A detailed summary of the previous research about fermentation of pyrolytic sugars and the strategies applied to improve the yield of ethanol

Strategies/methods used to improve ethanol yield	Substrate (g/l or %)	Type of microorganisms	Target inhibitors	Scale of fermentation	Time for maximum ethanol concentration (h)	Maximum ethanol titer (g/l)	Ethanol yield (g ethanol/g glucose)	Productivity (g/l/h)	References
H_2SO_4 hydrolysis + $Ca(OH)_2$ neutralization	Glucose in hydrolysate	*S. cerevisiae*	Detection not done	Flask	24	14.2	0.45	0.59	[195]
H_2SO_4 hydrolysis + $Ca(OH)_2$ neutralization + urea	(31.6)			Flask	24	15.1	0.47	0.62[a]	
H_2SO_4 hydrolysis $Ca(OH)_2$ neutralization + urea + adaptation	(31.6)	*S. cerevisiae*	Detection not done	Flask	72	40.2	0.44	0.55[a]	[195]
	(95.8)	*S. cerevisiae*®	Detection not done						[195]
H_2SO_4 hydrolysis + $Ca(OH)_2$ neutralization + diatomite shaking	Glucose in hydrolysate (35.9)	*S. cerevisiae-CCTCC 2.399*	Detection not done	Flask	24	16.1	0.45	0.67[a]	[196]
	Glucose in hydrolysate (35.9)	*Pichia sp. YZ-1*	Detection not done	Flask	110	15.1	0.42	0.14[a]	[196]
Water extraction + H_2SO_4 hydrolysis + NaOH neutralization + higher inoculum concentration + microaerophilic conditions	20 % hydrolysate solution	*S. cerevisae* T2	Detection not done	Flask	NM	NM	0.46	0.55[a]	[11]
Water extraction + H_2SO_4 hydrolysis + $Ca(OH)_2$ over-liming + extraction with organic solvents + adaptive evolution	40 % hydrolysate solution	*S. cerevisae* T2	Acetic acid	Flask	NM	NM	0.45	NM	[19]
Biodiesel or ethyl acetate extraction + H_2SO_4 hydrolysis + $Ba(OH)_2$ neutralization + activated carbon	Glucose in hydrolysate (70)	*S. cerevisiae* ATCC 200062	Acetic acid, phenols	Flask	20	32–35	0.47	1.6	[99]
$Ca(OH)_2$ over-liming	2 % pyrolytic sugar (levoglucosan)	*E. coli* KO11 + *lgk*	Phenols	Flask	NM	NM	0.24	0.01[a]	[25]

Table 4 continued

Strategies/methods used to improve ethanol yield	Substrate (g/l or %)	Type of microorganisms	Target inhibitors	Scale of fermentation	Time for maximum ethanol concentration (h)	Maximum ethanol titer (g/l)	Ethanol yield (g ethanol/g glucose)	Productivity (g/l/h)	References
Water extraction + ethyl acetate extraction + H_2SO_4 hydrolysis + $Ba(OH)_2$ neutralization	Hydrolysate glucose + pure glucose (40 g/l)	*S. cerevisiae* DSM 1334	Acetic acid, hydroxyacetaldehyde Furans and monophenols	Microtiter plate wells	15	20	0.49	1.33	[106]
Water extraction + n-butanol extraction + H_2SO_4 hydrolysis + NaOH and $CaCO_3$ neutralization	Hydrolysate glucose and mannose (20 g/l)	*S. pastorianus* ATCC 2345	Acetic acid, furfural and HMF	Flask	36	12.12	0.5	0.34[a]	[174]
Water extraction + NaOH over-liming	2 % pyrolytic sugar (levoglucosan)	*E. coli* KO11 + lgk	Acetic acid, formic acid, furfural, phenols, and HMF	Sterile centrifuge tubes	NM	NM	0.06		[163]

NM not mentioned

/ Denotes the value was zero or negligible

[a] Denotes that the values not given in the original papers were calculated

glucose have been accomplished. Results of all these studies are quite promising in the context of pyrolysis-based biorefinery for producing bio-ethanol. Most of the studies regarding the indirect fermentation of pyrolysis oil into lipids and ethanol follow the basic general scheme as shown in Fig. 1.

Yu and Zhang [196] pioneered a comprehensive detoxification study in order to efficiently produce ethanol from the acid-hydrolyzed cellulosic pyrolysate of waste cotton. Their work employed ten detoxification strategies, either alone or in combination with other detoxification methods. Among all detoxification strategies, neutralization + diatomite shaking gave hydrolysate that was almost completely fermented by *Saccharomyces cerevisiae* 2.399, and *Pichia* sp. YZ-1, with *S. cerevisiae* produced the highest ethanol yield 0.45 g/g glucose (Table 4). In another study, Yu and Zhang [196] aimed to find the optimal concentration of sulfuric acid that would produce the highest glucose yield from pyrolysis oil of waste cotton. The highest glucose yield (17.4 %) was obtained by hydrolyzing the bio-oil with 0.2 mol/l Sulfuric acid at 121 °C for 20 min. After dilution, the acid-hydrolyzed pyrolysate was successfully fermented by *S. cerevisiae*, giving ethanol yield higher than the pure glucose control solution which was a bit unusual. Their explanation for this phenomenon was there might be some other unknown substances also fermented along with pyrolysate glucose. However, Prosen et al. [151] also observed the same phenomenon with wood pyrolysate. In their work, they also investigated the effect of different nitrogen sources on fermentation of the hydrolysate to ethanol using *S. cerevisiae*. Among the different nitrogen sources tested, the highest ethanol titer (15.1 g/l) was obtained with single urea. Their work also evaluated a preadaptation strategy to improve the tolerance of yeast to the toxic chemicals present in hydrolysate medium. An adapted yeast strain was obtained after 12 times of recycling, which fermented much higher hydrolysate glucose concentration (95.8 g/l), confirming that the yeast acquired tolerance to fermentation inhibitors present in hydrolysate and showed 47 % increase in ethanol yield compared to its parental yeast strain (Table 4). However, they did not provide any explanation for the mechanism of tolerance which the yeast acquired after the preadaptation step.

Bennet [10] investigated the phase separation of levoglucosan using water, the hydrolysis of levoglucosan into glucose, and finally the fermentation of the hydrolysate into ethanol from pyrolysis oil prepared from Scots Pine feedstock. An optimal levoglucosan yield up to 7.8 % of the initial bio-oil was obtained at 34 °C and 62 wt.% (total) water. Hydrolysis with 0.5 M H_2SO_4 at 125 °C for 44 min, produced the highest glucose yield of 216 % (based on original levoglucosan), concluding that other derivatives of glucose are also converted into glucose during hydrolysis reaction. Hydrolysis solutions up to 20 % were successfully

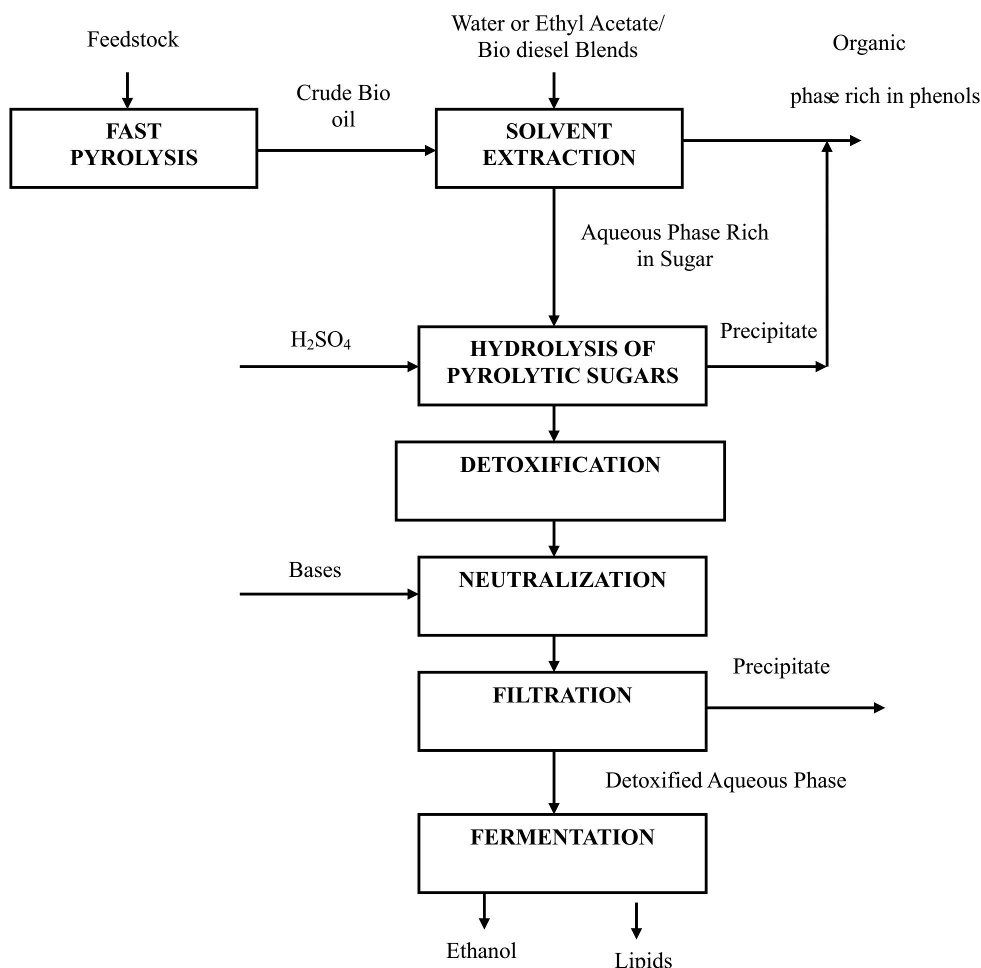

Fig. 1 Flow chart of the scheme studied for the conversion of lignocellulosic materials into ethanol and lipid via indirect fermentation (Source: separation, hydrolysis, and fermentation of pyrolytic sugars to produce ethanol and lipids by Lian et al. [99])

fermented to ethanol by *S. cerevisiae* T2, using high yeast inoculum (1 g/l in flask) under micro-aerophilic conditions. The ethanol yield (0.46 g ethanol/g glucose) in this study was very near to the theoretically calculated yield for pure glucose (0.51 g ethanol/g glucose), suggesting that ethanol production from pyrolysis oil is as efficient as producing ethanol from pure glucose (Table 4).

Chan et al. [19] investigated the fermentation of the hydrolyzed aqueous fraction from bio-oil provided by VTT the Technical Institute of Finland with *S. cerevisiae* T2 under both aerobic and anaerobic conditions. They applied a strategy involving the extraction of levoglucosan, over-liming, extraction with organic solvents, and adaptive evolution of yeast. The optimal ratio for levoglucosan extraction was 100 wt.% of water-to-bio-oil that produced levoglucosan yield of 4.98 wt.% (g levoglucosan/g bio-oil). Hydrolysis in this study was performed as described by Bennet et al. [11]. Bio-oil hydrolyzate treated with over-liming $(CaOH)_2$ and fermented at a concentration of 40 % produced the highest ethanol yield (0.45 g ethanol/g glucose). Ethanol yield (0.24 g ethanol/g glucose) was achieved with 25 vol.% tri-*n*-octylamine in 1-octanol. The adapted yeast produced 39 and 26 % better ethanol yields compared to the unadapted yeast under aerobic and micro-aerophilic conditions (Table 4). Over-liming applied as a detoxification technique in this study proved to be very promising for the removal of fermentation inhibitors.

Lian et al. [99] developed a comprehensive strategy to ferment pyrolytic sugars into lipids and ethanol. Their scheme first employed ethyl acetate as a solvent to separate pyrolytic sugars from phenols, followed by acid hydrolysis to produce glucose from the anhydrosugars. The hydrolyzed aqueous phase containing glucose was neutralized with $Ba(OH)_2$ and further detoxified with activated carbon to remove the fermentation inhibitors. Finally, the detoxified glucose syrup was fermented with yeasts, *Cryptococcus curvatus* and *Rhodotorula glutinis* to produce lipids, while *S. cerevisiae* to produce ethanol.

Yield of ethanol as high as 0.473 g ethanol/g glucose was achieved which was much higher than the 0.167 g lipids/g sugar of lipids (0.266 g ethanol equivalent/g sugar), suggesting that fermentation of pyrolytic sugars to ethanol is more efficient than lipid production. Their detoxification strategy involving extraction with ethyl acetate followed by hydrolysis and detoxification with activated carbon became the basic scheme of detoxification for many studies after that.

Fermentation of sugar-based substrates, such as glucose, sucrose, fructose, lactose, whey, and xylose have been widely studied; however, research on lipid production from organic acids is still very limited [26, 28, 53, 70]. In another study, Lian et al. [100] investigated the fermentation of carboxylic acid present in the aqueous fraction of pyrolysis oil as a fermentation substrate for oleaginous yeasts to produce lipids. Three oleaginous yeasts, *C. curvatus*, *R. glutinis*, and *Lipomyces starkeyi* were evaluated for their potential to ferment acetate, formate, hydroxyacetaldehyde, phenol, and acetol. After neutralization and detoxification, acetate was the main carboxylic acid present in the aqueous phase. *C. curvatus* was selected to ferment an aqueous fraction containing 20 g/l of acetate due to its high pH and acetol resistance, giving lipid yield of 2.2 g/l, while dry biomass was 6.9 g/l. This study proved that acetic acid could be successfully utilized for lipid production via yeast fermentation.

Lipid production by oleaginous microorganisms has attracted attention for the production of bio-diesels [142]. The lipids produced by these microorganisms have high carbon-to-heteroatom ratios, and are mostly in the form of triacylglycerols (TAGs), while some in the form of free fatty acids (FAs) [78]. The two most common biofuels produced from lipids are biodiesel, produced via transesterification of TAGs, and renewable diesel via hydrotreatment [84]. An oleaginous microorganism is the one that accumulates more than 20 % of its dry biomass as oil, mainly in the form TAGs and FAs lipids [78, 155]. A detailed study by Subramanium et al. [173] reported the following: 14 genera of microalgae with highest reported oil accumulation ranging from 20 to 77 % of the dry biomass, the highest noted for *Schizochytrium* sp. 50–77 %; four genera of bacteria with oil contents 24–78 % of dry cell weight, most notably *Arthrobacter* sp. 78 % from glucose [86]; four genera of yeast with oil accumulation ranging from 58 to 72 % of dry weight, the highest achieved by strains of *R. glutinis*; and four genera of molds with oil amounts ranging from 57 to 86 %, the highest level accumulated by *Mortierella isabellina* [78]. The theoretically calculated lipid yield is 0.32 g/g sugar from glucose and 0.34 g/g sugar from xylose [141]. However, the practical yield after biomass production and other products is mostly considered to be around 0.22 lipid/g glucose [97, 189].

Direct fermentation of pyrolytic sugars

However, it has been found that many eukaryotic and prokaryotic microorganisms can directly metabolize levoglucosan to valuable products through their direct LG utilization pathways [82, 124, 125, 202]. Searches for microorganisms exhibiting levoglucosan-utilizing pathways have identified many microorganisms that can metabolize levoglucosan as the sole carbon and energy source [82]. Studies have proved that the levoglucosan utilization pathway that exists in eukaryotic organisms is more developed and advanced than the one found in prokaryotic microorganisms. Prokaryotic microorganisms such as bacterium, *Arthrobacter* sp. can metabolize levoglucosan in at least three enzymatic steps using NAD^+ as a cofactor. This was rather a novel enzyme discovery which followed a three-step reaction as shown in Fig. 2b. In the first step, levoglucosan is dehydrogenated into 3-keto-levoglucosan, then to 3-keto glucose, and lastly to D-glucose by using NAD^+ as an electron acceptor [125, 202]. In contrast, eukaryotic organisms such as filamentous fungi and yeast strains convert LG directly into glucose-6-phosphate via levoglucosan kinase (LGK) as shown in Fig. 2a [82, 125, 202]. The levoglucosan kinase breaks the 1,6-anhydro bond and phosphorylate it into glucose-6-phosphate in the presence of magnesium ion and ATP [82]. A series of oxidation reactions take place to convert glucose-6-phosphate into pyruvate and finally to acetyl-CoA via pyruvate dehydrogenase complex [165]. During anaerobic conditions, the pyruvate is converted into acetaldehyde by pyruvate decarboxylase, and finally acetaldehyde is converted into ethanol by alcohol dehydrogenase using NADH as cofactor [145]. Due to the presence of LGK, a variety of fungal and yeast strains have the ability to use levoglucosan directly as their primary source for carbon and energy [124, 125, 151]. *Aspergillus terreus* K26 ferment levoglucosan to itaconic acid [124], while *Aspergillus niger* CBX-209 can grow on levoglucosan-containing medium and convert levoglucosan into citric acid [202]. The exciting thing in both of these examples was the similar fermentation rate and product yield compared to pure glucose, suggesting that levoglucosan can be consumed by microorganisms as efficiently as conventional 6-carbon sugars (glucose or fructose).

Biochemical characterization of LGK from *A. niger* CBX-209 has shown, LGK exhibit a strict preference for levoglucosan, the optimum temperature and pH for enzyme activity are 30 °C, and 9.3, respectively [202]. Unlike other hexokinases, LGK is inhibited by $HgCl_2$, $CoCl_2$, and Mg-ADP, but not by glucose-6-phosphate in micromolar concentrations; however, high concentrations of glucose-6-phosphate up to 10 mM might have a mild effect on LGK activity [202]. The K_m value of LGK for levoglucosan is 71.2 mM in *A. niger* CBX-209 [202] and 68 mM

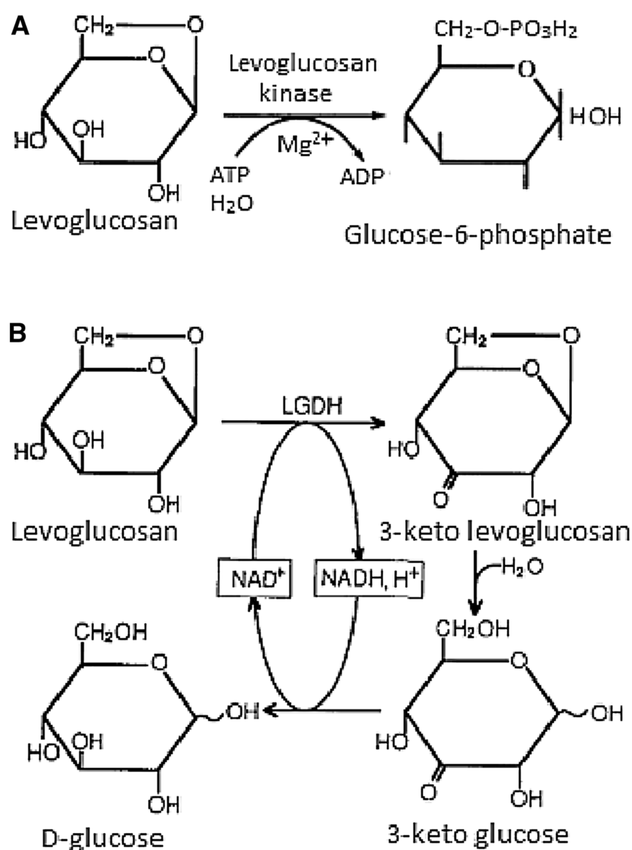

Fig. 2 Enzymatic reactions for the levoglucosan conversions to glucose-6-phosphate (**a**) and to D-glucose (**b**) [82, 125]

in *L. starkeyi* [32]. The comparatively higher K_m value of LGK for levoglucosan results in lower product yield due to incomplete substrate utilization [32, 92].

Genetic engineering for direct levoglucosan utilization

Nature itself explains to us the conversion of biomass into useful products such as ethanol by giving us the gift of many organisms possessing the innate capacity. These organisms, which are isolated from nature, have been found to be not as efficient as the microorganisms used in industrial processes. However, often these native microorganisms lack some genetic and biochemical pathways, and therefore huge scientific investment is required to make them useful biological factories [1]. Previous studies have shown that *S. cerevisiae* and *Escherichia coli* are the preferred industrial microorganisms for genetic and metabolic engineering manipulations due to their well-established genetic systems and a long track record of industrial applications [1].

Before fermentation, the pyrolytic sugars, mainly levoglucosan and cellobiosan present in bio-oil must be hydrolyzed into glucose using sulfuric acid, which is not only expensive but also produces heat and results in the formation of additional inhibitors [63]. Previously, researchers have shown that yeast strains also have the potential to ferment activated charcoal-treated pyrolysis tar to ethanol [151]. However, the unfortunate thing about the naturally existing levoglucosan utilizers is the fact that most of these microorganisms do not produce ethanol from levoglucosan or even if some produce ethanol, the yield is extremely low. For example, yeasts species such as *Candida utilise, Saccharomyces diastaticus*, and *Schwanniomyces castellii* fermented partially purified levoglucosan into ethanol with a yield of only 10 % compared to 48 % with pure glucose [151].Therefore, bio-catalysts that have the ability to utilize levoglucosan directly are more desirable for industrial applications.

The two best possible strategies could be to search for new organisms having the capabilities of utilizing levoglucosan directly, but it seems quite laborious and time consuming work. The second option is to exploit the genetic engineering tools for importing the direct levoglucosan pathway into existing fermentation organisms. A novel yeast strain *L. starkeyi* has the enzyme levoglucosan kinase that shows high catalytic activity for levoglucosan and converts it into glucose-6-phosphate [126]. A little research has been done on engineering the direct levoglucosan utilization pathway into other fermentation organisms. Dai et al. [32] identified, cloned, and expressed a novel cDNA of levoglucosan kinase gene from yeast *L. starkeyi* YZ-215 in *E. coli,* the resulting strain used levoglucosan as the sole carbon source on minimal media. In a more recent study, an engineered ethanologenic *E. coli* was produced by cloning the LGK gene after codon optimization. The engineered *E. coli* not only consumed levoglucosan as a source of carbon and energy but also fermented it into ethanol [92]. These studies show that existing bio-catalysts can easily be transformed into direct levoglucosan utilizers by application of genetic engineering tools.

Fermentation inhibitors in bio-oil

Despite the fact that bio-oil contains various fermentable substrates such as levoglucosan, acetic acid, glycolaldehyde, and hydroxyacetone, however the fermentation of these substrates to useful chemicals is not so easy and straightforward. The pretreatment of biomass during fast pyrolysis, and the acid hydrolysis (to produce glucose from levoglucosan) step later result in the production of various substances that are not fermentation friendly. These substances inhibit or reduce the rate of fermentation by inhibiting the growth of the microorganisms through different mechanisms. To date, bio-oil has not been thoroughly

characterized, and potential fermentation inhibitors are not well known. The fermentation inhibitors found in bio-oil include furans, phenols, aldehydes, and organic acids [77]. Research has been conducted in the past and still going on to identify the specific inhibitors, reduce the bio-oil toxicity, and improve the fermentation of fermentable substrates in bio-oil. Studies have also shown that organic acids (formic acid, acetic acid, and levulinic acid,), furfural, and 5-hydroxymethylfurfural (HMF) inhibit yeast fermentation [64, 83, 90, 133, 139, 140]. Research by Lian et al. [99, 100] also confirmed that the aforesaid compounds were inhibitory to yeast growth during ethanol production from pyrolytic sugars. Their findings show that not only the non-fermentable chemicals found in bio-oil are fermentation inhibitors, but the fermentable substrate such as acetic acid also shows inhibitory effects [100]. The mode of inhibition for various compounds in bio-oil is different from each other. In order to address the inhibition problem in an efficient way, the possible mechanism of action of these inhibitors need to be understood.

Weak acids such as formic acid, acetic acid, and levulinic acid are formed due to the de-acetylation of hemicelluloses present in biomass or due to the breakdown of lignin and sugars at the time of pretreatment. These acids are near their p*K*a values when fermentation pH is 5.5, and found in equal proportions of associated and dissociated forms. Formic acid has been found to negatively affect cell replication in *Rhodosporidium toruloides* and *Debaryomyces hansenii* [48, 139]. A possible explanation as to how weak acids inhibit fermentation process is that un-dissociated weak acids can diffuse across the cell membrane and dissociate in cytosol due to neutral pH, causing a drop in intracellular pH resulting in a decrease in ATP synthesis for the microorganism to survive. The production of weak acids is difficult to prevent as they are intrinsic to biomass, but optimizing pretreatment conditions can significantly reduce their formation [71, 83, 139, 200]. Acetic acid is not only a major fermentation substrate but also a fermentation inhibitor [100], exhibiting a similar mechanism of inhibition with formic acid [71, 147].

During extreme pretreatment conditions, furfural and 5-hydroxymethylfurfural are produced from pentose and hexose sugars respectively. At low concentrations, yeast can metabolize furfural to furfuryl alcohol via aldehyde reductases which is less inhibitory and produce NAD^+ resulting in a decrease glycerol synthesis. However, furfural and HMF in high concentrations interfere with cell replication, causing a prolong lag period as well as inhibiting anaerobic growth and ethanol productivity [46, 83, 104, 138]. Phenolic compounds such as vanillin, ferulates, and syringaldehyde, produced due to lignin decomposition are hydrophobic in nature making them capable of attaching to cell membrane of microorganisms resulting in loss of cell integrity, denaturation of membrane-associated enzymes, and ultimately reduce ethanol productivity [91]. Low molecular weight phenolic compounds act in a similar way to weak acids leading to disruption of normal cellular acidity [62, 83, 195]. Inhibition of fermentation is not only caused by the inhibitors found in bio-oil, but other substances such as high concentration of sugars, ethanol, and salts also negatively affect microbial fermentations. Higher amounts of sugars in media cause an osmotic stress for yeast leading the water to flow out of the cells and hence prolonging the lag phase at the beginning of fermentation [9, 66].

To overcome this problem, continuous or fed-batch fermentation should be applied in order to supply the sugars at the rates at which the yeast metabolize it. High salt concentrations which are formed due to pretreatment and neutralization of biomass before hydrolysis and fermentation can also lead to inhibition of fermentation. This problem can be addressed with the use of a salt-tolerant organism or using pretreatment conditions that do not require significant pH adjustments. Although yeast are robust organisms and are more ethanol resistant than bacteria, but even then higher concentrations of ethanol can also inhibit yeast growth due to damage of the cell membrane. Latest studies have shown that many inhibitor compounds interact synergistically leading to microbial growth inhibition [83, 139, 140, 188].

Improving fermentability of bio-oil hydrolysates

The challenge for fermentation of pyrolytic sugars obtained from bio-oil is the presence of various inhibitory compounds that severely inhibit microbial fermentation. Various strategies have been reported in previous research, such as solvent extraction, adsorption on adsorbents (activated carbon, bentonite, zeolites and diatomite), over-liming, distillation, and air stripping that can be used to remove the toxic inhibitors from bio-oil hydrolysates. Another approach is to develop microorganisms that can grow well even in presence of inhibitors and can resist toxic compounds present in bio-oil. Table 4 shows a detailed summary of the previous research work on ethanol production from pyrolysis oil and the strategies used to improve the fermentation of pyrolytic sugars.

Solvent extraction

The first approach is to use solvents to fractionate bio-oil into two distinct fractions (aqueous and oil fraction) depending on their relative solubilities. Various polar and non-polar solvents can be used to remove the inhibitor chemicals from bio-oil hydrolysate. The aqueous phase

will be rich in fermentable sugars and the oil phase will contain most of the inhibitors [150]. Various organic solvents have been used so far. Lian et al. [99] used ethyl acetate to remove most of the phenolic compounds and acetic acids successfully from bio-oil, and the aqueous phase rich in sugars was used for fermentation by three different yeasts obtaining a yield of 0.473 g ethanol/g glucose of the theoretical yield of ethanol that could be obtained from glucose. Their results clearly demonstrated that the fermentation of pyrolytic sugars for ethanol production is more efficient than producing lipids. A co-solvent system of tri-*n*-octylamine and 1-octonal was used by Chan and Duff [19] resulted in 90 % removal of acetic acid, while 100 % glucose was retained, and the ethanol production was increased by 0.24 (g ethanol/g glucose) using 40 % hydrolysate. Wang et al. [187] used non-polar hexane and polar linoleic acid to remove the inhibitors from bio-oil hydrolysate, and their method was moderately successful. In more recent study, Luque et al. [106] used a 1:2 wt.% of bio-oil filtrate and ethyl acetate to remove the yeast growth inhibitors e.g., phenolic compounds, furans, and aldehydes obtaining 96 % of the theoretical yield in microtiter plate wells. According to Luque et al. [106], extraction with ethyl acetate is relatively selective, largely removes the other organic compounds, and there is a no significant loss of levoglucosan. In another study, Hassan et al. [61] compared the efficiency of *n*-butanol and ethyl acetate on removing phenolic compounds and other enzymatic inhibitors from aqueous fraction bio-oil and they found that *n*-butanol solvent was more effective than ethyl acetate in removing such compounds. Sukhbaatar et al. [174] recently used an 1.8:1 ratio of *n*-butanol and bio-oil, fermentation inhibitors (acetic acid, furfural and HMF) were successfully removed, and ethanol yield reached up to 98 % of the theoretical yield in shake flask fermentation. These studies indicate solvent extraction is a useful method for removal of inhibitors and has wide scope of applications in bio-ethanol production from bio-oil, but on the other hand some organic solvents are expensive, their dissolution in bio-oil can create a further inhibition issue, multiple extractions might be required, time consuming, and generates large volume of organic wastes.

Adsorption on absorbents

Activated carbon is a common method used to remove toxic compounds from liquids. Factors such as temperature, duration of contact, and dosage of carbon affect adsorption on activated carbon [122]. It has been reported in previous research that the removal of lignin degradation products, especially phenolic compounds were increased sixfold when temperature of hemicellulose hydrolysate was raised from 20 to 40 °C, while applying low pH [121]. Adsorption on activated carbon was used by Prosen et al. [151] to remove phenolic compounds and improve fermentability. They noticed that variety of fungal and yeast strains can utilize bio-oil rich in levoglucosan for ethanol production. Their study indicated that some of the fungi and yeast could ferment activated charcoal-treated liquid and acid hydrolysate of bio-oil; however, ethanol yield was much higher for acid-hydrolyzed bio-oil than the un-hydrolyzed bio-oil liquid suggesting the acid hydrolysis of levoglucosan to glucose which is a preferred substrate for ethanol fermentation. Yu and Zhang [197] carried out an extensive work on the fermentation of cellulosic pyrolysate of waste cotton containing high amount of levoglucosan while applying ten detoxification strategies. They basically applied neutralization with $Ca(OH)_2$ and over-liming with $Ca(OH)_2$ in combination with activated carbon, diatomite, bentonite, and zeolite (10 % w/v) shaking for 80 min, respectively. According to their study, neutralization plus diatomite shaking produced the best ethanol titer and yield (16.1 g/l ethanol, 0.45 g ethanol/g glucose) followed by neutralization plus activated carbon shaking (15.4 g/l ethanol, 0.45 g ethanol/g glucose) and neutralization separately (15.1 g/l ethanol, 0.45 g ethanol/g glucose). Over-liming plus diatomite shaking was also proved to be good option. Lian et al. [99, 100] also used a combination strategy by extraction with ethyl acetate followed by adsorption on activated carbon and neutralization to remove acetic acids and phenols. In another study Lian et al. [99] used an activated carbon/bio-oil hydrolysate ratio of 1:1 and kept the mixture at 4 °C overnight, a colorless liquid was obtained after filtration. The GC/MS analysis of the bio-oil aqueous phase after detoxification showed no peaks of the inhibitors. The detoxified aqueous solution was colorless indicating that colored compounds were also eliminated, but their study does not indicate whether there was also any loss of fermentable sugars. The detoxified aqueous phase was fermented with three different yeasts for ethanol and lipid production obtaining yields of 0.473 g ethanol/g glucose and 0.167 g lipids/g sugar, respectively. Wang et al. [187] used a 1 % (w/v) ratio of activated carbon and the aqueous solution, the resulting slurry was kept overnight at 4 °C and obtained a colorless liquid after filtration. Their detoxification procedure resulted in a 92 % decrease in acetic acid concentration and 78 % decrease in the furfural concentration but they also saw a 3.8 % loss in glucose level which is a significant loss of fermentable sugars. Liang et al. [101] used a combined approach of activated and metabolic evolution to improve microalgae growth significantly on bio-oil rich in acetic acid concentration to produce lipids. All these studies indicate that the application of activated carbon to remove fermentation inhibitors seems very promising, however further studies are required to find the optimal concentration of activated carbon along with the effect of

contact time as well temperature that would largely remove the inhibitors while maintaining maximum sugar level.

Over-liming

Over-liming is an extensively used method for detoxification of cellulose hydrolysate [71, 87, 131]. Martinez et al. [109] applied over-liming for detoxification of hemicelluloses hydrolysate, and observed that furans (51 %) and phenolic compounds (41 %) were removed along with 9 % sugar. During the process of over-liming, the pH of bio-oil hydrolysate, which is usually around 2–3, is adjusted to around 10–11 with the addition of bases that result in the formation of a solid precipitate containing phenol- and furan-derived compounds that are later removed by filtration. Over-liming utilizes high temperatures around (80 °C) and a relatively long treatment time (usually 3 h) that can result not only in the removal of fermentable sugars, but also cause a twofold to threefold increase in the concentrations of acetic and formic acid [69, 197]. As some of the inhibitors found in cellulose hydrolysate are similar to the inhibitors found in bio-oil hydrolysate, the same method was also used to carry out detoxification of bio-oil hydrolysate. Yu and Zhang [197] carried out an extensive work on reducing the effect of inhibitors using cellulosic pyrolysate of waste cotton. They employed over-liming alone and in combination with activated carbon shaking, diatomite shaking, bentonite shaking, and zeolite shaking that increased ethanol yield by 5–13 %, with the loss of 0.6–1.03 % glucose which is not very significant. However, another study reported a 20 % loss of sugars by applying over-liming treatment to bio-oil hydrolysate, and this was supposed to be due to the calcium ion-catalyzed degradation of sugars in an alkaline environment [69]. Chan and Duff [19] used $Ca(OH)_2$ over-liming to remove furans along with phenols, and found that over-liming improved the ethanol yield by 0.19 ± 0.01 and 0.45 ± 0.05 (g ethanol/g glucose) at 50 and 40 % bio-oil hydrolysate concentration respectively. Chi et al. [25] also examined the effect of $Ca(OH)_2$ over-liming for detoxification of pyrolytic sugar syrup and noticed that over-liming treatment removed phenolic compounds either partially or completely. The detoxified sugar solution was later subjected to fermentation by an engineered *E. coli* resulting in a tenfold improvement in fermentability of the pyrolytic syrup. These studies indicate that over-liming with $Ca(OH)_2$ is a useful method for detoxification, however a recent study conducted by Rover et al. [161] observed that there was 7.0 ± 0.2 % loss of sugars due to over-liming, further the precipitate formation makes it a complicated procedure. In their study, they carried out over-liming in three different ways [$Ca(OH)_2$, NaOH, and NH_4OH], and all three were equally successful in eliminating furans, but over-liming by NaOH was more favorable showing no loss of sugars, removing three times more phenol and more vanillin along with guaiacol compared to the other three methods. Ethanologenic *E. coli* successfully fermented the detoxified sugars (2 wt.%), however this little sugar consumption is still not commercially attractive for ethanol production.

Air stripping and distillation

Air stripping is a common method used to remove volatile organic compounds (VOCs) by converting them into gaseous forms. This method is commonly employed to remove VOCs from wastewater. Volatile organic compounds have high vapor pressure and low aqueous solubilities and therefore easy to be removed by air stripping. Henry's law governs the removal of VOCs by air stripping. The removal of VOCs by air stripping is directly proportional to the Henry's constant, the higher the Henry's constant the greater the removal of VOCs. Higher temperatures increase Henry's constant and therefore air stripping becomes more efficient. Other factors like pH and residence time can also affect air stripping efficiency. Low pH causes the removal of volatile acids, while at high pH (pH 10) ammonium can also be removed, however, this method could cause the evaporation of water as well [63, 64]. Wang et al. [187] used air stripping to remove acetic and formic acids at temperatures of 25 and 60 °C by forcing air into flasks containing bio-oil solution. The concentration of glucose, acetic acid, and furfural was supposed to decrease but in contrast it increased. They found a 2.2 and 87 % increase in glucose concentration at 25 and 60 °C, respectively. Acetic acid concentration increased by 16.7 and 39.6 %, while furfural increased by 39.6 and 117.9 % at 25 and 60 °C, respectively. This increase might be due to the evaporation of water from bio-oil. They concluded that air stripping is not an effective method for bio-oil detoxification. The method of distillation separates various compounds on the basis of differences in their boiling points, but due to the complex chemical nature of bio-oil, distillation cannot be used alone for bio-oil detoxification. Distillation has been used in combination with solvent extraction or activated carbon to remove inhibitors [19, 100, 184].

Microbial removal

This method of detoxification is more desirable as it involves the removal of fermentation inhibitors by exploiting the capabilities of microbes to digest phenolic and furan containing compounds without consuming glucose. The bright aspect of this method is that it would not introduce any additional inhibitors to the aqueous fraction, while maintaining glucose level at the same time but the limitation with this method is that only few microorganisms are

known to exhibit such a remarkable potential. However, recently a bacteria *Cupriavidus basilensis* has been discovered that has the potential to eliminate the toxic compounds without consuming glucose. The bacteria have the capability to consume furfural, hydroxymethyl furfural, toluene, dichlorophenol, phenol, and benzene without utilizing glucose. Additionally the bacteria has been found to produce 2,5-furandicarboxylic acid from furan-derived compounds [191]. This compound can replace terephthalic acid used to produce aromatic polymers and polyesters due to its renewable nature. Further research in this area would have dual benefit resulting in the production of a useful building block chemical and at the same time detoxify the aqueous bio-oil fraction. Due to its positive attributes, 2,5-furandicarboxylic acid is viewed as a priority chemical for future green energy by the US Department of Energy [13].

Adaptive evolution for increasing tolerance in bio-catalysts

In order to efficiently remove the microbial growth inhibitors from bio-oil hydrolysate, a combination of different detoxification methods is required. Therefore, developing microorganisms exhibiting tolerance to the toxic inhibitors is more desirable and cost effective to the multi-treatment detoxification procedures. Adaptive evolution refers to mutations that occur in the DNA of an organism upon exposure to environmental challenges. This approach can be used to develop microorganism having abilities to grow in the presence of inhibitors present in bio-oil hydrolysate. There would be no need of additional detoxification steps if the adapted microbe acquires the ability to grow and produce ethanol in the presence of inhibitors [110]. The factors affecting the growth of microorganisms are temperature, substrate limitation, and pH [64, 113]. The process of adaptation is still not clearly understood, but several models have been proposed to explain the process. The first one is the directed mutation model in which mutations directly ease the stresses posed by the environmental conditions. Next is the cryptic growth model, here the mutations take place in a random manner and are not caused by the environmental conditions. In the hyper-mutation model, mutations occur across the genome which stimulate adaptive and non-adaptive evolution. Most of the mutations are not helpful in the adaptation process but some are useful and help the organism to survive [51, 110].

These models can be applied to microbial growth cultures. Microorganisms can be cultured for several generations under non-stop environmental stresses to obtain resistant strains to furan and phenol derivatives [51]. The growth of microorganisms in toxic bio-oil can be greatly improved if they are initially grown on media containing small quantity of bio-oil and then slowly increasing the dose of bio-oil in the media for several generations. This process is known as directed evolution as it speeds up the survival of the fittest according to Darwinian law of natural selection [95]. The process of directed evolution typically takes place in three phases; diversification, selection, and amplification. The first one is diversification in which random mutations can be used to speed up the process of diversification. In the second step, mutants with desired traits are selected and screened [136, 156]. During the final step, the screened microorganism is grown in bulk quantities for characterization and maintenance [136]. These steps are repeated again and again for many times in order to obtain microbes with desirable traits [136, 197]. Yu and Zhang [197] carried out adaptation of *S. cerevisiae* to the hydrolysate medium for 12 times and observed 47 % increase in ethanol production compared to its parental strain which clearly explains the potential of adaptive evolution in order to improve fermentation of bio-oil hydrolysate. In another study, adaptive evolution was applied to increase the tolerance of yeast to inhibitors in bio-oil hydrolysate. The adapted yeast produced 39, and 26 % better bio-ethanol yields under aerobic and micro-aerophilic conditions, respectively [19]. But, however, there was no explanation for the mutations that provided the yeast with resistance to inhibitors.

Economic feasibility of pyrolysis technology

Many developing countries have already started using food crops such as maize, sugarcane, and soybean for the production of biofuels and ethanol but this seems to be unsustainable in the long run [38, 105]. A vast amount of scientific research is focusing on developing methodologies with which biomass can be converted into hydrogen. Some other areas of research have also been discovered, but no such economically attractive option has been found yet [164, 194]. Currently, the widely available lignocellulosic feedstock has attracted much attention as a renewable and sustainable source for the production of bio-ethanol via fast pyrolysis [65]. There are some technical and non-technical challenges that need to be addressed before the commercial applications of pyrolysis processes for producing bio-based products. The commercialization of pyrolysis technology on an industrial scale primarily depends on its economic viability [179]. Currently, the production cost of pyrolytic products is more than the production of fossil fuel. Pyrolysis technology involves two types of costs: (1) fixed or capital cost that includes facilities development such as land, road, transportation, feedstock storage, handling, and the necessary equipment. The capital cost is about 10–20 % mainly due to the pyrolysis reactors; (2) the variable cost includes biomass harvesting, collecting, cutting, drying,

grinding, and storage [76, 119]. Studies in the recent past have shown that fast pyrolysis has a great potential to replace the dependence on fossil fuels. Recently, a comparative cost study has revealed that fast pyrolysis is more cost effective than gasification or hydrolysis technology for the production of biofuels. In this study, transportation fuels in the near-future (5–7 years) were approximated to cost $2–3/gallon gasoline equivalent (gge) for pyrolysis, $4–5/gge for gasification, and $5–6/gge for cellulosic ethanol through enzymatic hydrolysis [3]. Sandvig et al. [162] found fermentation of bio-oil derived sugars to be economically fascinating when combined into the production of both bio-power and bio-based chemicals. These studies attract an increased interest in the production of pyrolytic sugars via fast pyrolysis for generating bio-ethanol. A recent comparative cost analysis showed fast pyrolysis to be an attractive means of biofuels production relative to both enzymatic hydrolysis and gasification [3]. But the pyrolysis technology still needs to combat some challenges as the technology for the conversion of lignocellulosic biomass to ethanol is not well established as the technology for converting edible crops to ethanol. Further research is required to understand the pyrolysis processes better for converting biomass to ethanol on a large scale. Many pyrolysis technology companies are close to getting commercial status. In North America, Ensyn, and DyanaMotive are the prominent ones, using forest residues, agriculture waste, etc., as the source material. Despite the rapid progress in recent times, the pyrolysis technology still has to overcome some techno-economic and social challenges in order to compete with fossil fuels [76].

Conclusion

Due to the vast availability of lignocellulosic biomass, pyrolysis-based conversion of pyrolytic sugars into biofuel and other value added chemicals exhibit promising potential for commercial applications due to its socio-economic advantages. Extensive scientific research is required to address the issue of detoxification by optimizing the pretreatment conditions in order to reduce the formation of inhibitors, new strategies of bio-oil detoxification with minimal loss of fermentable sugars need to be explored and developed. Metabolic engineering and adaptive evolution of microorganisms could be extremely helpful in combating the detoxification problem and improving fermentability of the bio-oil hydrolysate. New microorganisms having direct levoglucosan-utilizing pathways should be searched and isolated from the environment. Modifying suitable fermentation biocatalysts for direct levoglucosan utlization along with tolerance engineering could not only let these microorganisms grow in the presence of inhibitors but also convert the anhydrosugars directly into ethanol and lipids.

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