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125th Anniversary Review: Fuel Alcohol: Current Production and Future Challenges

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ABSTRACT

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Global research and industrial development of liquid transportation biofuels are moving at a rapid pace. This is mainly due to the significant roles played by biofuels in decarbonising our future energy needs, since they act to mitigate the deleterious impacts of greenhouse gas emissions to the atmosphere that are contributors of climate change. Governmental obligations and international directives that mandate the blending of biofuels in petrol and diesel are also acting as great stimuli to this expanding industrial sector. Currently, the predominant liquid biofuel is bioethanol (fuel alcohol) and its worldwide production is dominated by maize-based and sugar cane-based processes in North and South America, respectively. In Europe, fuel alcohol production employs primarily wheat and sugar beet. Potable distilled spirit production and fuel alcohol processes share many similarities in terms of starch bioconversion, fermentation, distillation and co-product utilisation, but there are some key differences. For example, in certain bioethanol fermentations, it is now possible to yield consistently high ethanol concentrations of ~20% (v/v). Emerging fuel alcohol processes exploit lignocellulosic feedstocks and scientific and technological constraints involved in depolymerising these materials and efficiently fermenting the hydrolysate sugars are being overcome. These so-called second-generation fuel alcohol processes are much more environmentally and ethically acceptable compared with exploitation of starch and sugar resources, especially when considering utilisation of residual agricultural biomass and biowastes. This review covers both first and second-generation bioethanol processes with a focus on current challenges and future opportunities of lignocellulose-to-ethanol as this technology moves from demonstration pilot-plants to full-scale industrial facilities.

Key words: bioethanol (fuel alcohol), first and second generation feedstocks, lignocellulose, pentose-fermenting yeasts, *Saccharomyces cerevisiae*.

GENERAL INTRODUCTION TO BIOETHANOL PRODUCTION

Ethanol: characteristics and advantages as a biofuel

Bioethanol, or fuel alcohol, refers to ethyl alcohol produced by microbial fermentation (as opposed to petrochemically-derived alcohol) that is used as a transportation biofuel. It is produced through distillation of the ethanolic wash emanating from fermentation of biomass-derived sugars and can be utilised as a liquid fuel in internal combustion engines, either neat or in petrol blends (see section 4). Table I summarises some of the important characteristics of ethanol as a fuel source.

The high octane rating (99) of ethanol (as a measure of a fuel's resistance to pre-ignition) means that engines combusting ethanol exhibit a high compression ratio and provide a higher power output per cycle. Ethanol's higher octane rating – compared with that of petrol (gasoline) with an average rating of 88 – increases resistance to engine knocking. Nevertheless, vehicles running on pure ethanol have fuel consumption (miles per gallon or kilometres per litre) 10–20% less than petrol. Information on ethanol-petrol blends employed in different countries (e.g., E10, E85, etc.) is discussed in Section 4.

It should be borne in mind that the use of ethanol as an internal combustion fuel is not new technology. For example, in the early 1900s, Henry Ford designed his famous Model T-Ford (the world's first mass-produced car) to run on ethanol. On a similar vein, Rudolf Diesel designed his 1898 prototype diesel engine to run on peanut oil (the first *biodiesel*).

The primary beneficial aspect of fermenting biomass-derived sugars to ethanol as a fuel source is that it can be produced from renewable plant material that is able to

Table I. Physico-chemical characteristics of ethanol as a liquid fuel.

Parameter	Characteristic properties
Molecular formula	C_2H_5OH
Molecular mass	46.07 g/mol
Appearance	Colourless liquid (between –117°C and 78°C)
Water solubility	∞ (miscible)
Density	0.789 kg/L
Boiling temperature	78.5°C (173°F)
Freezing point	–117°C
Flash point	12.8°C (lowest temperature of ignition)
Ignition temperature	425°C
Explosion limits	Lower 3.5% (v/v) Upper 19%(v/v)
Vapour pressure @ 38°C	50 mm Hg
Higher heating value (at 20°C)	29,800 kJ/kg
Lower heating value (at 20°C)	21,090 kJ/kg
Specific heat	Kcal/Kg 60°C
Acidity (pK_a)	15.9
Viscosity	1.200 mPa·s (20°C)
Refractive index (n_D)	1.36 (25°C)
Octane number	99

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photosynthetically re-fix CO_2 produced during bioethanol production and combustion. Therefore, unlike fossil fuels, bioethanol is not a net contributor to greenhouse gas or toxic gas emissions. Additional environmental and health benefits of bioethanol production include: removal of toxic gasoline additives such as methyl tertiary-butyl ether (MTBE) and lead; ethanol (containing 35% O_2) as an oxygenate reduces harmful exhaust pipe emissions due to more complete fuel combustion; and ethanol is readily biodegradable. Nevertheless, products from ethanol combustion do include carcinogenic formaldehyde and the ozone precursor, acetaldehyde[53,54]. Other disadvantages of bioethanol usage relate to adverse impacts on food security if agricultural land is diverted to biomass production specifically for biofuels. However, these drawbacks can be ameliorated if *second generation* feedstocks (e.g., from waste lignocellulosic material) are employed. Additionally, ethical and sustainability concerns can be addressed by securing land for biofuel production without decreasing the overall land area employed for food crops.

Although ethanol for fuel can be produced by alternative routes, such as hydration of petrochemically-derived ethylene[87] and thermochemical biomass-to-liquid (BTL) processes, such technologies have a high demand for fossil fuel energy compared with biochemical routes to ethanol[42,58]. The latter process involves pyrolysis/gasification technologies to produce "syngas" ($CO + H_2$) which acts as a progenitor for bioethanol production (using anaerobic *Clostridium* spp.).

Worldwide production of bioethanol

The main drivers for production of renewable transportation fuels such as bioethanol include maintenance of future fuel security, enhancement of the rural economy, and safeguarding the environment/reducing greenhouse gas emissions. The combustion of fossil fuels in the transportation sector currently contributes around 20% of global CO_2 emissions, and this is increasing due to expanding economies such as India and China[65].

Linked to environmental concerns and climate change issues, national governmental obligations and international directives on biofuels are acting as stimuli for the bioethanol industrial sector. For example, the United States Energy Policy Act of 2005 created a Renewable Fuel Standard (RFS) that was expanded when the US Congress passed the Energy Independence and Security Act of 2007 which will see renewable fuels grow to more than 57 billion litres by 2012, 136 billion litres by 2022 and (according to the US Department of Energy Roadmap) ~150 billion litres by 2030. However, over 750 billion litres of biofuels would be needed to totally replace liquid fossil fuels in the US[2]. In 2010, there were 187 operational bioethanol plants in the US[95], with several new facilities under construction. It is important to note that in the US, which is the world's largest producer of bioethanol, a limit 56.8 billion litres has been set for the amount of bioethanol that can be produced from maize and the increasing targets will be met from other feedstocks (such as sugar cane) as well as cellulosic feedstocks. For example, according to the US Environmental Protection Agency it is anticipated that by 2022 around ~57 billion litres of American bioethanol will be sugar cane-derived.

The Brazilian government's *Proalcool* programme was initiated in 1975 to exploit sugar cane fuel alcohol as a gasoline substitute in response to rising oil prices[41,79,129]. Brazil is the world's second largest producer of fuel alcohol, with around 400 sugarcane bioethanol plants. Brazilian production by 2012/13 is expected to reach 37 billion litres/year, from 728 million tons of sugar cane[8,14].

In Europe, bioethanol is increasing year-by-year, primarily in response to national obligations and European Commission directives. Under the 2009 EU Renewable Energy Directive, European nations have been set a target of ensuring that by 2020, 20% of its energy consumption comes from renewable sources, and that biofuels should account for 10% of transportation sector energy. The

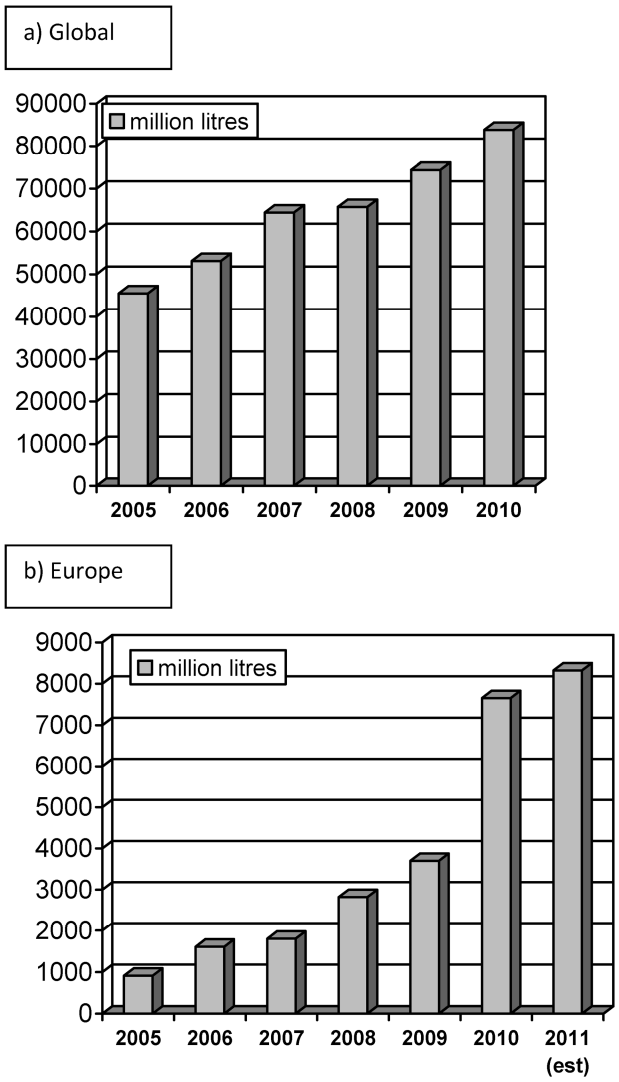

Fig. 1. Global (a) and European (b) fuel alcohol production. Information from: Biofuel and Industrial News[20] www.hgca.com; eBIO, the EU ethanol industry body; FO Licht. Further production statistics are available from[35,90,119] and Renewable Fuel Association (http://www.ethanolrfa.org/industry/statistics/), 'Global Biofuel Market Analysis' http://www.marketresearch.com). Biofuel & Industrial News from www.hcga.com; www.ethanolproducer.com; http://domesticfuel.com; News@All-Energy; bio@smartbrief.com; www.biofuelreview.com; www.distill.com; www.best-europe.org).

European Commission are promoting only biofuels with greenhouse gas emission savings of at least 35% compared with fossil fuels, rising to 50% in 2017 and to 60% by 2018.

In the UK, the RTFO (Renewable Transport Fuels Obligation, see http://www.renewablefuelsagency.gov.uk) means that biofuel-fossil fuel blends will rise to a maximum of 5% by 2013/14[116]. Biofuels pertinent to the RTFO include bioethanol, biodiesel, pure plant oil, biogas (methane), biobutanol, bio-ETBE and HVO (hydrogenated vegetable oil, also referred to as renewable diesel), as long as they meet environmental sustainability standards. There will be a further review of UK biofuel targets in 2011/12 to coincide with the EU's review of member states' progress on biofuel targets, but targets higher that 5% beyond 2013/14 will only be implemented if biofuels are shown "to be demonstrably sustainable (including avoiding indirect land-use change)".

Bioethanol is the principal global biofuel and production will soon exceed 100 billion litres (Fig. 1), with the US and Brazil being the dominant industrial players, accounting for over 80% of total production. Worldwide, bioethanol production has been predicted to double between 2007–2017 reaching 125 billion litres (www.oecd.org), with significant growth potential for biofuels in India and China.

European bioethanol production, predominantly from wheat and sugarbeet, is increasing markedly with the current main producers being France, Germany and Spain (Fig. 2). Projections for EU bioethanol in 2011[35] show an increase to 8.3 billion litres as new distilleries come into production, (see The European Bioethanol Fuel Association (www.ebio.org) and The European Union of Ethanol Producers (www.uepa.be).

UK bioethanol capacity is predicted to grow from 70 million litres in 2009, to 470 million litres in 2010 and to 890 million litres in 2011 as more plants come on stream (further information from www.britishbioethanol.co.uk; www.adas.co.uk[35]). Currently the largest European wheat biorefinery is in the UK, where the Ensus facility (Wilton, Teeside) produces 400 million litres of bioethanol and 350,000 tonnes of animal feed (DDGS) from an annual intake of 1.2 million tonnes of wheat[96]. It has been reported (Renewable Energy Association, 2009) that the UK has potential to deliver up to 80% of its biofuels needs to fulfil European obligations without decreasing overall land used for arable crops.

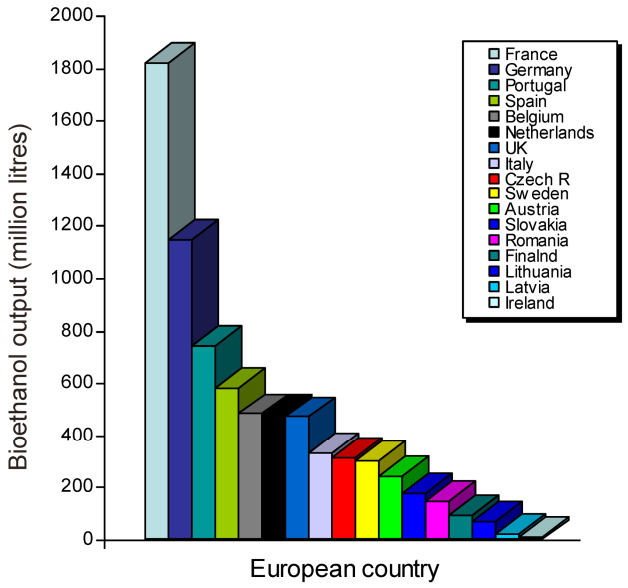

Fig. 2. European bioethanol producing countries (2010). See online version for colour figure. Information from: Biofuel & Industrial News[20].

Economic, energy and environmental aspects

Economic aspects. Regarding economic aspects, Table II indicates that bioethanol production costs vary depending on the biomass source. Very simplistically (due to oil price fluctuations and biomass feedstock costs) if petrol production costs are assumed to be 0.25 Euro/L, then only a few biomass sources used for bioethanol come close to closing the price gap between biofuels and fossil fuels. For example, current and future feedstocks, such as Brazilian sugarcane and waste lignocellulose, respectively, are competitive with other biomass sources only marginally so. Nevertheless, increasing oil prices will prove to be positive economic drivers for continued production and future development of bioethanol. For bioethanol to compete economically with petrol, production costs should be no greater than ~0.2 Euro/litre[123].

The costs of feedstocks represent the predominant expenditure in bioethanol production (e.g., first and second generation feedstocks generally 50–80% and 40% of total costs, respectively[89]). As technological improvements continue, lignocellulose-to-ethanol production costs would be expected to become lower in the future and the total value of US second-generation bioethanol is estimated to grow from 380 million Euro in 2010 to over 13,000 million Euro by 2020 (Walker[123]).

Energy balances of bioethanol production. Regarding energy aspects, bioethanol production and consumption requires exhibiting a positive Net Energy Balance (NEB), this being the ratio of the ethanol energy produced to the total energy consumed (in biomass growth, processing and biofuel production).

NEB values <1 mean that bioethanol production is unfeasible from an energetic standpoint and it is evident

Table II. Bioethanol production costs compared with petrol[a].

Biomass source	Production costs [€/litre]
Petrol (gasoline)	0.25
US corn	0.42
Corn stover	0.45–0.58
EU wheat	0.27–0.43
EU sugarbeet	0.32–0.54
Brazil sugarcane	0.16–0.28
Molasses (China)	0.24
Sweet sorghum (China)	0.22
Corn fibre (US)	0.41
Wheat straw (US)	0.44
Spruce (softwood)	0.44–0.63
Salix (hardwood)	0.48–0.71
Lignocellulose (biowaste)	0.11–0.32

[a] Information from www.eubia.org; Sassner[100]; Abbas, *personal communication*; Gnansounou[39].

from Table III that sugar cane represents the most favourable first-generation feedstock with respect to energy balances. Brazilian bioethanol distilleries that combust residual sugar cane bagasse for electricity generation have very favourable energy balances[8,14,74] (http://bioenergytrade.org/downloads/sustainabilityofbrazilianbioethanol.pdf; http://english.unica.com.br/).

Environmental aspects. Regarding sustainability issues with bioethanol, it is apparent that fossil fuel combustion is contributing to an elevation of greenhouse gas (GHG) emissions (especially CO_2) and consequentially is causing changes to the Earth's climate[110]. Road transport fuel combustion is currently responsible for around 20% of GHG emissions. Table IV highlights the benefits of utilizing bioethanol, at the expense of petroleum fuels, in reduction of GHG emissions. For example, Pilgrim[90] has reported that combustion of 18.5 billion litres of bioethanol can save ~8 million tons of CO_2, equivalent to the removal of 1.2 million automobiles. Cellulosic-derived bioethanol can reduce GHG emissions in excess of 60% (Renewable Fuels Association[95]).

Table III. Energy balances for bioethanol production from some first-generation feedstocks (petroleum production may be assumed to be around 6, for comparison).

Feedstock	Net energy balance
Sugar cane	6.5–9.5
Sugar beet	1.1–2.3
Sweet sorghum	0.9–1.1
Maize	1–2

Similarities and differences with potable alcohol

Whilst there are many similarities in ethanol production processes for potable and fuel use, several salient differences exist between them as outlined in Table V. For example, through yeast strain improvements and careful attention to fermentation nutrients, it is now possible to produce very high levels of ethanol in fuel alcohol plants (e.g., over 20% (v/v) – see references[50,117]). Mashing methods and starch saccharification approaches also differ, particularly regarding application of exogenous amylolytic enzymes (including glucoamylase) as discussed in Section 2.1. The production of certain potable spirits, most notably Scotch whisky, prohibits the use of such enzymes. Another key difference lies in the final ethanol concentrations achieved in the distillation/final purification of fuel alcohol (i.e., 99.9% (v/v), compared with ~95% in potable alcohol stillhouses).

Table IV. Greenhouse gas (GHG) emission savings by using bioethanol.

Bioethanol production	Typical GHG gas emissions[a] (g CO_2 eq/MJ)	Typical GHG emission saving[b]
Sugar beet ethanol	33	61%
Sugar cane ethanol	24	71%
Wheat ethanol (process fuel not specified)	57	32%
Wheat ethanol (natural gas as process fuel)	46	45%
Wheat ethanol (natural gas in CHP plant)	39	53%
Wheat ethanol (straw as process fuel in CHP plant)	26	69%
Maize ethanol (natural gas in CHP plant)	37	56%
Wheat straw ethanol	11	87%
Waste wood ethanol	17	80%
Farmed wood ethanol	20	76%

[a] Figures represent total CO_2 emissions for cultivation, processing, transport and distribution.
[b] Savings compared to fossil fuel (e.g., petroleum) combustion.
Adapted from DIRECTIVE 2009/30/EC OF THE EUROPEAN PARLIAMENT AND OF THE COUNCIL of 23 April 2009 amending Directive 98/70/EC). This Directive provides information on GHG emissions and savings (compared to fossil fuel combustion) of bioethanol (L 140/88 EN Official Journal of the European Union 5.6.2009).

Table V. Comparison between fuel and potable alcohol production. The processes considered refer to fuel alcohol from sugar cane (juice, molasses) as conducted in Brazil, and Scotch whisky (malt and grain) as conducted in Scotland.

Parameter	Fuel alcohol from sugar cane	Potable alcohol from cereals
Scales of operation	Large bioethanol plants typically ~500 m litres/a	Grain distillery output ~100 m litres/a; Scotch malt distillery ~20 m litres/a
Fermentation media	1. Variable gravity (using sugarcane juice or molasses) 2. pH 4–5 3. Sucrose is released without need for enzymes, then yeast invertase produces fermentable glucose and fructose	2. Wort gravity ~1,060 OG (15°Plato) 2. pH 5 3. Maltose and glucose (mainly) are generated using malt enzymes to convert cereal starch
Yeasts & fermentation	1. *Saccharomyces cerevisiae*. Baker's yeast at start (then indigenous yeasts predominate) 2. Yeast pitching rate: 8–17% wet weight 3. Yeast recycling with acid-treatment? Yes 4. Fermentation temperature/time: 30–35°C/6–10 h 5. Final ethanol: 6–11% (v/v). Newer processes aim for >15% (v/v) ethanol	1. *Saccharomyces cerevisiae*. Specially selected distiller's strains 2. Yeast pitching rate: 10–20 × 10^6 cells/mL 3. Yeast recycling with acid treatment? No 4. Fermentation temperature/time: Starts at ~20°C rises to ~32°C/24–48 h 5. Final ethanol: 8-10% (v/v)
Distillation	1. Anhydrous ethanol (99.9% (v/v)) obtained via molecular sieves 2. Fusel oil fractions are removed from stills to facilitate ethanol purification, but can be blended back into bioethanol for fuel use	1. For Scotch whisky, ethanol collected below 94.8% (v/v) (to retain some flavour congeners) 2. In Scotch whisky distillation, fusel oils are separated from distilled spirit
Lactic acid bacteria	Undesired throughout fermentation and controlled with antimicrobial agents (antibiotics now limited).	Undesired at start, desired at end of fermentation (for flavour development). No antimicrobials applied.

FEEDSTOCKS FOR BIOETHANOL PRODUCTION

First generation feedstocks

Carbohydrate material for bioethanol production can come from sugary, starchy or cellulosic biomass sources.

First-generation bioethanol feedstocks come from agricultural cereal and sugar crops that are also sources of human (and animal) food (see Fig. 3 and references[71,88]).

Sugar-rich crops predominantly refer to sugar cane (*Saccharum* sp.) and sugar beet (*Beta vulgaris* L.), whilst starch-rich crops mainly refer to the cereals maize (*Zea mays*) and wheat (*Triticum* spp.). The former crops contain a readily fermentable sugar source, namely sucrose (Table VI); whilst cereal starches require pre-hydrolysis prior to sugar fermentation. Thus, production of bioethanol from sucrose-containing feedstocks is the easiest, most efficient and economical compared with starchy feedstocks.

Sugar cane processing for bioethanol production is dominated by Brazil, where a continuous sugar cane harvest season takes place over a period of 200 days. Sugar cane juice (~15% sucrose), or the residual molasses (~50% sucrose) from sugar refining processes, is readily fermented by yeasts such as *Saccharomyces cerevisiae*.

The juice can be processed either into crystalline sugar or directly fermented to ethanol, as per many Brazilian industrial plants (see Fig. 4).

For sugar production, sugar cane juice is clarified with lime and evaporated to form crystalline sucrose[103]. This process leaves molasses (a syrupy brown by-product) which represents a very good fermentation medium comprising sugars (sucrose, glucose, fructose), minerals, vitamins, fatty acids, organic acids etc. (see Table VI). For alcohol fermentations, additional yeast nutrients may be supplemented to molasses (e.g., nitrogen in the form of di-ammonium phosphate). When more sucrose is processed for crystalline sugar production, the residual molasses will be of poorer quality containing excess levels of salts and browning reaction products (e.g., furfurals, formic acid) that may inhibit fermentation. For bioethanol fermentations, molasses is typically diluted to 20–25% total sugar (measured in °Brix), treated with sulphuric acid (which will precipitate excess calcium) and heated to 90°C prior to cooling, centrifugation, pH adjustment and addition of yeast. Instead of being processed to crystalline sugar, cane juice can either be directly fermented, clarified following heat (105°C) treatment, or mixed with molasses in different proportions. Mixing clarified juice with molasses improves yeast nutrition and fermentation performance. Ethanol yields are also improved following

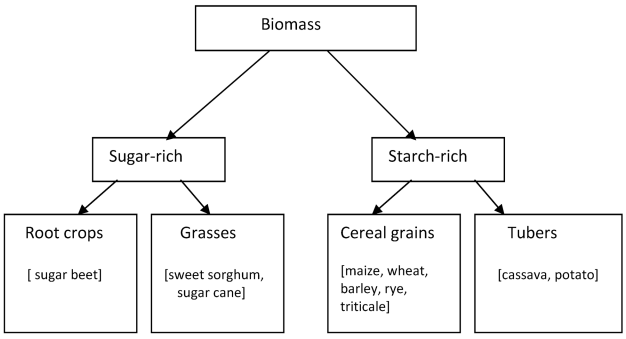

Fig. 3. Bioethanol from first generation feedstocks.

Table VI. Composition of sugar-based feedstocks for bioethanol production.

Composition	Sugar cane juice (g/L)	Sugar cane molasses (g/Kg)	Sugar beet molasses (g/Kg)
Total solids	140–190	735–875	759–854
Total sugars	105–175	447–587	477–530
Sucrose	98–167	157–469	443–530
Reducing sugars	6–11	97–399	1.2–10
Raffinose	-	-	4.7–21
Nitrogen	0.08–0.3	0.25–1.5	1.3–2.3
Phosphorus	0.02–0.1	0.3–0.7	0.15–0.52
Potassium	0.7–1.5	19–54	15–52
Calcium	0.1–0.5	6–12	0.75–3.8
Magnesium	0.1–0.5	4–11	0.1–2.7

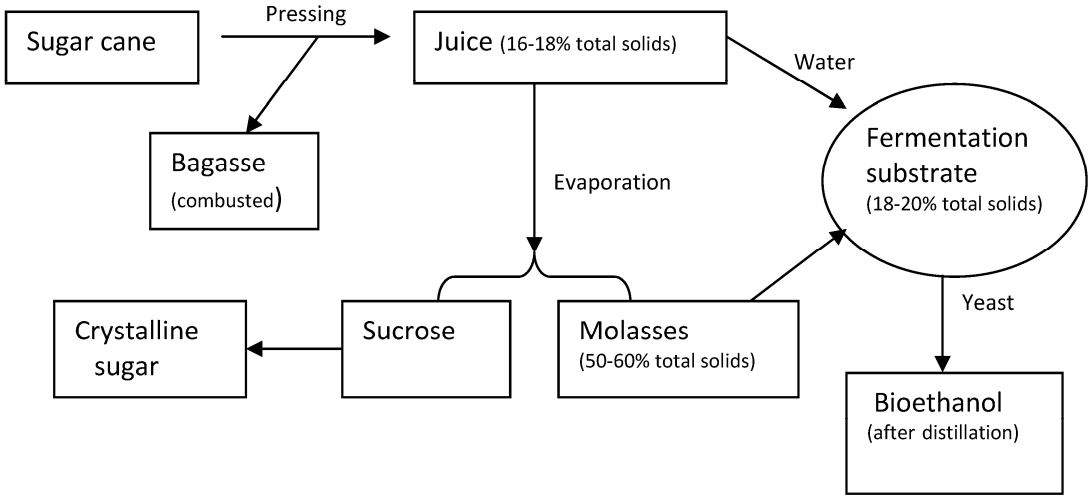

Fig. 4. Sugar cane processing for sugar and bioethanol production.

Table VII. Main constituents of starch-based feedstocks for bioethanol. (Adapted from Monceaux[71])

Constituent (% w/w)	Maize	Wheat	Barley	Sorghum	Rye	Cassava	Potato
Starch	65–72	57–70	52–64	72–75	55–65	65–82	14–24
Sugar	2.2	-	-	-	-	0.25	1.5
Protein	9–12	12–14	10–11	11–12	10–15	2–3	0.6–3.5
Fat	4.5	3	2.5–3	3.6	2–3	0.8	0.1
Cell wall	9.6	11.4	14	-	-	-	2
Fibre	-	-	-	-	-	4.6	-
Ash	1.5	2	2.3	1.7	2	2–5	0.6–1.1

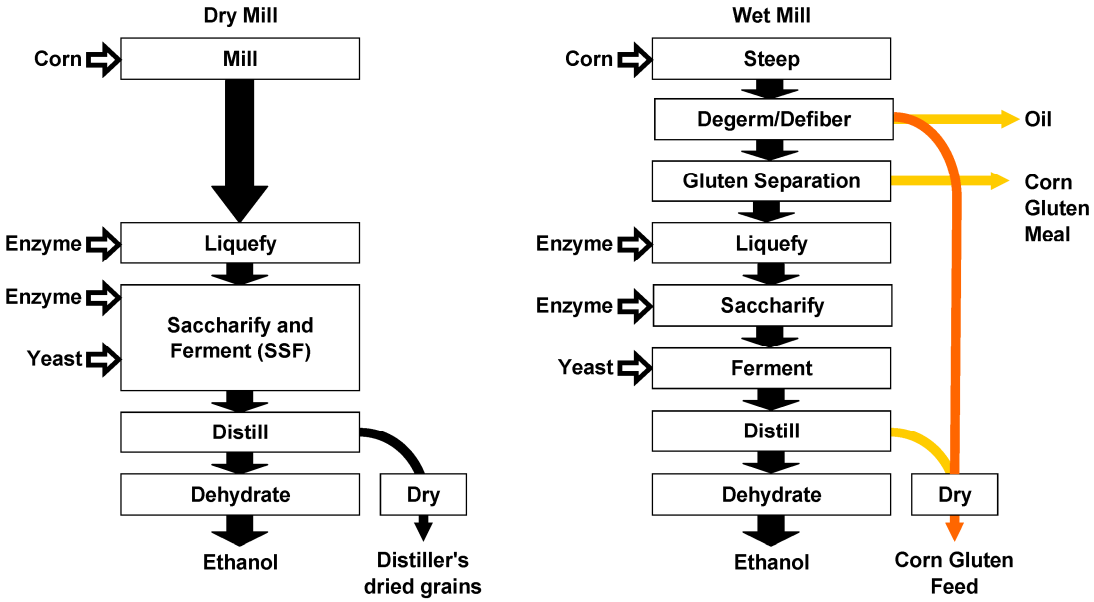

Fig. 5. Dry and wet milling maize processes for bioethanol (Reproduced with permission from Abbas[2]).

heat treatment and clarification of juice/molasses to reduce impurities and bacterial and wild yeast contaminants.

Regarding starchy crops for bioethanol production, Table VII summarises the main macromolecular constituents of feedstocks. In Canada and the US, bioethanol is produced predominantly from *Zea mays* (maize or corn), whilst European processes utilise wheat. Cereal conversion to bioethanol basically comprises: milling, starch liquefaction and hydrolysis, yeast fermentation and distillation.

Maize-to-bioethanol processes in the US are differentiated into 2 main types: dry and wet milling (see Fig. 5 and references[1,81]). Dry milling processes are used to produce most American bioethanol and involve fine grinding of maize kernels, which are further processed without fractionation. In contrast, wet milling processes firstly soak maize in water (or dilute acid) which separates the cereal into starch, gluten, protein, oil and fibre. In both dry and wet milling processes, the maize starch is liquefied and saccharified with amylolytic enzymes prior to fermentation.

Of course, starch is not directly fermented by yeasts such as *S. cerevisiae* and requires the following pretreatments and hydrolysis prior to fermentation: cereal cooking, starch liquefaction and amylolysis. In potable alcohol fermentations (e.g., brewing) starch conversion is accomplished using endogenous malt enzymes, but for bioethanol production, more complete starch hydrolysis is required. This is accomplished using exogenous amylolytic enzymes, including: α- and β-amylases (for liquefaction); amyloglucosidase (or glucoamylase) required to debranch amylopectin fractions (comprising 75–90% of starch, depending on cereal source) and glucanases (for viscosity reduction). Industrial enzymes used in starch-to-ethanol bioconversions are produced by specialist companies from microbial fermentations using bacteria such as *Bacillus* spp. and fungi such as *Aspergillus* spp. (see Nair et al.[77]).

Wheat-to-bioethanol processes share similarities with these maize processes (see Fig. 6).

Although wheat yields a greater level of ethanol when compared to sugar beet on a weight basis (374 cf 100 L/t, respectively), on an acreage basis, sugar beet is more productive 5,500 cf 3,141 L/ha, respectively.

Second generation feedstocks

Exploitation of first generation feedstocks for future biofuel production is ultimately unsustainable due to food security and land-use issues. *Second-generation* bioethanol refers to fuel alcohol produced from non-food biomass sources, such as lignocellulose, the most abundant form of carbon on the Earth. Lignocellulosic biomass encompasses two main categories of bioethanol feedstocks:

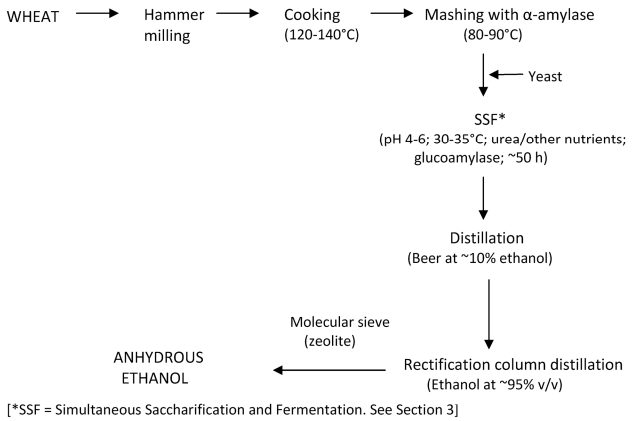

Fig. 6. Typical wheat-to-bioethanol production process.

Table VIII. Composition of lignocellulosic biomass (% dry weight).

Biomass or waste	Cellulose	Hemicellulose	Lignin
Trees			
Poplar	45–50	17–19	18–26
Eucalyptus	50	13	28
Pine (spruce)	44	23	28
Salix (hardwood)	43	22	26
Grasses			
Switch grass	31–45	20–30	12–18
Bermuda grass	25	36	6
Rye grasses	25–40	35–50	10–30
Paper			
Office paper	69–99	0–12	0–15
Newspaper	40–55	25–40	18–30
Paper pulp	60–70	10–20	5–10
Food/agriculture wastes			
Corn cobs	45	35	15
Corn stover	38–40	22–28	18–23
Corn fibre	14	17	8
Wheat straw	30–38	21–50	15–23
Rice husk	24	27	13
Bagasse	38	27	20
Nut shells	25–30	25–30	30–40
Leaves	15–30	80–85	0
Cattle manure	1.6–4.7	1.4–3.3	2.7–5.7
Miscellaneous sorted refuse			
Wastewater solids	60	20	20
Municipal solid	8–15	NA	24–29
Waste (MSW)	33	9	17
MSW paper pulp	62	5	11

Information from[42,73,100,112]; bio-process.com/wp-content/uploads/2009/12/MSW.pdf

1. Biowaste materials (straws, corn residues (stover, fibres and cobs), woody wastes/chippings, forestry residues, old paper/cardboard, bagasse, spent grains, municipal solid waste (MSW), agricultural residues (oilseed pulp, sugar beet pulp).
2. Energy crops such as short rotation coppice, SRC (e.g., basket willow *Salix viminalis*) and energy grasses *Miscanthus* × *giganteus* (hybrid of *M. sinensis* and *M. sacchariflorus*), alfalfa (*Medicago sativa* L.), switchgrass (*Panicum vigratum*), reed canary grass (*Phalaris arundinaceae* L.), giant reed (*Arundo donax*), ryegrass, etc).

Residual cellulose-based agricultural and industrial biomass (or biowastes) represent the most sustainable and ethically acceptable materials for bioethanol production, and also offer greater cost reductions compared to utilization of starch and sugar crops[134]. The use of so-called energy crops is also advantageous in this regard (including GHG emission reductions), especially as such crops can be cultivated on degraded/contaminated land for bioethanol production[11,28,38,44,64,90,92,99,104,131].

Table IX. Xylan and arabinan in selected lignocellulose sources[34].

Feedstock	% Xylan	% Arabinan
Ryegrass	16	5
Corn stover	19	3
Wheat bran	19	15
Wheat straw	21	3.4
Barley husks	20	9
Hardwood	15	1
Softwood	5	2
Bagasse	26	1.5
Newspaper	4.3	0.8

Table VIII lists the composition of major lignocellulosic biomass sources. A more detailed analysis of the major components of lignocellulosic agricultural residues has been provided by Yang et al.[132] A typical lignocellulosic material, woody biomass, is comprised of the following (with empirical formulae): cellulose $C_6H_{10}O_5$; hemicellulose $C_5H_8O_4$; and lignin $C_6H_{11}O_2$. The former two macromolecules can both be hydrolysed to fermentable sugars, but lignin cannot. Cellulose is a glucose polymer (in β-(1,4)-linkages, with an average molecular mass of ~100,000 Da) and hemicellulose is a highly branched heteropolysaccharide (average molecular mass of 30,000 Da) comprising pentose sugars (xylose and arabinose) and hexose sugars (glucose, mannose and galactose). The hemicellulose sugar backbone in softwoods is mannose with glucose and galactose side-chains; whilst in hardwoods and grasses, the backbone is xylose with side chains of arabinose and glucuronic acid. In hardwoods (e.g., *Salix*), some of the xylose moieties are acetylated (OH groups replaced by O-acetyl groups) and pre-treating this material can produce high levels of acetic acid, which may be inhibitory to yeast fermentation performance.

Xylose and arabinose are polymerised in the form of xylan and arabinan, respectively to form arabinoxylan (see Table IX).

Lignin is a very tough, recalcitrant plant cell wall material which is comprised of di- and mono-methoxylated, and non-methoxylated phenylpropanoid units (derived from the corresponding *p*-hydroxycinamyl alcohols) in a three-dimensional network. Acid hydrolysis of lignocellulosic biomass will leave behind acid-insoluble lignin, but some acid-soluble lignin may be released into the hydrolysate liquor. For bioethanol production processes, acid-soluble lignin components include phenolic degradation products that can inhibit cellulase activity and yeast fermentation. The amount of non-utilisable (and potentially inhibitory) lignin in corn stover is high and varies between 17–26% dry wt.

Other minor components of lignocellulosic biomass for fuel alcohol production include: ash (inorganic minerals), pectins (highly-branched polysaccharides of galacturonic acid and its methyl esters), acids and extractives (extracellular, non-cell wall material).

Lignocellulose pretreatments. Compared with sugary and starchy biomass sources for bioethanol production,

Table X. Pre-treatment technologies for lignocelluloses. Further information from[6,12,36,56,73].

Pre-treatment methods	Examples
Physical	Milling (mechanical comminution), microwave irradiation, ultrasound, thermal processes (pyrolysis at >300°C, steam explosion using 160–260°C, 0.69–4.83 MPa pressure, followed by rapid decompression), thermochemical processes (weak acid, high temperature), extrusion.
Chemical	Alkali-pretreatment, ammonia fibre expansion (AFEX) technologies, organosolv (ACOS), liming (calcium hydroxide), sulphur dioxide, liquid hot water (LHW) and wet oxidation (hot water plus oxygen at 200°C), CO_2 explosion, SO_2 explosion, ozonolysis, H_2O_2 delignification, supercritical fluid and ionic liquid pre-treatments (e.g. n-butyl-methy-lilidazolium chloride ~300°C).
Biological	Microbial (e.g., white-rot fungi such as *Phanerochaete chrysosporium, Trametes versicolor*) and enzymatic (e.g., peroxidase and laccase) pretreatments (delignification).

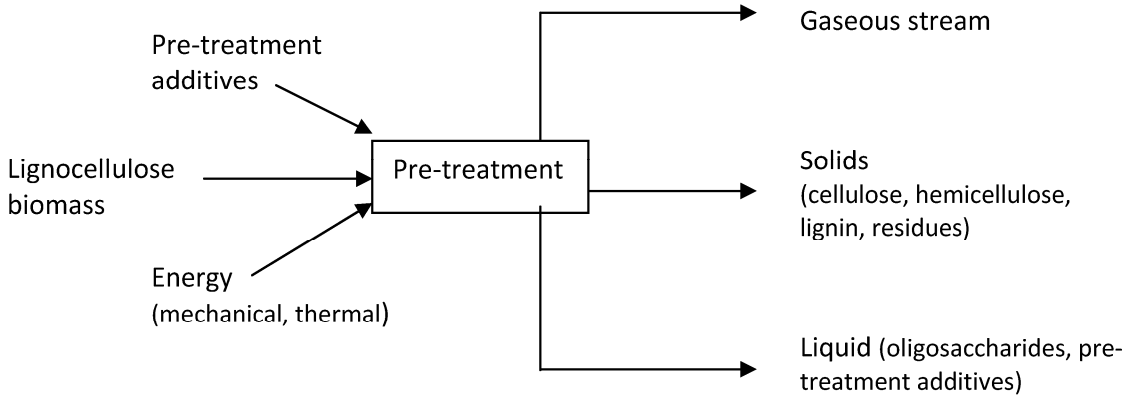

Fig. 7. Basic features of lignocellulose pre-treatments.

lignocellulose-based material demands more complex pretreatment and hydrolysis technology Lignocellulosic recalcitrance is due to sheathing of cellulose crystals by hemicellulose, together with lignin acting as a "sealant". The following are the principal stages in generating free pentose and hexose sugars from lignocellulose material:

1. Pre-processing (mechanical removal of dirt, debris and shredding into smaller particles)
2. Pre-treatment (see Table X)
3. Solid-liquid separation (hemicellulose sugars are separated from solid fibrous material comprising cellulose and lignin)
4. Cellulose hydrolysis (cellulase attack on crystalline cellulose to liberate glucose)

Figure 7 outlines the basic features of lignocellulosic pre-treatment processes, but a single process does not exist for all types of biomass material. Successful methods will preserve pentose sugars from the hemicellulose fraction, render cellulose more amenable to enzymolysis and limit lignin degradation[6,60,73,85,86,114,135].

Table X summarises physical, chemical and biological lignocellulose pre-treatment technologies available. The major problems from the yeast fermentation perspective are the generation of inhibitory chemicals (acids, furans, phenols), high particle load, and efficient separation of soluble sugars from solid residues. In addition, to keep net energy balances favourable, energy input needs to be minimised. The use of ultrasound has potential as a low-energy pretreatment[17,51]. Lignocellulose pre-treatment methods are also one of the most expensive steps in the overall conversion to bioethanol[6,73].

Once the lignocellulose material has been pretreated, it must then be hydrolysed to liberate fermentable sugars. Different hydrolytic approaches have been discussed by Mousdale[74] and Anish and Rao[9], but typical hydrolytic methods subject hemicellulose fractions to mild acid hydrolysis (followed by cellulolysis with enzymes). For example, treatment with 0.7% H_2SO_4 at 190°C for 3 min may be adopted to release pentose sugars from softwoods, but more concentrated acid treatments (e.g., 30–70% H_2SO_4) can be employed at lower temperatures (40°C) for longer time (2–6 h). Lignocellulose acid hydrolysis tends to degrade sugars and release chemicals (e.g., hydroxymethylfurfural (HMF) from glucose and furfural and acetic acid from xylose) that can inhibit yeast in the subsequent fermentation stages.

Following acid hydrolysis, cellulolysis takes place using cellulase enzymes that act to degrade the β-1,4-D-glucan bonds in cellulose to yield predominantly glucose, and also some cellobiose (glucose disaccharide) and cello-oligosaccharides[16,111]. This is conducted with commercial industrial enzymes (usually at pH 4.8 and 45–50°C) derived from bacteria (e.g., *Cellulomonas fimi, Clostridium thermocellum, Bacteriodes cellulosolvens*) or fungi (e.g., *Trichoderma reesei*). These "cellulases" belong to a family of glycoside hydrolases[16] and comprise the following types of celluloytic enzyme activity:

1. Endo-β-1,4-glucanase (expose reducing and non-reducing ends within cellulose)
2. Exoglucanases (acting on reducing and non-reducing ends of cellulose)
   - Cellodextrinases (liberating glucose)
   - Cellobiohydrolases (liberating cellobiose and cello-oligosaccharides)
3. β-Glucosidases (liberates glucose from cellobiose)

The inhibition of cellulase activities by cellobiose and glucose may be minimised using high enzyme concentrations; supplementary β-glucosidases; ultrafiltration to re-

Table XI. Co-products from bioethanol production processes.

Feedstock	Co-product	Applications
Cereals (maize, wheat)	Cereal residues (spent grains)	Animal feeds (DDGS), drying and combustion, bioconversion to biofuels.
	Backset (stillage) residues from distillation	Re-cycling options for mash preparation and supplements to fermentation media.
Sugar cane	Bagasse (sugar cane processing residues)	Combustible energy source (distillery plant power, and surplus to electricity grid).
	Vinasse (stillage)	Used as agricultural fertilizer.
Sugar beet	Pulp (residue of milling process)	Fibre-rich animal feed component.
Lignocellulose	Lignin (residue from lignocellulose bioconversion is ~40% lignin)	Combustible energy source (formulated into dry pellets or thermally gasified to synthetic natural gas, SNG).
All	Fusel oil (higher alcohols fraction from distillation)	Chemical commodities (cosmetics, paints/inks).
	Carbon dioxide	Liquefied CO_2 for carbonated drinks, use in greenhouses and potentially microalgal bioreactors.
	Spent yeast	Animal feeds (directly and incorporation with other co-products).

move produced sugars and simultaneous saccharification and fermentation (SSF).

Dilute acid pretreatments and enzyme hydrolysis can convert hemicellulose and cellulose fractions of lignocellulosic material to glucose, xylose and arabinose. This cocktail of hexose and pentose sugars released represents a challenge for fermentation to ethanol (see Section 4).

Co-products from bioethanol production

Bioethanol production processes generate a variety of co-products, including CO_2, fusel oils, cereal residues, bagasse, stillage and spent yeast (see Table XI). From cereal (maize, wheat) bioethanol production, the main co-products are DDGS (distillers' dried grains with solubles, with ~30% protein) and DWG (distillers wet grains, lower in protein) which are used as feeds for livestock (beef and dairy cattle) and non-ruminants (poultry and swine)[26,72,90]. Animal feeds in the form of DDGS represent profitable co-products for bioethanol producers and it can be assumed that for maize and wheat processes 0.75 and 0.8 kg DDGS from 2.4 and 2.7 kg, respectively, are obtained from each litre of ethanol produced from these cereals[107].

Food and beverage processing residues and co-products represent potential biomass sources for bioethanol[57]. For example, spent grains (SG) that remain following brewers or distillers wort extraction, may provide lignocelluose-rich biomass for fuel ethanol fermentations. Dilute acid and enzyme treatments can convert hemicellulose and cellulose fractions of SG to glucose, xylose and arabinose and these sugars can be fermented by non-*Saccharomyces* yeasts such as *Pichia stipitis* and *Kluyveromyces marxianus* resulting in favourable ethanol conversion yields[130].

BIOETHANOL FERMENTATIONS

Microbes for bioethanol fermentations

The yeast, *Saccharomyces cerevisiae,* is the predominant microorganism responsible for ethanolic fermentations and is the major cell factory in industrial bioethanol production processes. Other yeasts (e.g., genetically manipulated or GM, variants of *S. cerevisiae, Pichia, Candida* and *Kluyveromyces* spp.) and certain bacteria (e.g., *Zymomonas mobilis, Thermoanaerobacterium* spp.) have future potential in this regard. Table XII summarises some ethanologenic microbes for use in bioethanol fermentations.

Many yeasts, but few bacteria, express the key fermentative enzyme, pyruvate decarboxylase (PDC). This enzyme decarboxylates pyruvate to acetaldehyde in the penultimate step to ethanol. *Zymomonas* spp. (*Z. mobilis* and *Z. palmae*) are some of the very few bacteria that naturally (i.e., without genetic engineering) produce ethanol under anaerobic fermentation conditions. *Saccharomyces* yeasts and *Zymomonas* bacteria both produce ethanol via *homoethanol* pathways, by the Embden-Meyerhof-Parnas (EMP) and Entner-Doudoroff pathways, respectively[55].

The EMP (glycolytic) pathway may be summarised as follows:

$$\text{Glucose} + 2\text{ADP} + 2\text{Pi} + 2\text{NAD+} \rightarrow 2\text{Pyruvate} + 2\text{ATP} + 2\text{NADH} + 2\text{H}^+$$

S. cerevisiae reoxidizes the reduced co-enzyme NADH to NAD^+ in terminal fermentative step reactions emanating from pyruvate:

$$2\text{Pyruvate} + 2\text{NADH} + 2\text{H}^+ \rightarrow 2\text{NAD}^+ + 2\text{Ethanol} + 2\text{CO}_2$$

The intermediate compound, acetaldehyde, acts as the electron acceptor and is generated following pyruvate decarboxylation:

$$\text{CH}_3\text{COCOOH (Pyruvate)} \xrightarrow{\text{Pyruvate decarboxylase}} \text{CH}_3\text{CHO} + \text{CO}_2 \text{ (Acetaldehyde + CO}_2\text{)} \xrightarrow{\text{Alcohol dehydrogenase}} \text{CH}_3\text{CH}_2\text{OH (Ethanol)}$$

NAD^+ is re-generated by alcohol dehydrogenase which requires zinc as an essential co-factor for its activity. Fermentation thus maintains the redox balance by regenerating NAD and keeps glycolysis proceeding. In doing so, yeast gets energy for its own maintenance by generating 2ATP.

The theoretical (stoichiometric) conversion to ethanol from glucose is as follows:

$$\text{C}_6\text{H}_{12}\text{O}_6 \text{ (Glucose, 180 kg)} \rightarrow 2\text{C}_2\text{H}_5\text{OH (Ethanol, 92 kg)} + 2\text{CO}_2 \text{ (Carbon dioxide, 88 kg)}$$

This means that for each kilogram of glucose fermented, around 470 g of ethanol can be produced (i.e., <50%) representing a yield of 92% of theoretical maximum. In industrial fermentation practice, however, the best yields are only around 90% of this theoretical conversion due to the diversion of fermentable carbon to new yeast biomass and minor fermentation metabolites (organic acids, esters, aldehydes, fusel oils etc).

Regarding the nature of pitching yeast for bioethanol fermentations, it is important to maintain strains as pure cultures free from wild yeast and bacterial contaminants.

Table XII. Microbes for bioethanol fermentations.

Microbe	Characteristics
Saccharomyces cerevisiae (yeast)	Predominant bioethanol microbe capable of fermenting the main sugars derived from first-generation feedstocks (e.g., glucose, fructose, sucrose, maltose) under large-scale industrial production conditions. Incapable (unless genetically modified) of fermenting pentose sugars (e.g., xylose, arabinose) derived from second generation lignocellulose feedstocks. Ethanol productivities of GM strains fermenting xylose are quite low 0.23–0.34 g/g sugar).
Pichia stipitis, Candida shehatae, Kluyveromyces marxianus, Pachysolen tannophilus (yeasts)	Non-*Saccharomyces* yeasts capable of fermenting pentose sugars (e.g., xylose, arabinose) derived from second generation lignocellulose feedstocks. Not particularly ethanol-tolerant yeasts and await exploitation for large-scale industrial fermentation processes (although *K. marxianus* is used in whey lactose fermentations).
Hansenula polymorpha (yeast)	High temperature xylose fermentations[52], Untested on an industrial scale
Dekkera bruxellensis (yeast)	"Wild" yeast found in distillery fermentations that may be capable of ethanol production under stressful conditions. *D. bruxellensis* is one of the very few yeast species known to outcompete *S. cerevisiae* in high ethanol fermentations, but it awaits further research prior to industrial exploitation.
Candida krusei (yeast)	Ethanologenic yeast producing low levels of secondary fermentation metabolites such as succinic acid. Untested on industrial scale.
Non-GM bacteria	Numerous ethanologenic bacteria are known, some of which (e.g., *Zymomonas mobilis*) produce ethanol more effectively than yeast. *Klebsiella oxytoca* also has potential. May not survive the stressful environment in large-scale bioethanol plants, and ethanol productivities are generally quite low. Typical ethanol productivity (g/g sugar): *Z. mobilis* 0.46; *K. oxytoca* 0.34–0.42
GM bacteria	*Geobacillus stearothermophilus* is a thermophile that ferments C5 and C6 sugars including short polymers at temperatures in excess of 60°C with yields ~80% theoretical maximum. It has been genetically modified to produce ethanol rather than lactate and formate (see www.tmo-group.com). Not particularly ethanol tolerant (~5% (v/v)). Attributes discussed by Candy[23]. *Escherichia coli* (with *Z. mobilis* genes encoding pyruvate decarboxylase and alcohol dehydrogenase) and *Erwinia chrysanthemi* (with pyruvate decarboxylase genes) also have potential. Typical ethanol productivities (g/g sugar): *G. stearothermophilus* 0.40; *E. coli* 0.41; *E. chrysanthemi* 0.45.
Microalgae	Certain species of blue-green algae (cyanobacteria) can be metabolically engineered to produce ethanol, potentially from CO_2, sunlight and seawater[24,31].

Table XIII. Typical stresses experienced by bioethanol production yeasts.

Stress factor	Examples
Chemical	Lignocellulosic hydrolysate inhibitors (acids, phenols, furans); sulphite >100 mg/L; sodium >500 mg/L; low free amino nitrogen <150 mg/L; low zinc <0.1 ppm; high sugar ~30% (w/v); high ethanol >10% (v/v); high CO_2; acetic acid >0.05% (w/v); pH <3–4.
Physical	Mechanical shear; hydrostatic pressure; anaerobiosis; temperature >35°C; cold-shock; dehydration/osmostress.
Biological	Contaminant bacteria (e.g., lactic acid >0.8% (w/v)); wild yeasts (e.g., killer strains).

Poor yeast viability and vitality and the presence of *Lactobacillus* spp. can significantly reduce ethanol yields. For example, every molecule of bacterial lactic acid produced in a fermenter equates to the loss of an ethanol molecule. Fuel alcohol production is a non-sterile process, and contamination control and good hygienic operations are of utmost importance in bioethanol plants. In addition to heat treatments of raw materials, air, water, vessels, pipework etc, bioethanol distilleries may acid-wash (e.g., H_2SO_4) recycled yeast slurries, apply preventative antibiotics such as penicillin and virginiamycin (these are now restricted[75]), use various chemical cleaners, sanitisers and sterilants (e.g., chlorine dioxide, ammonium bifluoride, potassium metabisulphite, urea hydrogen peroxide, hop acids) to control microbial contamination. Various strategies for control of microbial (wild yeast and bacterial) contaminants in bioethanol fermentations have been discussed by Muthaiyan et al.[75]). Selection of specific starter cultures of *S. cerevisiae* with bactericidal activity to control contaminants in bioethanol distilleries is an interesting development[30].

Some distilleries operate yeast recycling (see below) and this circumvents the need to regularly purchase new yeast batches, whilst other plants aerobically propagate their own yeast in order to boost biomass required as starter cultures for fermentation. Otherwise, different distilling strains of *S. cerevisiae* are available from yeast manufacturers as cream, compressed (cake) and dried preparations.

Physiological characteristics of bioethanol yeasts. For existing and emerging industrial bioethanol fermentations, most of the desirable characteristics of the producing microbes are met by the budding yeast, *S. cerevisiae*[121,122,124]. These characteristics especially include abilities of yeast strains to tolerate stresses due to physico-chemical and biological factors during the rigours of industrial fermentation processes (Table XIII). There is a need to develop stress-resistant yeasts for fuel alcohol fermentations, especially strains able to withstand substrate and product toxicity[18]. Some commercially available bioethanol yeast strains can produce ethanol at >10% (v/v) in high solids >20% (w/v) mashes. However, it is now possible (through correct yeast nutrition) to produce over 20% (v/v) ethanol in high gravity wheat fermentations.[50,117]

In addition, several cell physiological approaches can be adopted to improve stress-tolerance of yeasts for bioethanol production. These do not involve genetic manipulation strategies and include: sterol pre-enrichment (pre-oxygenation, mild aeration); mineral preconditioning of yeast (Mg, Zn enrichment); ethanol adaptation (in chemostats); pre heat-shocks to confer thermotolerance; and salt preconditioning to confer osmotolerance[63]. Some naturally robust indigenous yeast (e.g., distillery resident) can

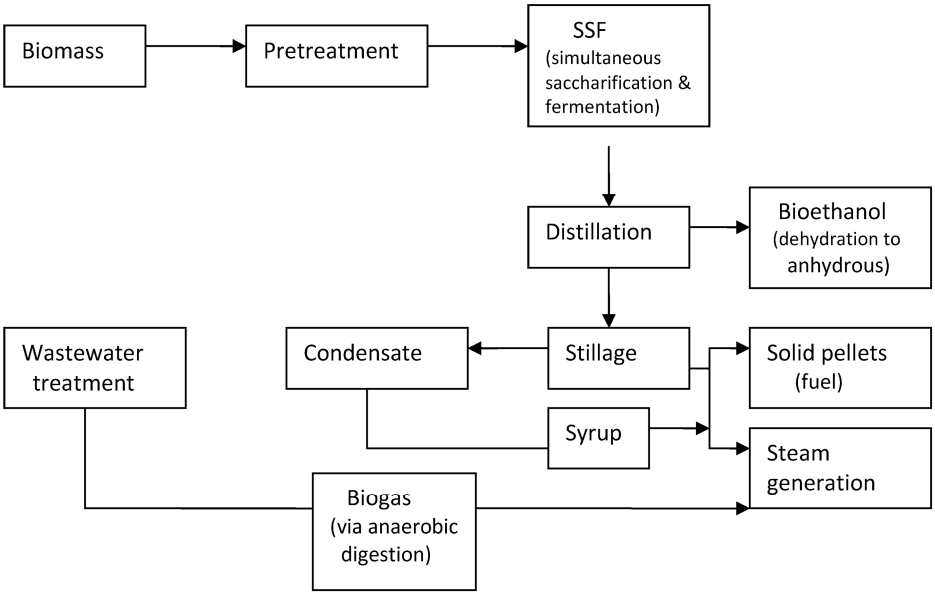

Fig. 8. Generalised lignocellulose-to-bioethanol process (adapted from Sassner et al.[100]).

be isolated and selected for industrial fermentations[7,15,25]. Some of the wild yeast strains isolated from Brazilian semi-continuous fermentation operations have proved to be particularly well suited to survive the stressful environment of large-scale bioethanol processes[15].

S. cerevisiae grows well in many industrial feedstocks, such as sugar cane juice and molasses and in starch hydrolysates. Some supplementary nutrients (minerals, vitamins, growth factors) may prove beneficial in stimulating fermentation of certain bioethanol feedstocks. Industrial *S. cerevisiae* strains grow best from 20–30°C and between pH 4.5 and 5.5. Thermotolerant yeasts are sought after, especially for fuel alcohol plants in tropical countries. *S. cerevisiae* is not, strictly speaking, a facultative anaerobe and is unable to grow well under completely anaerobic conditions because oxygen is needed for membrane biosynthesis (specifically for fatty acid (e.g., oleic acid) and sterol (e.g., ergosterol) biosynthesis). For this reason, some bioethanol fermentation processes may benefit from mild aeration.[4,27] Under ideal (laboratory-optimised) conditions, *S. cerevisiae* reproduces quickly (approx. every 90 min), but in industrial fermenters this takes considerably longer due to the stressful physico-chemical environment. In Brazilian semi-continuous fermentations, yeast growth and budding is greatly restricted, but this is mitigated by the very high cell densities employed.

Sucrose and starch hydrolysate fermentations

For first generation bioethanol fermentations, the principal fermentable sugars are sucrose, glucose and fructose (e.g., in sugar cane juice and in molasses) and glucose and maltose (in cereal starch hydrolysates). These are all readily fermented by *S. cerevisiae*. No extraneous enzymes are required to liberate sugars from sugar-rich crops (cane, beet, sweet sorghum) and *S. cerevisiae* produces the enzyme invertase to hydrolyse sucrose into readily-fermentable glucose and fructose).

Bioethanol producers aim to achieve fast and efficient conversion of available sugars to ethanol. For starch-based fermentations such as maize dry-mill operations (see Fig. 5), it is possible to produce >400 litres of ethanol per tonne of maize (at 63% starch), whereas for wheat, typical values would be 385–400 litres/tonne.

Table XIV. Examples of typical ethanol yields from first-generation crops.

Crop	Ethanol yield (tonnes ethanol per hectare)
Sweet sorghum	4.0–6.5
Wheat	4.8
Sugar beet	3.3–3.8
Potato	2.0–2.9
Chicory	2.0–3.9
Jerusalem artichoke	4.0–4.7

Sugars fermented by yeast are converted to ethanol and carbon dioxide (the principal metabolic products), and other fermentation metabolites fusel alcohols (e.g., isoamyl alcohol); polyols (e.g., glycerol); esters (e.g., ethyl acetate); organic acids (e.g., succinate); vicinyl diketones (e.g., diacetyl); and aldehydes (e.g., acetaldehyde). These are important for beverage (beer, wine, spirits) flavour development but are undesirable for bioethanol production due to loss of ethanol yield. For example, the production of glycerol in bioethanol plants can significantly detract from fuel alcohol yields[33] and efforts are made to dissipate this, including simultaneous saccharification and fermentation (SSF) processes and construction of yeast strains with reduced glycerol[46].

Agronomically speaking, bioethanol crops can be ranked according to potential ethanol yields per hectare of cultivable land (Table XIV and references[2,10,37]).

Lignocellulosic hydrolysate fermentations

Following pretreatments of lignocellulosic feedstocks (discussed in Section 2), the material may subsequently be hydrolysed and fermented via simultaneous saccharification and fermentation (SSF). Figure 8 outlines the general scheme for producing bioethanol with this approach, and also shows further utilisation of distillation residues

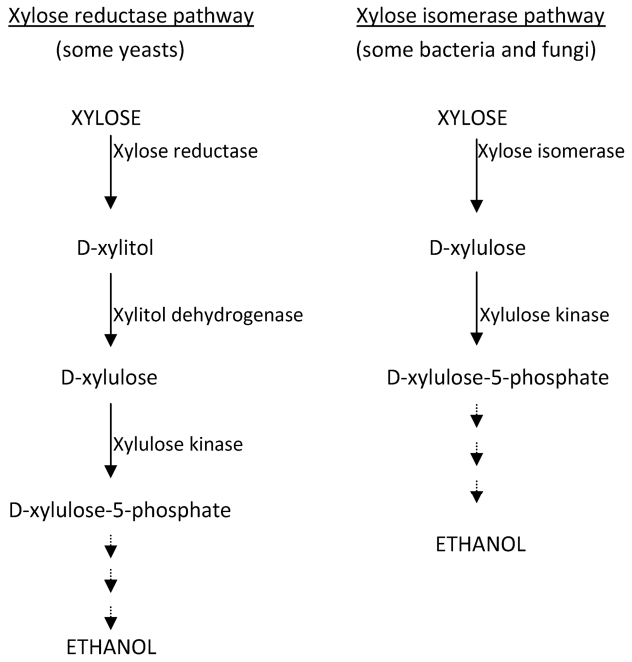

Fig. 9. Pathways for microbial xylose fermentation.

and wastewater to provide fuel (e.g., biogas) to run the facility (and improve net energy balances).

Sugars derived from second generation feedstocks are glucose, xylose and arabinose (in lignocellulose hydrolysates). However, although *S. cerevisiae* can ferment glucose without difficulty, this yeast cannot ferment the pentose sugars xylose and arabinose. This has led to various microbiological and molecular genetic approaches to enable efficient fermentation of these compounds. Yeasts and bacteria with lignocellulosic hydrolysate fermentation capabilities are currently subject to intense research activity[74].

The inability of *S. cerevisiae* to metabolise resultant C5 (pentose) sugars in lignocellulose hydrolysates represents a significant microbiological challenge. If this premier industrial microorganism could be engineered to ferment xylose (the predominant pentose in hydrolysates) then this would provide distinct industrial benefits in the production of second generation fuel alcohol. Although some non-*Saccharomyces* yeasts (e.g., *Candida shehatae var. lignosa, C. tenuis, Cryptococcus albidus, Kluyveromyces marxianus, Pachysolen tannophilus* and *Pichia stipitis*) are able to ferment xylose (via the pathway outlined in Fig. 9), they do so inefficiently. Such yeasts cannot convert xylose to ethanol under anaerobic conditions[106] and are also regarded as being not very alcohol tolerant for use in bioethanol production.

Genetic manipulation strategies with bioethanol microbes aim to:

- expand metabolic pathways
- alleviate metabolic blocks
- circumvent sugar transport limitations (e.g., glucose repression, new sugar transport permeases)
- overcome lignocellulosic hydrolysate toxicity, and
- reduce recycling of process water in fermentation make up (high gravity fermentations).

Table XV. Some engineered bacteria with thermophilic, cellulolytic and ethanologenic characteristics.

Geobacillus thermoglucosidasius
Thermoanaerobacterium saccharolyticum
Thermoanaerobacter mathranii
Clostridium thermocellum
Clostridium thermohydrosulfuricum

Various approaches have been adopted to overcome the yeast xylose-fermentation dilemma, including: co-fermentations with C6 and C5-fermenting yeast species (e.g., *S. cerevisiae* + *P. stipitis*); metabolic engineering of *S. cerevisiae* to enable it to ferment xylose; use of genetically engineered bacteria (e.g., *E. coli, Zymononas, Klebsiella oxytoca, Thermoanaerobacterium, Geobacillus* (with xylose-utilising genes); immobilisation of xylose isomerase with *S. cerevisiae*.

Regarding recombinant DNA approaches to construct strains of *S. cerevisiae* able to ferment pentose sugars, successful cloning of xylose isomerase genes from the following organisms into this yeast has been achieved:

- fungi (e.g., *Piromyces* – a fungus isolated from elephant dung!)
- bacteria (e.g., *Clostridium phytofermentans*)

The expression of xylose isomerase genes, rather than xylose reductase and xylitol dehydrogenase avoids accumulation of xylitol and an imbalance of the co-factors NADP and NAD. Further information on this genetic engineering approach is available from references [18,21,47,59,120,134]. The yields of ethanol from xylose by GM strains of *S. cerevisiae* have been reported at 0.43 g/g, with maximum ethanol concentrations achieved at 46.5 g/L[101]. Further research is ongoing to improve ethanol productivities from pentose sugars by recombinant yeasts.

Bacteria have also been engineered to ferment lignocellulose hydrolysates, some at high temperature (Table XV and [61,67,115]). For example, a recombinant a *Geobacillus* spp. has been developed (see www.tmo-group.com) to ferment straw hydrolysate at 70°C. Although such thermophilic bacteria possess some key advantages over yeast-based processes, compared with yeasts, these bacteria are not particularly ethanol tolerant.

For both yeast and bacterial processes, significant technological challenges remain for commercial lignocellulose-to-bioethanol processes. For example, the presence of toxic chemicals in hydrolysates (see Fig. 10) can seriously inhibit fermentative microbial activity[84].

Pretreatment and hydrolysis of woody wastes, corn cobs/stover, switchgrass, spent grains, paper waste, municipal solid waste etc. all produce cocktails of inhibitory chemicals that act to suppress the activities of yeast (and bacteria) in converting hydrolysate sugars to ethanol. Various approaches have therefore been adopted to alleviate the deleterious effects of these inhibitors[98,126–128,136] For example, these can be reduced using steam stripping, nanofiltration membranes, supercritical fluid extraction, or polymeric adsorbent materials (e.g., amberlite resins).

Bioethanol fermentation systems

Industrial bioethanol fermentation processes may adopt batch, continuous, semi-continuous or (potentially) immobilised systems. For sugar-based bioethanol production

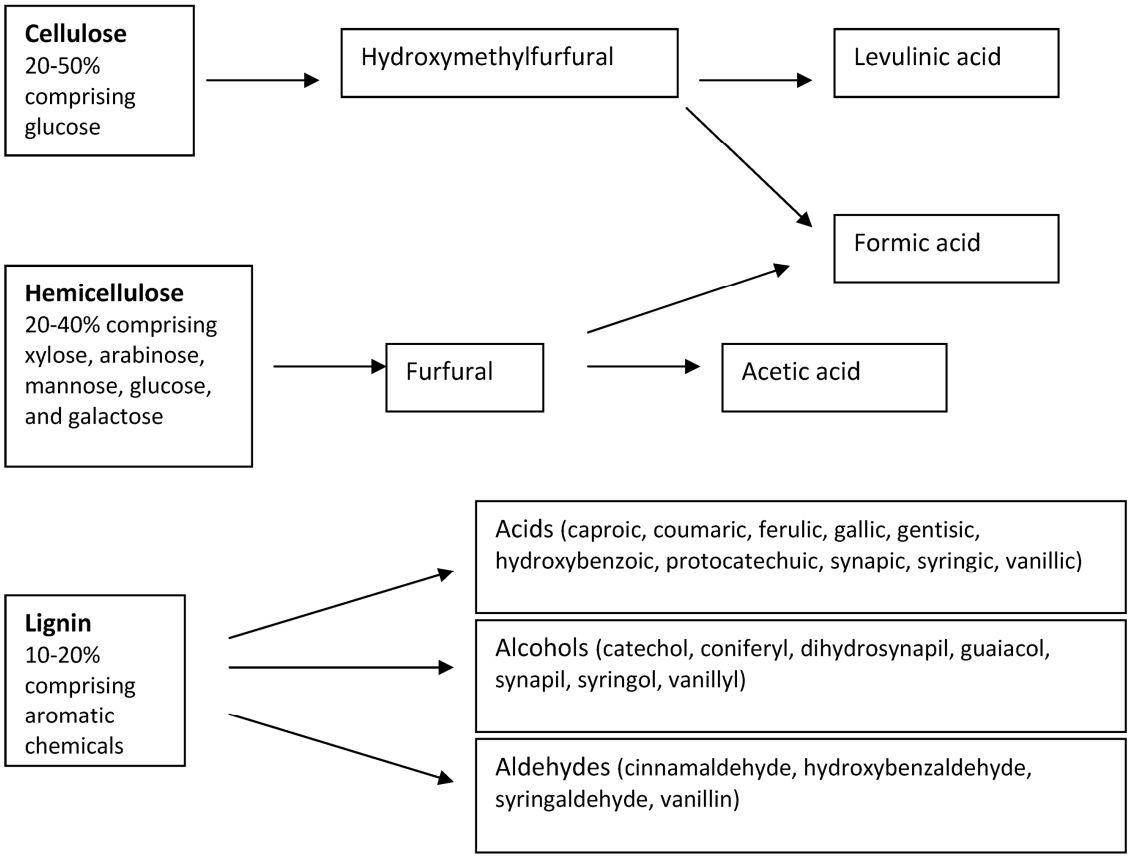

Fig. 10. Derivation of chemical inhibitors from lignocellulose components.

processes, two basic fermentation systems are employed[8,15,72]:

1. Fed-batch addition of substrate with yeast propagation
2. Fed-batch addition of substrate with yeast recycle

In the first system, fermenters are pitched with freshly-grown yeast followed by controlled addition of sugar substrate. The second system, which is employed in many Brazilian distilleries, uses a semi-continuous (modified Melle-Boinot) fermentation process[15,129]. This system uses very high yeast densities and produces alcohol concentrations (6–11% (v/v)) in very short fermentation times (6 to 10 h). After the end of each fermentation cycle, the yeast cells are recycled following centrifugation and treated with diluted sulphuric acid (pH 2.2 to kill contaminant bacteria). The fermented "beer" is distilled and subsequently dehydrated to produce anhydrous bioethanol for use as an internal combustion engine biofuel (see Section 4).

The behaviour of yeasts in Brazilian fuel alcohol plants employing yeast recycling has been discussed by Basso et al.[15] It appears that distillery-resident yeast strains in such systems exhibit higher stress tolerances compared with cultured strains and these indigenous yeasts have potential as selected starter cultures for bioethanol processes.

Regarding fermentation systems for lignocellulose-to-ethanol operations, the following approaches can be employed, depending on the nature of the feedstock: simultaneous saccharification and fermentation (SSF)[82], simultaneous saccharification and co-fermentation (SSCF), separate hydrolysis and fermentation (SHF), and direct microbial conversion (DMC). These processes all require the hydrolysis of pre-treated biomass (with cellulase and hemicellulase enzymes or microbes); and fermentation of resultant hexose (glucose, mannose, galactose) and pentose (xylose, arabinose) sugars[66,67,98,112]. Fermenters may be operated in batch, fed-batch, batch fill-and-draw or continuous operation modes. Figure 11 outlines SSF and SHF processes. Regarding DMC approaches, these may en-

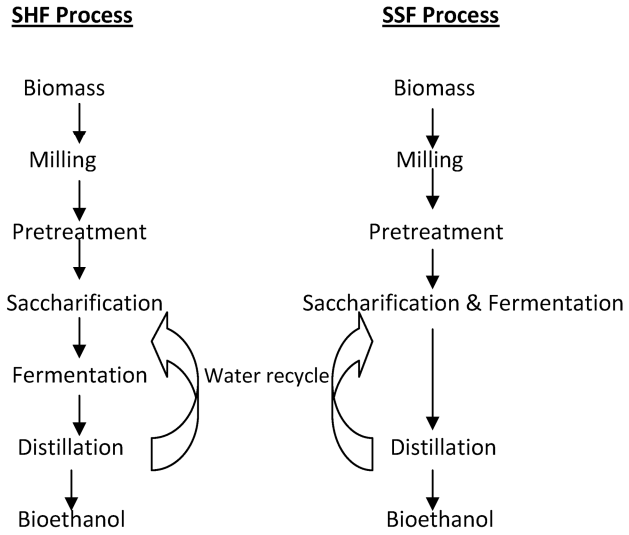

Fig. 11. SHF and SSF processes for conversion of lignocellulosic biomass to bioethanol.

compass cellulose hydrolysis and fermentation in a single integrated step without the need for a production stage for cellulolytic enzymes. This has been termed consolidated bioprocessing (CBP – see Lynd et al.[66,67]).

Irrespective of the system employed, it is important to monitor (and control) the following parameters during fermentation: yeast cell density, sugar consumption, pH, temperature, and alcohol production. Of particular importance are calculations of ethanol yield and conversion efficiencies (of sugar to ethanol). Table XVI provides some data on ethanol yields from selected second-generation feedstocks and developments in industrial-scale lignocellulosic bioethanol production and the uses of recombinant yeasts have been discussed[22,123] (http://biofuels.abc-energy.at/demoplants).

DISTILLATION AND FUEL ALCOHOL FORMULATIONS

Anhydrous ethanol methods

Fuel ethanol needs to be almost completely dry (anhydrous) because even small amounts of water can lead to poor vehicle performance and potentially engine damage. Anhydrous bioethanol can also be used for the production of other fuel additives, such as the high-octane gasoline component bio-ETBE (a mix of ethanol and isobutylene). Standard distillation practices only produce around 95% (v/v) ethanol due to the formation of constant boiling ethanol-water azeotropes. Therefore, additional approaches are needed to completely dehydrate ethanol destined for blending with petrol and use as a transportation biofuel (see Table XVII). Molecular sieve desiccants are commonly employed in bioethanol distilleries for this purpose[113]. "Sieving" is accomplished by synthetic aluminium silicate zeolite resins with pore sizes small enough (0.3 nm) that permit water molecules (0.28 nm diameter) to penetrate, but not ethanol molecules (0.44 nm diameter).

Fermented wash does not solely comprise ethanol due to numerous yeast secondary fermentation metabolites (congeners) that are also distilled. Those of low volatility include higher alcohols or fusel oils and fatty acids (e.g., propionic, isobutyric, isovaleric, hexanoic, octanoic). Fusel oil constituents (percentage by weight: iso-amyl alcohol 87.3%; iso-butyl alcohol 0.7%; and n-propanol 0.3%) are separated to recover ethanol from the water-alcohol stream. For bioethanol plants (e.g., those processing wheat), separated fusel oils can be blended back into the alcohol vapour and incorporated into the final biofuel (due to their combustibility).

Fuel alcohol formulations, denaturation requirements

Various petrol (gasoline)-ethanol blends are used as fuel for internal combustion engines, and Table XVIII summarises some blends used in different countries together with relative energy contents compared with fossil fuels. To ensure ethanol for fuel use is unfit for human consumption it is "denatured" by supplementation with hydrocarbon denaturants, which include petrol, diethyl phthalate and isopropanol[68].

In Brazil, where bioethanol currently accounts for ~50% of the transport fuel market, petrol-ethanol blends are mandatory (E20 to E25). In addition, more than 20% of cars (and some light aircraft) in Brazil use E100

Table XVI. Ethanol yields from selected second-generation materials[a].

Biomass	Ethanol yield (litres/dry metric ton)
Hardwood	350
Softwood	420
Corn stover	275–300
Wheat straw	250–300
Sugar cane bagasse	314
Municipal solid waste	170–486

[a] Figures are estimated yields from the hexose fraction, which theoretically is represented as: $(C_6H_5O_5)n$ (cellulose) + nH_20 (water) → $nC_6H_{12}O_6$ (glucose) → $2nCH_3CH_2OH$ (ethanol) + $2nCO_2$ (carbon dioxide). In practice, such conversions are inefficient and improving the overall cellulose-to-ethanol process remains a technological challenge. More information from[100,104] and www.bioenergy.novozymes.com; www.dialogue4s.de/_media/Prince_Bioethanol_Preparation_from_Organic_Waste_Residues.pdf

Table XVIII. Some international petrol-ethanol blends, with energy contents. (E = ethanol and number represents % in petrol)

Country	Blend	Energy content (MJ/L)
USA	E10[a]	33.7
	E70–E85	25.2 (for E85)
Brazil	E25–E75	
	E100	23.5
Europe	E5	
	E85	
Global	Petrol (regular gasoline, no ethanol)	34.8
Global	Aviation fuel (no ethanol)	33.5
Global	Diesel (no ethanol)	38.6

[a] In October 2010, the US Environment Protection Agency (EPA) increased the level of ethanol blended in petrol to 15% (i.e., E15) for cars built from 2007.

Table XVII. Dehydration of ethanol for fuel use.

Method	Description & comments
Azeotropic distillation	Addition of a solvent (e.g., benzene, cyclohexane or monoethylene glycol) to break the ethanol-water azeotrope. When the additive is more volatile than water, separation is called azeotropic distillation, and when it is less volatile than water, it is called extractive distillation. Now seldom used due to solvent carcinogenicity/toxicity.
Molecular sieves	Examples include zeolite resins ("molsieves"), and synthetic zeolites (based on aluminium silicates) that act as desiccants to selectively adsorb water from aqueous ethanol streams[19].
Vacuum distillation	Anhydrous ethanol obtained under pressures of 10 kPa.
Membrane pervaporation	The use of membranes to recover ethanol by "pervaporation" (ethanol removal by vacuum applied at the permeate side of a membrane) conserves energy by abolishing energy-expensive distillation. It is possible to concentrate ethanol from 80 to 99.5% by pervaporation.[87] It can also reduce yeast ethanol (and inhibitor) toxicity problems if applied during fermentation.
Miscellaneous	e.g., Liquid extraction, supercritical fluid extraction, intermediate heat pumps and optimal sidestream return (IHOSR) technique using an inorganic salt (potassium acetate) as entrainer[102].

(anhydrous ethanol) as fuel, and there are around 6 million *flex-fuel* vehicles which are able to run with either neat ethanol, neat gasoline, or any mixture of both. In the US, bioethanol is blended in more that 80% of motor fuels, and the "blend wall" has recently been increased from 10 to 15% (i.e E10 to E15).

Table XIX shows the ASTM (American Society for Testing and Materials International, see www.astm.org) analytical specifications for bioethanol transportation fuel performance quality.[29] The specifications for denatured fuel ethanol are regulated in the US by the Alcohol and Tobacco Tax and Trade Bureau (TTB). All formulations should be clear and bright and visibly free of suspended or precipitated matter.

FUTURE CHALLENGES FOR BIOETHANOL

Emerging trends in bioethanol production

Renewable fuels have been forecast to account for 8.5% of global energy use by 2030 with bioethanol predicted to replace around 20% of gasoline usage by that year.[125] For developing countries, biofuels in general offer new economic opportunities in terms of lessening dependence on energy imports. However, feedstocks for bioethanol production must be sustainable and must not threaten biodiversity or food security. First generation feedstocks, particularly cereal crops, have somewhat limited roles in decarbonising our energy needs and reducing greenhouse gas emissions. Of course, such technologies may also impact negatively on food prices (International Energy Agency, www.iea.org and Sims et al.[105]). Only certain sugar cane processes (especially in Brazil) may be regarded as environmentally sustainable and socially acceptable for long-term first generation bioethanol production. Therefore, although significant technological challenges remain, the future for bioethanol lies in exploiting second-generation (non-food) substrates for bioethanol production, mainly those based on lignocellulosic biowastes generated from agriculture, industry and forestry activities[13,76,85,86,91,95,97,108].

If 20% of gasoline is to be displaced by ethanol by 2030, this will necessitate significantly increased production of bioethanol from lignocellulosic materials. By integrating both first and second generation ethanol technologies, existing bioethanol facilities that currently use cereal starch or sugar crops can be adapted to *biorefineries* that process the entire biomass (including lignocellulosic residues) to biofuels and other industrial commodities[43,45,91,94]. For example, if sugar cane bagasse-to-ethanol conversion technologies became fully industrialised, Brazil could potentially produce up to 750 billion litres of bioethanol, representing a substantial proportion of global transportation fuels[108]. In the US, 136 billion litres of biofuels mandated by 2022 could be met by cultivating energy crops (e.g., *Miscanthus* spp.) on marginal land (see also Fig. 12). However, to replace all US transportation fuels with ethanol, an estimated 800 billion litres would be required[2]. This clearly cannot be met by growing first generation feedstocks such as maize as this would require 500 million acres of cultivable land (current area is 473 million acres).

In Europe, strategic research agenda for deployment of sustainable biofuels are being drawn up under the auspices of the EBTP (European Biofuels Technology Platform, see www.biofuelstp.eu), and recent EU renewable energy directives (e.g., Energy Directive 2009/28/EC) have specifically stipulated the usage of non-food cellulosic and lignocellulosic material for future bioethanol production.

Technological challenges

It is clear from the above discussion that the future for fuel alcohol lies with lignocellulosic biomass. Nevertheless, there are significant scientific and technological challenges facing second generation bioethanol production (see Table XX). In addition to such challenges, Walker[123] has discussed important geo-political and ethical chal-

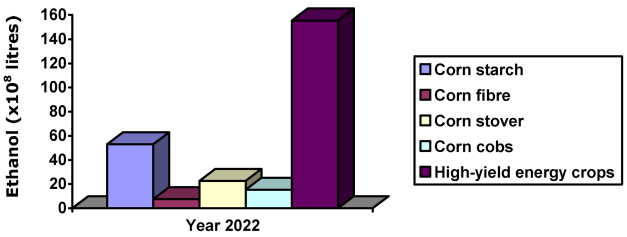

Fig. 12. Bioethanol in the USA – year 2022 projections. See online version for colour figure (From Charles Abbas, *personal communication*).

Table XIX. ASTM quality parameter specifications (2007) for bioethanol.

Quality parameter	Limits for denatured fuel ethanol	Limits for E85
Ethanol, % (v/v) min	92.1	74[a]
Methanol, % (v/v) max	0.5	0.5
Water, % (v/v) max	1.0	1.0
Acidity (as acetic acid), mass% (mg/L) max	0.007 (56)	0.005 (40)
pHe	6.5–9.0	6.5–9.0
Copper, mg/kg max	0.1	0.07
Inorganic chloride, mass ppm (mg/L) max	40 (32)	1 (mg/kg)
Solvent-washed gum, mg/L max	5.0	5.0
Sulphur, mass ppm max	30	
Sulphate, mass ppm max	4	
Denaturant, % (v/v)	1.96 (min); 5.0 (max)	
Hydrocarbon/aliphatic ether, % (v/v)		17–26

[a] Plus higher alcohols.

Table XXI. Unconventional biomass sources with potential for bioethanol production.

Biomass source	Comments
Triticale	A hybrid of wheat (*Triticum*) and rye (*Secale*).
Sorghum bicolour	For some developing countries, sorghum has distinct advantages over sugar cane for bioethanol production[78].
"Sugarcorn"	This is a hybrid cross between sugar cane and maize under development (www.ethanolproducer.com, January 2009).
Jerusalem artichoke	Polyfructan (inulin-rich) root crops can be grown in nutrient-poor soils. Hydrolysed by inulinases to fermentable fructose, or directly fermented by certain yeasts (e.g., *Kluyveromyces marxianus*).
Cheese whey	Whey residues from cheese making comprise around 5% (w/v) lactose (disaccharide of glucose and galactose) that can be directly fermented by certain yeasts (e.g., *Kluyveromyces marxianus*).
Marine macroalgae	Macroalgal seaweeds grow much quicker than terrestrial plants. Brown seaweeds (*Phaeophyta*) such as *Laminaria* and *Macrocyctis* spp., contain high amounts of carbohydrates such as alginic acid (structural) and laminarin and mannitol (storage) that can potentially be fermented to ethanol (see [3,48,49]; www.ba-lab.com). Compared with conventional (first-generation) feedstocks, macroalgae (third-generation[40]) have greater potential ethanol productivities (see Table XXII).
Chitin	The exoskeletons of crabs, lobsters and shrimps are comprised of chitin, a polymer of N-acetyl glucosamine that resembles cellulose in structure and has potential for bioconversion into chemical commodities, including ethanol.
Glycerol	Glycerol is a major co-product of biodiesel production which has the potential to be converted to ethanol by certain bacteria and yeasts (e.g., *Candida magnoliae, Zygosaccharomyces rouxii* and *Pachysolen tannophilus* - see[32,133]; www.glyfinery.net).
Municipal solid waste (MSW)	MSW (comprising various combinations of paper/cardboard, kitchen and vegetation organic waste) is a very low-cost feedstock sources for cellulosic bioethanol production[62]; www.biofuelstp.eu/spm2/pdfs/poster_PERSEO.pdf; www.biofuelstp.eu/bioethanol). From one ton of MSW, 320 litres of ethanol can be produced and Shi et al.[104] have reported that >80 billion litres of MSW paper-derived bioethanol can be produced worldwide (this would replace over 5% of global gasoline consumption).

Table XXII. Potential productivities of first generation and third generation bioethanol feedstocks (adapted from Adams et al.[3])

Parameter	Wheat	Maize	Sugar beet	Sugar cane	Macroalgae
Annual yield, average (kg/ha/year)	2,800	4,815	47,070	68,260	730,000
Carbohydrate (kg/ha/year)	1,560	3,100	8,825	11,600	40,150
Potential ethanol (L/ha/year)	1,010	2,010	5,150	6,756	23,400

lenges facing future bioethanol production that remain to be overcome.

Regarding improvement of yeast strains for lignocellulose hydrolysate fermentations, major advances in *S. cerevisiae* metabolic engineering have been made in recent years. For example, the following characteristics have been conferred on *S. cerevisiae* for bioethanol production: expression of cellulolytic activity; expression of xylose (and arabinose) fermenting enzymes; reduction of glycerol, xylitol and arabitol biosynthesis; tolerance of chemical inhibitors and reduced glucose repression[5,69,80]. Research is ongoing to develop robust GM yeasts that will be able to survive the rigours of large-scale lignocellulose fermentations.

Future challenges in bioethanol technologies also centre on bioconversions of feedstocks other than conventional first and second generation biomass sources. Some of these are outlined in Tables XXI and XXII.

Third-generation bioethanol refers to fuel alcohol produced from non-terrestrial feedstocks such as macroalgae, particularly the giant brown seaweeds (e.g., kelp). The growth rate of these marine plants far exceeds that of terrestrial plants and macroalgal cultivation does not encroach on land required for food crops. Another primary advantage is that macroalgae only need seawater, sunlight and carbon dioxide for their growth[40]. They also have much greater ethanol production potential compared with more conventional (e.g., first-generation) bioethanol feedstocks[3].

Finally, although this review has focused on bioethanol production, primarily from yeast fermentations, it should be mentioned that recent research has also shown potential for *S. cerevisiae* to produce other types of biofuel. For example, n-biobutanol and isobutanol[109] can be produced by GM *S. cerevisiae* that express solventogenic *Clostridium* spp. genes (see also Gevo Inc – www.gevo.com/; Butalco – www.butalco.com). Butanol (a C4 alcohol) exhibits several advantages over ethanol as a fuel, including better combustibility, amenability to storage and transportation and miscibility with diesel. Several companies are focusing efforts to commercialise ethanol and/or butanol production, specifically from cellulosic feedstocks[93]. *S. cerevisiae* can also be engineered to produce hydrocarbons (e.g., farnesene) with potential to be used as "biodiesel" (e.g., see www.amyris.com).

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