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Review of Second Generation Bioethanol Production from Residual Biomass

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SUMMARY

In the context of climate change and the depletion of fossil fuels, there is a great need for alternatives to petroleum in the transport sector. This review provides an overview of the production of second generation bioethanol, which is distinguished from the first generation and subsequent generations of biofuels by its use of lignocellulosic biomass as raw material. The structural components of the lignocellulosic biomass such as cellulose, hemicellulose and lignin, are presented along with technological unit steps including pretreatment, enzymatic hydrolysis, fermentation, distillation and dehydration. The purpose of the pretreatment step is to increase the surface area of carbohydrate available for enzymatic saccharification, while minimizing the content of inhibitors. Performing the enzymatic hydrolysis releases fermentable sugars, which are converted by microbial catalysts into ethanol. The hydrolysates obtained after the pretreatment and enzymatic hydrolysis contain a wide spectrum of sugars, predominantly glucose and xylose. Genetically engineered microorganisms are therefore needed to carry out co-fermentation. The excess of harmful inhibitors in the hydrolysate, such as weak organic acids, furan derivatives and phenol components, can be removed by detoxification before fermentation. Effective saccharification further requires using exogenous hemicellulases and cellulolytic enzymes. Conventional species of distiller's yeast are unable to ferment pentoses into ethanol, and only a very few natural microorganisms, including yeast species like *Candida shehatae*, *Pichia* (*Scheffersomyces*) *stipitis,* and *Pachysolen tannophilus*, metabolize xylose to ethanol. Enzymatic hydrolysis and fermentation can be performed in a number of ways: by separate saccharification and fermentation, simultaneous saccharification and fermentation or consolidated bioprocessing. Pentose-fermenting microorganisms can be obtained through genetic engineering, by introducing xylose-encoding genes into metabolism of a selected microorganism to optimize its use of xylose accumulated in the hydrolysate.

Key words: second generation bioethanol, biofuel, lignocellulosic biomass, biomass pretreatment, enzymatic hydrolysis, co-fermentation

INTRODUCTION

Chief among the many challenges facing the modern world are the interconnected issues of global warming, reliance on fossil fuels, and food and energy security. Population growth and increasing industrial development lead to greater demand for energy, but conventional fossil fuels, including petroleum, are a both a finite resources and emit greenhouse gasses (GHG) when combusted. Sustainable and environmentally friendly energy sources are required in order to meet the world's future energy needs (*1*,*2*). Biofuels, namely cellulosic bioethanol, butanol and biodiesel, are therefore of considerable interest to researchers, industrial partners and governments (*3*,*4*). In particular, bioethanol is considered a promising drop-in fuel, which could provide an alternative to petrol in the transport sector.

The use of ethanol in gasoline in 2016 reduced CO_2-equivalent GHG emissions from transportation by 43.5 million metric tonnes — the equivalent of removing 9.3 million

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cars from the road for an entire year (*5*). Use of bioethanol further decreases reliance on crude petroleum, which is usually imported from overseas, increasing energy security and diversifying energy supplies. It can also help to increase employment and stimulate the economy in rural areas (*6*,*7*). Among the first bioethanol-producing countries were Brazil and the United States. In the US, where corn starch is the main raw material used, 6.4 billion L of bioethanol were produced in 1998 (*8*). By 2007, output had risen almost fourfold to 24.71 billion L, by 2010 it had more than doubled to 50.41 billion L. In 2013, production decreased slightly to 50.37 billion L, but in 2016 it climbed again to 57.8 billion L. Currently, there are more than 200 biorefineries in the US, with the combined capacity of producing approx. 60.64 billion L of ethanol per year (*5*). In Brazil, where sugarcane juice (specifically sucrose) is the main raw material used, production reached around 13.5 billion L in 1998 (*8*). In Europe, Sweden, Germany, the UK, France and Italy are involved in bioethanol production. Collectively, European Union countries produce annually more than 2 billion L of bioethanol.

This review provides a summary of the process for converting recalcitrant, lignocellulosic biomass components (cellulose, hemicellulose and lignin) into renewable second generation liquid bioethanol. It examines each step individually, including pretreatment of the lignocellulosic feedstock, enzymatic hydrolysis/saccharification to produce fermentable sugars, fermentation and distillation steps and finally dehydration, which yields anhydrous, extremely pure bioethanol. This paper also discusses the biocatalysts used in the process of alcoholic fermentation, as well as the development of fermentative microorganisms with the required properties for effective industrial production of bioethanol (*i.e.* ability to effectively utilize pentoses and hexoses in co-fermentation, tolerance towards inhibitory compounds in the hydrolysate and tolerance towards high ethanol concentrations). The importance of using genetic engineering techniques to optimize bioethanol yield (by introducing pathways for the fermentation of pentoses by selected strains) is emphasized.

BIOETHANOL GENERATIONS

First generation bioethanol

First generation bioethanol is a liquid biofuel designed for road vehicles, generated from food crops with high levels of starch and sugar (*9*). Both starchy and lignocellulosic materials require hydrolysis, because complex carbohydrates are not broken down by *Saccharomyces cerevisiae* (*10*). Production of first generation bioethanol uses food feedstock, mainly starchy materials (*e.g.* corn, maize, wheat, barley, cassava, potato) and sucrose-containing feedstock (*e.g.* sugarcane, sugar beet, sweet sorghum). This has led to serious concerns regarding the socio-economic and environmental consequences of large-scale production (*11*). First generation biofuel production competes with food production for water and arable land, and may also contribute to resource depletion such as water shortages, and soil and water degradation due to over-fertilization (*12*). It may push up the price of food commodities such as cereals, crops and vegetable oils and livestock feed. Moreover, the GHG emissions from biofuels are reduced compared to those released from the combustion of fossil fuels (*13*). Apart from food-based bioethanol, another first generation biofuel is biodiesel, which is produced from vegetable oils through transesterification (*9*).

Second generation bioethanol

Generally, second and subsequent generations of biofuels including bioethanol do not compete against food supplies as they are based on non-food raw material (*14*). Second generation bioethanol is typically produced from lignocellulosic biomass, but it is also possible to use industrial byproducts, such as whey (*10*) or crude glycerol, as feedstock. Such biomass is usually relatively inexpensive as well as readily and locally available (*15*). Lignocellulose is considered a renewable and sustainable carbon source, and occurs in many plant raw materials (*16*). The amount of available lignocellulosic biomass depends on climatic conditions. The conversion of lignocellulose into reducing sugars is more difficult than the conversion of starch. Various types of plant biomass have been considered by researchers for use in the production of biofuels (**Fig. 1**). These include dedicated energy crops which grow on low-quality soil (*e.g.* herbaceous crops and perennial grasses such as *Miscanthus sinensis* and *M. giganteus* (*17*) or switchgrass (*18*). Agricultural wastes, such as cereal straw (stover (*18*), wheat straw (*19*), corn cob (*20*,*21*), rice husk (*22*)) and bagasse from processing sugar cane (*23*) have also been examined as potential sources of lignocellulosic biomass. Other research has focused on forest-based woody wastes and forest biomass (bark (*24*), sawdust (*25*), softwood trimmings (pine) (*26*) and hardwood chips (oak) (*27*)), or on waste from parks and gardens (leaves (*21*), grasses (*28*), branches (*29*)). Industrial wastes, such as brewer's spent grains (*30*) and spent grains from distilleries (*31*), and municipal solid wastes such as food waste, kraft paper and paper sludge containing cellulose (*32*) have also been considered.

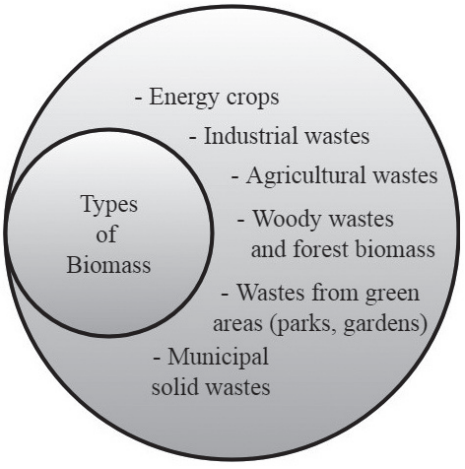

Fig. 1. Types of biomass. Data taken from Sims *et al.* (*7*)

Because of its high organic load, the whey obtained as a byproduct of the cheese industry is toxic to the environment and requires treatment before removal as waste. The use of whey as a substrate for the production of biomediated ethanol can reduce the costs associated with the treatment of effluent in dairies (*33*). Crude glycerol, which is generated during the transesterification of animal fats and vegetable oils, is a significant byproduct of the biodiesel industry. The fermentation of crude glycerol obtained from waste enables this surplus to be reduced. Fermentation of glycerol is performed by conversion to phosphoenolpyruvate (PEP) or pyruvate, leading to increased content of reducing equivalents and higher bioethanol yield than the fermentation of glucose and xylose from biomass (*34*). Lignocellulosic bioethanol generates lower levels of greenhouse gases than first generation bioethanol and causes less air pollution (*35*). However, the production of lignocellulosic bioethanol requires feedstock preparation prior to fermentation and the finding/developing of ethanol producers able to ferment sugars from cellulose and hemicellulose breakdown.

Third generation bioethanol

The third generation of biofuels is based on the cultivation of microalgae or unicellular microorganisms derived from eukaryotes and prokaryotes (cyanobacteria, such as *Cyanidium caldarium* or *Synechococcus*) (*36*). Live biocatalysts in the form of active microalgal biomass are able to use nutrients (carbon, nitrogen, phosphate or sulfur) from industrial waste streams as substrates to create high concentrations of biomass. These waste streams include effluent gases from industrial power plants, wastewater, products of hydrolysis of organic waste and digestate (waste from biogas production). Producing third generation biofuels can therefore help minimize waste streams from many industries. Biological sequestering of CO_2 from the combustion of fossil resources by microalgae and conversion of CO_2 to biofuels contributes to the reduction of levels of GHGs in the atmosphere, helping to meet global targets for preventing climate change (*36*). Some strains of microalgae, such as *Chlorella*, *Nanochloropsis* or *Botryococcus*, are capable of producing biofuels (*36*). The microalgal strain *Botryococcus braunii* produces monounsaturated and polyunsaturated hydrocarbons that can be converted into gasoline-like fuels *via* cracking (*36*). As a result of the assimilation of carbon from CO_2, certain strains of microorganisms are capable of intracellular production of high-value carbohydrates, pigments or lipids (such as polyunsaturated fatty acids; PUFA), which are then separated from the cells of the residual algal biomass (*36*). Alternative feedstocks such as algae have high lipid and carbohydrate contents. The oils obtained from microalgae offer an alternative raw material to the vegetable oils used conventionally for the production of biodiesel *via* alkaline transesterification with methanol (*34*). The production of biodiesel from microalgae requires optimization of technologies, including improving the extraction of oil from the cellular biomass. Residual biomass from microalgae can be converted to valuable ecological and sustainable energy carriers (biofuels), such as bioethanol and biogas, or used for the fermentation of methane or biohydrogen production (*36*). Bioethanol from algal biomass is obtained from the fermentation of starch and starch-like polysaccharides under anaerobic conditions. Starch is a reserve material in species of microalgae including *Chlorophyta* and *Cryptophyta* (*36*). The production of bioethanol from biomass gives low yields, despite the complexity of the process. Biohydrogen is a byproduct obtained during the production of methane from organic acids, which are formed in the acidogenic phase from the transformation of organic wastes under conditions of anaerobic digestion (*36*). However, the amount of biohydrogen that can be produced from algae is still low, so the process of obtaining biohydrogen requires improvement (*36*). Cultivation of algae is unproblematic and can take place in a wide variety of water environments (*37*).

ETHANOL PRODUCTION

Composition of lignocellulosic feedstock for bioethanol production

Lignocellulosic biomass is a promising substrate for bioethanol production, as it is unlikely to become depleted or suffer permanent damage (*15*). The composition of lignocellulosic material depends on its species, variety, growth conditions and maturity (*16*,*38*). Ethanol yield and conversion productivity depend on the type of biomass, requiring a high content of cellulose and hemicellulose and low lignin content (*38*). Other factors which affect ethanol yield include the development of efficient technologies and the selection of appropriate or potential recombinant or non-recombinant microorganisms (*6*). It is widely believed that the structure of lignocellulose is resistant to degradation due to its compositional heterogeneity, consisting of cellulose, hemicellulose and lignin (*39*). The elements of plant cell walls are connected strongly through covalent and hydrogen bonds. These bonds make lignocellulosic material resistant to different methods of pretreatment (*40*). Cellulose with hemicellulose forms a holocellulose, which comprises more than half of the entire dry biomass (*41*).

Cellulose

Cellulose is surrounded by lignin. In terms of chemical structure, it is a β-glucan linear polymer of D-glucose linked by β-1,4-glycosidic bonds. The cellulose structure is difficult to break without enzymatic hydrolysis, due to its crystalline character (*42*). The linear cellulosic chain is made up of 500-14 000 D-glucose units. Around 36 hydrogen-bonded glucan chains form insoluble microfibrils in secondary cell wall (*43*). Conditions of high temperature and pressure, at 320 °C and 25 MPa respectively, are needed to turn this rigid crystalline structure into an amorphous structure in water. These requirements are higher than for starchy raw materials (liquefaction step: 95-105 °C and pH=6.0–6.5, following saccharification step: temperatures of 60–65 °C and the pH adjusted to 4.0–4.5) (*15*,*44*).

Hemicellulose

Hemicellulose is made up of different sugar units. Hemicellulose is a heteropolymer of short, branched chain sugars. Apart from monosaccharides, there are sugar acids called uronic acids in the hemicellulose fraction (*45*). Typical sugar acids in the hemicellulose structure include D-glucuronic, 4-O-methylglucuronic and D-galacturonic acids (*46*). All monosaccharides in hemicelluloses are classified into pentoses (D-xylose and L-arabinose) and hexoses (D-mannose, D-galactose, D-glucose) (*47*). Meaningful quantities of L-arabinose are contained in corn fibre and specific herbaceous crops (*48*). C5 sugars such as xylose and arabinose are released from xyloglucan, xylan, arabinan and arabinogalactan (substructures of pectin), which are components of polysaccharides in the plant cell wall (*49*). Xylan is the largest hemicellulose component (*10*). The content of hemicellulose in hardwood is 35 % and in softwood 28 % (*10*).

Lignin

Lignin is not a desirable component in plant cell walls, as it is particularly difficult to biodegrade. Its recalcitrant character makes this three-dimensional polymer molecule a physical obstacle to the action of enzymes. It is the most common aromatic polymer, and is considered the 'glue' that holds plants together (*6*). Its structure is formed by phenolic and non-phenolic compounds (*50*). Lignin consists of phenylpropanoid units and is considered a heterogeneous polimer (*51*). It may be used directly through combustion, to supply electricity and heat to biorefineries (*52*). Significant amounts of this biopolymer are obtained from the commercial production of lignocellulosic ethanol (around 62 million tonnes), which is why new uses are being sought, such as the transformation of lignin into higher value compounds – also known as the valorization of lignin. Being a renewable feedstock, lignin can be used as a substrate for the production of commodity chemicals, replacement fuels, polymeric foams, thermoplastic elastomers, engineered plastics and lignin-based carbon fibres (such as for the production of lightweight and energy-saving vehicles made of inexpensive carbon fibre composites (*53*). However, lignin carbon fibres exhibit poor mechanical properties due to their porosity (*53*). For the lignin valorization to be effective, efficient extraction of lignin at the pretreatment stage is crucial (extraction can be improved by genetic engineering of the lignocellulosic material to reduce cross-linking with other biopolymers), as is its structure (structural carbon order, and monomer ratio) (*53*).

Conversion of biomass into ethanol

There are two processing routes by which lignocellulosic biomass can be converted into second generation ethanol and biofuels: thermochemical and biochemical. The thermochemical process converts biomass into an intermediate gas (synthesis gas) or liquid using non-biological catalysts (*e.g.* heat) in a reactor. The intermediate product is transformed into fuel options (methanol, lignocellulosic ethanol, other higher alcohols, hydrogen and synthetic diesel/Fischer-Tropsch (FT) diesel or aviation fuel) (*54*). Biological catalysts/microorganisms are not required in this process. The thermochemical process allows the conversion of any carbon material (*i.e.* lignocellulosic feedstock) into valuable products, including ethanol (*15*,*54*). In the thermochemical approach, it is possible to obtain ethanol and other biofuels through gasification or pyrolysis. Ethanol is formed *via* FT conversion. During gasification, biomass undergoes full depolymerization at temperatures between 800 and 1000 °C at 2 to 3 MPa with limited oxygen access, producing intermediate syngas (a synthesis gas) (*54*). Syngas is a mixture of carbon monoxide (CO), hydrogen (H_2) and other hydrocarbons. Pyrolysis occurs at lower temperatures than gasification (400-650 °C) and under zero oxygen conditions. The effect of pyrolysis is the depolymerization of biomass into liquid intermediates such as pyrolysis oil or biooil (*7*,*54*,*55*).

Biochemical conversion is a common technique for producing bioethanol, because of the high selectivity and efficiency of biomass conversion (*7*,*15*). The biochemical method involves pretreatment of lignocellulosic material, enzymatic hydrolysis, fermentation of sugars by specific strains of microorganisms and distillation of bioethanol with dehydration (**Fig. 2**) (*56*). In the biochemical route, biomass is subjected to biological, physical (heat) or chemical catalysts during pretreatment. Additionally, biocatalysts such as enzymes are applied for the hydrolysis of polysaccharides, and fermentative microorganisms (yeast or bacteria) for fermentation of mixed sugar streams (*15*).

Types of pretreatment

Lignocellulosic biomass from vegetable waste has a great potential for use in the production of bioethanol, but due to its complex structure, it requires pretreatment to improve the yield of reducing sugars in the hydrolysate during enzymatic hydrolysis from cellulose and hemicellulose. Without pretreatment during the enzymatic saccharification stage, the presence of almost non-biodegradable lignin in lignocellulosic material and the low digestibility of crystalline cellulose and hemicellulose are major obstacles to the use of complex lignocellulosic biomass (*57*). Pretreatment makes cellulose and hemicellulose more readily available to hydrolytic enzymes, such as cellulases and hemicellulases, which generate simple sugars. However, use of complex technologies at the pretreatment stage increases the cost of ethanol production. Pretreatment reduces cellulose crystallinity, leading to anamorphic cellulose, removes or degrades the lignin (delignification) and requires total or partial hydrolysis of hemicellulose (*47*). As reported by Brodeur *et al.* (*58*), the goals of pretreatment are: (*i*) the production of highly digestible solids that increase sugar yields during enzymatic hydrolysis, (*ii*) to avoid loss of sugars (mainly pentose sugars), including those derived from hemicellulose through degradation, (*iii*) to reduce the forma-

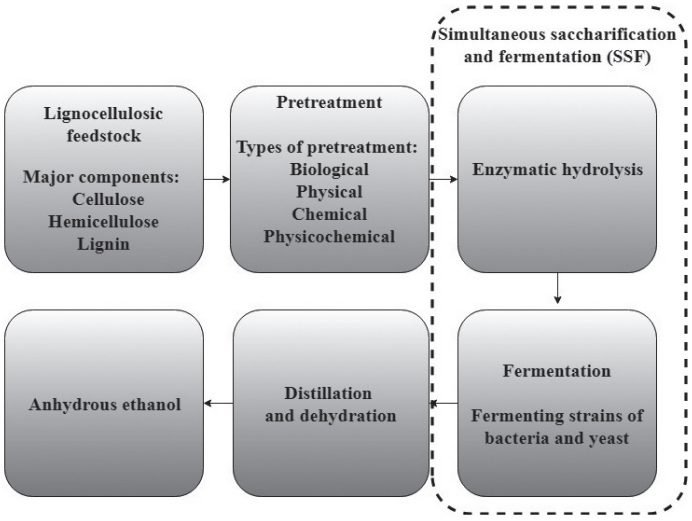

Fig. 2. Major steps in bioethanol production. Data taken from Kang *et al.* (*56*)

tion of inhibitors which can impede further fermentation steps, (*iv*) the recovery of lignin for modification into valuable coproducts, and (*v*) the reduction of heating and power costs. Pretreatment is part of the biochemical conversion of lignocellulose and is usually divided into biological, chemical and physical processes. The last two methods are often used in tandem in physicochemical treatments. Biological treatment uses microorganisms such as white, brown or soft rot fungi for the degradation of the biomass structure. Such pretreatment, particularly with white rot fungi, has the effect of improving the efficiency of enzymatic hydrolysis. Compared to other presaccharification treatments, the biological method is environmentally friendly, does not generate toxic products, does not require recycling of chemical substances and is energy-efficient (*59*). Delignification through white rot fungi occurs through the release of extracellular lignolytic enzymes, such as peroxidase (EC 1.11.1.7) and laccase (EC 1.10.3.2.), which break up the structure of lignin (*50*). Other pretreatment methods are based on fractionation, solubilization, hydrolysis and the separation of cell wall elements. Chemical treatments include treatment with bases (alkaline pretreatment), concentrated and diluted acids (acid pretreatment), pretreatment with ionic liquids (green solvents) and processing with oxygen as an oxidizer (wet oxidation). Mechanical methods are used to reduce the size of the raw material and are considered energy-intensive processes. Physical pretreatment reduces cell wall crystallinity and particle size by physical milling or grinding (*58*). Physicochemical pretreatment can involve steam explosion (autohydrolysis), liquid hot water (LHW), ammonia fibre explosion (AFEX), ammonia recycle percolation (ARP) or processing with a supercritical carbon dioxide (CO_2) (supercritical fluid) (*58*). Steam explosion causes hydrolysis of hemicellulose and improves cellulose digestibility. It is a hydrothermal method that uses both mechanical forces (temperature and sudden pressure reduction) and chemical factors (steam and catalyst concentration). The main purpose of steam explosion is the separation of fibres through explosive decompression after 1-5 min under high pressure and at high temperatures. Although it is the most commonly used method for processing herbaceous biomass and agricultural residues, steam explosion contributes to the partial degradation of sugars and lignin into soluble inhibitors in prehydrolysates, such as levulinic acid and phenolic products (*60*). The process of steam explosion is carried out by hydrolysis of glycosidic bonds in polysaccharides (hemicellulose or cellulose), mainly in hemicellulose. The process allows for more efficient recovery/separation of biopolymers by cleavage of hemicellulose and lignin bonds and subsequent solubilization of hemicellulose (in water) and lignin (delignification with organic or alkaline solvent). Cellulose with a reduced degree of polymerization is contained in the solid phase. Steam explosion has lower environmental impact (uses less dangerous chemicals), requires lower investment and consumes less energy than other methods of biomass fractionation (*61*). An impregnation agent (biomass pre-impregnation) is sometimes used before the pretreatment step. An acid catalyst is added before steam explosion to achieve higher sugar yield. Use of sulfuric acid and sulfur dioxide for impregnation increases the efficiency of enzymatic hydrolysis to glucose and xylose and contributes to lower enzyme consumption. This procedure is used firstly to improve enzymatic hydrolysis of the cellulose contained in the water-insoluble solid fraction and thereby obtain higher fermentable sugar concentrations, and secondly to reduce the time and temperature necessary for proper depolymerization of the feedstock (*62*). Steam explosion further contributes to delignification.

The choice of pretreatment technology depends on the composition of the lignocellulosic biomass. Thermal degradation is not recommended for agricultural and hardwood wastes with high contents of pentoses and low levels of lignin, due to the susceptibility of pentoses to degradation. Steam explosion is recommended for processing straw and bagasse.

Other methods for processing bagasse include pretreatment with ammonia water and wet oxidation (*63*).

Inhibitory compounds and their impact on microorganisms

Many inhibitors are generated during the pretreatment of lignocellulosic biomass, which can have a negative influence on ethanol production. Inhibitors create severe environments, seriously weakening fermentative microbes or causing their death (*64*). They increase the length of lag phase, cause loss of cell density and lower growth rates of fermenting microbes, decreasing ethanol yields as a consequence (*65*). Inhibitors consist of different compounds, mainly furan derivatives such as 5-hydroxymethyl-2-furaldehyde (HMF) and 2-furaldehyde (furfural), weak organic acids (formic, acetic and levulinic acids) and phenolic compounds (*66*). Phenolic compounds interfere with the function and integrity of cell membranes (*66*). *Saccharomyces cerevisiae* cell growth is inhibited by the intracellular process of accumulating anions of weak acids. Acetic acid is often found in hydrolysates and comes from acetyl side-chain groups in hemicellulose (*67*). Microbes affected by furan derivatives have been found to have lower cell mass yield, lower specific growth rates and lower volumetric ethanol productivity. Simple conversion of glucose into ethanol requires the removal of inhibitors (*7,68*). There are several methods used for detoxification. These include over-liming, extraction with organic solvents, ion exchange, use of molecular sieves, and steam stripping (*69*). The detoxification of lignocellulosic hydrolysates (*i.e.* removal of microbial inhibitors) can be performed using inhibitor sorbents such as excess of lime (overliming), or active carbon or lignite (brown coal), an alternative sorbent known from wastewater treatment. Overliming of hydrolysates destabilizes the inhibitors and causes them to be precipitated from the hydrolyzate. Advantages of using lignite instead of activated carbon include its relatively low price, its positive effect on the growth of microorganisms in the medium and the fact that it can be used to generate heat and energy after the detoxification process. However, lignite has lower sorption capacity than active carbon, meaning that it binds fewer inhibitors. The most commonly used detoxification method is overliming. The detoxification of hydrolysates is time-consuming and incurs additional expenses, especially related to the price of the sorbent (*70*). The removal of inhibitory compounds thus increases the price of second generation bioethanol. It is possible that ethanologenic microorganisms may become accustomed to living with inhibitor compounds during preconditioning at specific inhibitor concentrations. This might lead to improvement in resistance of microbes to inhibitors (*1*).

Enzymatic hydrolysis

The purpose of enzymatic hydrolysis is to release monosaccharides from polysaccharides located in the plant cell wall. The hydrolysis of polysaccharides, such as cellulose and hemicellulose generates fermentable sugars, which can be converted into ethanol during fermentation. The quantity of sugars in the hydrolysate depends on the type of raw material used (mainly lignocellulosic wastes) and the pretreatment methods applied (*1*). Monosaccharides are a source of carbon for the microorganisms responsible for the fermentation process (*49*). Glucose and xylose are the main products of the breakdown of lignocellulose found in hydrolysates after pretreatment and enzymatic hydrolysis. Enzymatic hydrolysis applies hydrolytic enzymes to break down cellulose and hemicellulose. Untreated biomass (in its native form) is difficult to digest by enzymes, and for this reason requires higher doses of hydrolytic enzymes (*50*). Enzymes derived from microorganisms can be used in a wide range of industrial applications, including for the production of biofuels, detergents, paper and pulp, as well as in the food, feed and beverage industries.

Enzymes used in industrial applications are produced mainly by the filamentous fungi such as *Aspergillus nidulans*, *Aspergillus niger*, *Penicillium* spp. and *Trichoderma reesei* (*49*). *T. reesei* has the ability to produce cellulases, specifically endoglucanase (EG), exo-cellobiohydrolase (CBH) and β-glucosidase (BGL). Endoglucanases attack the internal parts of the amorphous cellulose regions, causing depolymerization of the cellulose structure. The function of exoglucanase is to further break down β-glucan molecules, by releasing the cellobiose units from its ends. In contrast, β-glucosidase attacks cellobiose and contributes to the creation of two glucose units (*71*). The costs of a number of cellulases remain high. Some are produced by wood-rotting fungi, employed for hydrolysis of the interconnected matrix of cellulose and hemicellulose (*7*). Reusing hydrolysis enzymes offers an opportunity to reduce costs (*7*). The use of hemicellulases, such as endoxylanases, xylosidases, exoxylanases and other accessory enzymes, such as esterases and arabinosidases, improves the efficiency of enzymatic hydrolysis of lignocellulosic biomass (corn stover, herbaceous biomass, barley straw), contributes to the reduction of enzyme loading and lowers costs. The interaction of cellulases with hemicellulases and accessory enzymes makes the entire process of enzymatic hydrolysis effective (*72*). Various strains of yeasts and bacteria are being investigated with the goal of developing a consolidated process of hydrolysis and co-fermentation of glucose and xylose, without the need for adding exogenous cellulases (*31*).

Fermentation of lignocellulosic hydrolysates

The purpose of fermentation is to achieve efficient conversion of hexose and pentose sugars into ethanol by fermenting microorganisms such as yeasts. Ethanol-producing microorganisms are susceptible to lignocellulosic hydrolysate, depending on their strain and fermentation conditions (temperature, pH, aeration and nutrient supplementation) (*69*). There is a tendency for microorganisms that ferment xylose efficiently in laboratory media to produce poorer yields in lignocellulosic hydrolysates. Fermentation leads to stress conditions, such as ethanol accumulation, gradual decreases in pH, a shift to anaerobic growth and nutrient limitation (*73*). Both osmotic stress (hyperosmotic stress), caused by the ions and sugars in the hy-

drolysate, and alcohol accumulation are known to inhibit yeast growth and viability (*74*). High sugar levels in the wort at the beginning of fermentation can result in osmotic shock (*75*,*76*). Severe conditions, such as oxidative and ethanol stress, affect the performance of bioethanol fermentation.

Enzymatic hydrolysis/saccharification and fermentation

Enzymatic hydrolysis of cellulose and hemicellulose, and fermentation in the presence of fermentative microorganisms may be performed separately in separate hydrolysis and fermentation (SHF) or at the same time in simultaneous saccharification and fermentation (SSF). These processes may also be carried out at the same time by a single strain that is additionally capable of producing saccharification enzymes in the process of consolidated bioprocessing (CBP) (*58*,*77*).

Ethanol production using SSF is more cost-effective and has therefore been the preferred approach. Significant progress has been made with regard to increasing feedstock loading, decreasing inoculum loading and ensuring co-fermentation of both hexoses and pentoses during SSF (*37*,*78*). Although the integration of hydrolysis and fermentation reduces investment costs by reducing the number of vessels, there is an inevitable mismatch between the optimal temperatures for the enzymes (fungal cellulases and hemicellulases) on the one hand, and microbial biocatalysts on the other. The temperature optima for saccharifying enzymes are higher than those for fermenting mesophilic culture. Mesophilic yeasts exhibit slower growth rates at higher temperatures than thermotolerant and thermophilic yeasts. For this reason, thermotolerant and ethanologenic yeasts are suggested for use in SSF processes. Optimizing the conditions for simultaneous saccharification and fermentation can improve the efficiency of both stages. The application of the SSF requires lowering the optimum temperature for cellulase activity (50-55 °C) to the temperature of fermenting organisms. The optimal temperature for yeasts is below 35 °C. The action of enzymes at lower than optimal temperatures results in higher cellulase loading and may increase costs.

Using genetically modified microorganisms with the ability to ferment at higher temperatures (50-55 °C) could improve the SSF process and avoid the costs associated with extra loading of enzymes during enzymatic saccharification (*6*). Efficient bioethanol production by SSF requires the use of thermotolerant ethanologenic yeast. Some isolated yeasts have been found to be thermotolerant. These microbes, including *Saccharomyces*, *Candida*, *Pichia* and *Wickerhamomyces,* are able to grow at temperatures of 40 °C and also have the ability to ferment sugars at higher temperatures (*1*).

In order to prevent feedback inhibition, in SSF process glucose molecules are fermented immediately by the fermentative microbes. In the production of lignocellulosic bioethanol by this route, there is a shortage of end-product feedback inhibition, because sugar monomers released during the saccharification are immediately fermented by the microorganisms. The risk of microbial contamination during SSF is decreased, as glucose is fermented instantly into ethanol (*79*). Contamination of the fermentation vessel can affect the efficiency of bioethanol production. Contaminating microorganisms may disrupt fermentation by absorbing monosaccharides in the hydrolysate, as well as by producing toxic metabolites which inhibit fermentative microorganisms.

Simultaneous saccharification and co-fermentation (SScF) of lignocellulosic material is not a fully developed technology, and requires further research at biorefineries and in biotechnology centres. No feedback inhibition occurs in this process. Advances in genetic engineering enable both enzyme hydrolysis of lignocellulosic material and fermentation of mixed C5 and C6 sugars, giving higher ethanol productivity. Introducing the pentose metabolic pathway into microorganisms enables the use of C5 sugars by microbes that do not ferment them earlier, even if glucose is not present in the environment (*80*).

In SHF process, saccharification and fermentation take place in individual vessels. The increased number of vessels makes SHF uneconomical. It causes end-product inhibition of hydrolytic enzymes and has a negative influence on the efficiency of saccharification. End-product inhibition is caused by simple carbohydrates (such as cellobiose) and has an impact on the inhibition of the cellulolytic enzymes, for example cellulases or celobiases. Therefore, in order to prevent end-product inhibition, extra doses of β-glucosidase are needed together with the commercial cellulase preparations. The application of enzyme cocktails with additional β-glucosidase activity plays a greater role in SHF than in SSF processes (*81*).

The technology of consolidated bioprocessing (CBP) connects the three steps of lignocellulosic bioethanol production, namely enzyme production, enzymatic saccharification and sugar fermentation. CBP technology promises to eliminate costs associated with the production of enzymes and the purchase of additional infrastructure/apparatus (vessels), but more research is required into microbial biocatalysts (*82*).

FERMENTATIVE MICROORGANISMS

For microorganisms (mainly yeasts) to be considered for industrial bioethanol production using lignocellulosic biomass, they have to show thermotolerance and high fermentative activity for simple carbohydrates such as glucose and xylose (a suitable sugar utilization pattern) (*6*,*80*). They should also provide high ethanol yields and be resistant to environmental stressors, including inhibitors generated during the industrial process (specifically, furfural and 5-hydroxymethyl furfural) (*65*). Other requirements are the ability to grow on different media (frequently lignocellulosic substrates such as crop wastes or forestry residues), a fast growth rate and suitability for genetic modification. According to Dien *et al.* (*67*,*83*) and Zaldivar *et al.* (*84*) microorganisms used in the bioethanol industry based on lignocellulose should show high ethanol yield (above 90 % of theoretical yield), high tolerance to ethanol (above 40 g/L), low requirements for growth in the medium, high resistance to stressors in the medium such as inhibitors, and resistance to acidic pH and higher temperatures, which prevents microbiological contamination. Microbes may be derived from differ-

ent sources, such as distillery waste. Research has particularly focused on the isolation, identification and evaluation of yeast strains that exhibit potential for ethanol production from glucose and xylose (*85*,*86*). Microorganisms have a natural preference towards the consumption of certain sugars, often glucose without using xylose (*16*). Promising and suitable strains for fermentation are selected based on their behaviour in the presence of inhibitors in fermenting media and after the application of different pretreatment methods. The isolated strains *Saccharomyces cerevisiae* JRC6 and *Candida tropicalis* JRC1 are recommended for fermentation of lignocellulosic hydrolysates after alkali pretreatment and acid pretreatment, respectively (*1*). Certain strains, such as *Saccharomyces cerevisiae* and *Zymomonas mobilis*, are suitable for ethanol production, but their use with lignocellulosic hydrolysates is uneconomical. Other strains, *e.g*. *C. tropicalis* JRC1 and *C. tropicalis* JRC3 (*1*), are used to produce value-added products such as xylitol from glucose instead of bioethanol.

Saccharomyces cerevisiae

The conventional yeast *Saccharomyces cerevisiae* is incapable of using pentose sugars, as its metabolism does not generate the appropriate enzymes, and its pentose phosphate pathway does not work effectively (*87*). This strain is capable mainly of metabolizing glucose, as it has glycolytic pathway. Around 18 hexose transport proteins are involved in glucose uptake in *S. cerevisiae*. These hexose transport proteins exhibit affinity for xylose (*88*). Pentose-specific transporter proteins and enzymatic reactions determining the metabolism of pentoses such as L-arabinose and D-xylose have not been found in naturally occurring baker's yeast. There is a shortage of xylose-specific transporters in engineered strains, which are required for efficient xylose use without hampering fermentation of combining C5 and C6 sugars. In mixed fermenting sugars, glucose is thought to inhibit the absorption and use of xylose by cells (*6*,*67*). In *S. cerevisiae,* affinity for xylose uptake depends on the extracellular concentration of glucose (*81*). If the glucose concentration is high, the transporters show low affinity for xylose. Even if all the glucose in the medium has been used, the quantity of consumed xylose will still be lower than that of the consumed glucose. The reason for the low consumption of xylose in this case is that xylose alone cannot match the redox balance of the cell (*16*). *Saccharomyces cerevisiae* has not been found to have genes for encoding cellulases or the capacity to metabolize xylose.

Saccharomyces sp. yeasts are used in biorefineries to ferment monosaccharides released during starch degradation. Apart from glucose, they are capable of fermenting galactose and mannose. Only recombinant strains are capable of fermenting xylose and arabinose (*67*).

Zymomonas mobilis

Glucose, fructose and sucrose are the only carbon sources that are fermented by *Zymomonas mobilis* (*89*). Although they produce bioethanol from starch with high productivity and yield (*89*), these Gram-negative bacteria contain a narrow range of fermentable carbohydrates (excluding pentoses). This prevents them from being used in industrial production of bioethanol (*6*). These microorganisms metabolize glucose *via* the Entner-Doudoroff (ED) pathway and consequently ferment most of the carbon source into ethanol rather than into biomass (*67*). Significant properties of the *Z. mobilis* strain include resistance to ethanol concentration up to 120 g/L, capacity for the homofermentative production of ethanol, and low nutritional requirements for growth (*67*). As little as 2.5 g/L of acetic acid results in a decrease in ethanol productivity, indicating that even recombinant *Z. mobilis* strain AX101 cultures have low tolerance to acetic acid. The problem of intolerance to acetic acid can be resolved in two ways: by adapting the strain to the inhibitor (increasing tolerance) or by detoxifying the hydrolysate prior to the fermentation stage (*67*).

PENTOSE FERMENTATION AND XYLOSE METABOLISM

Efficient xylose fermentation depends on finding suitable xylose-fermenting microorganisms in the environment or using genetic engineering techniques (*69*). The lower ethanol yield obtained from xylose-fermenting strains may be a result of the production of xylitol or the reabsorption of ethanol (*69*). A list of microorganisms capable of fermenting xylose is provided by Olsson and Hahn-Hägerdal (*69*). Among the natural bacteria capable of fermenting xylose are strains such as *Bacillus macerans* DMS 1574, *Bacteroides polypragmatus* NRCC 2288 and *Erwinia chrysanthemi* B374. The most thoroughly researched natural xylose-fermenting yeast species are *Candida shehatae*, *Pichia stipitis* and *Pachysolen tannophilus* (*90*). *Candida* and *Pichia* strains exhibit greater tolerance to inhibitors than *S. cerevisiae*.

Specific fungal and bacterial species may metabolize xylose into xylulose *via* different enzymes such as the oxidoreductases xylose reductase (XR) and xylitol dehydrogenase (XDH) or isomerases such as xylose isomerase (XI) (*2*,*91*). Xylulose is then phosphorylated through a single enzyme, xylulose kinase (XKS), and enters the pentose phosphate pathway (PPP) (*31*). Genetically modified xylose-fermenting *Z. mobilis* has been constructed by introducing enzymes (such as transaldolase and transketolase) into the PPP and operons responsible for xylose adaptation through encoding xylose isomerase and xylulokinase (*6*).

RECOMBINANT FERMENTATIVE MICROBES

The environment is a rich source of fermentative microbes for researchers. Nevertheless, wild yeasts and other isolated microbes do not meet the requirements for industrial production of second generation bioethanol. These include, *inter alia*, high tolerance to elevated temperatures, the ability to grow in lignocellulosic hydrolysate and effective utilization of xylose after the initial transfer into microbes of a xylose metabolic

pathway (*64*). Non-recombinant/wild microorganisms do not produce high ethanol yields, as they are unable to utilize both pentoses and hexoses. They are therefore modified through genetic engineering. One of the aims of metabolic engineering of fermentative species is to improve their resistance to the conditions of fermentation. Genetic engineering has also been used to improve the resistance of microorganisms to inhibitors generated during pretreatment, as well as their tolerance to ethanol and high sugar concentrations (*87*).

Genetic modification is crucial for increasing the range of consumed sugars, making ethanol production using microorganisms more cost-effective (*16*). Encouraging progress has been made towards engineering microorganisms for the fermentation of mixtures of hexoses and pentoses. One fruitful strategy has been to add a pathway for the conversion of pentose or other sugars to a natural ethanol-producing yeast strain such as *Saccharomyces cerevisiae* or the bacteria *Zymomonas mobilis* (*13*). In other words, to create recombinant strain, genes for xylose metabolism are introduced into the host, often *S. cerevisiae*. Genetic engineering has led to improved ethanol yields from fermenting bacteria such as *E. coli* and *Z. mobilis* (*83*). The second mode of recombination involves the genetic modification of microorganisms that metabolize multiple sugars, to enable them to produce ethanol *via* the glycolysis pathway (*13*). Attempts to use genetic engineering to enable simultaneous use of mixed sugars for ethanol production have focused mainly on *Saccharomyces cerevisiae* yeast, the Gram-positive bacteria *Clostridium cellulolyticum* and *Lactobacillus casei* and the Gram-negative bacteria *Zymomonas mobilis, Escherichia coli* and *Klebsiella oxytoca* (*6*). Methods for obtaining microorganisms capable of simultaneous consumption of glucose and xylose include mutagenesis and the introduction a heterologous metabolic pathway for xylose utilization into well-known conventional strains such as *S. cerevisiae* (*92*). Cellulase-encoding genes may also be introduced into specific species during recombination (*93*). The aim of research into recombinant strains is to provide efficient and economical conversion of feedstock into bioethanol and to decrease the capital costs of processing (*e.g.* by not requiring the use of exogenous cellulases during the process). However, when cellulase-encoding genes for degrading cellulose were introduced into *S. cerevisiae*, the application of exogenous cellulases was still found to be necessary (*94*). The ratio of pentose to hexose utilized by recombinant yeasts is still relatively lower for xylose than for glucose during fermentation. Recombinant yeasts do not ferment hexoses and pentoses at the same time, a fact that explains the prolonged fermentation period required, and which means that the process remains uneconomical. They first consume glucose entirely, after which pentose fermentation occurs (*87*). Special genetic engineering techniques are required to design yeast strains able to co-ferment pentoses and hexoses for the production of lignocellulosic ethanol (*6*). Although genetic engineering has contributed to progress in this area, there is still a lack of both reaction intermediates and efficient pentose transporters. For these reasons, yields from the fermentation of pentoses are still limited (*87*).

DISTILLATION AND DEHYDRATION (DRYING) OF BIOETHANOL

Traditional distillation systems are typically based on countercurrent vapour/liquid mass transfer. Distillation enables the recovery of dilute volatile products, such as ethanol, from impure biomass-based streams (*95*). Residual solids, such as unconverted polysaccharides (cellulose and hemicellulose), ash and compounds from the breaking down of lignin, contaminate these streams (*95*). Ethanol obtained by fermentation is contaminated and in low volume fractions. During distillation, ethanol is separated from the other components of the mixture. Rectification thus enables the concentration and purification of ethanol. However, extremely high purity (99.7 %, by volume) ethanol can be achieved only after drying, as a result of dehydration (*96*).

NEW IMPROVEMENTS IN ETHANOL PRODUCTION FROM LIGNOCELLULOSIC BIOMASS

Unit operations such as pretreatment, enzymatic hydrolysis and distillation are responsible for much of the cost of producing bioethanol (*17*). Current research and development therefore aims to improve these unit processes to make them more economical. For example, the use of steam explosion instead of mechanical comminution can save energy. Pretreatment methods are also now selected with consideration for their environmental impact (*97*). Chemical methods require subsequent recycling of chemical compounds, for example ammonia recovery in the ammonia fibre explosion (AFEX) process (*17*,*97*). The parameters of pretreatment methods should be optimized for the adequate processing of the substrate. If pretreatment is effective, an easily digestible substrate with an increased specific surface area should be obtained. The cellulose is then better available for the action of hydrolytic enzymes obtained from fungi and bacteria. The efficiency of pretreatment is especially important, because it affects the reactivity of the enzyme substrate. Effective pretreatment minimizes reductions in enzyme activity, and thus improves the rate of biomass hydrolysis. The efficiency of ethanol production depends on effective depolymerization and delignification of polysaccharides in lignocellulosic materials, minimizing energy-intensive processes, and on fermentation of carbohydrates with 5 and 6 carbons in hydrolysates containing inhibitors (*20*).

Effective pretreatment improves the enzymatic digestibility of lignocellulosic biomass, providing the highest possible concentration of fermentable sugars and reducing the degradation of monosugars. The level of specific enzyme activity (U/mL), which depends on time, and the composition of the enzyme preparation, *e.g.* for cellulose: endoglucanase, exoglucanase and β-glucosidase affect the dosage of enzyme required (*81*,*98*). Current research aims to improve enzyme activity by searching for new organisms with cellulolytic and hemicellulytic activities (*3*) or to lower the enzyme dosage and the cost of converting lignocellulosic biomass to ethanol through protein

engineering (*80,98*). To reduce the cost of enzymatic hydrolysis, it may also be possible to reuse enzymes. The surface area of the lignocellulosic biomass accessible to the enzyme also has an influence on enzymatic hydrolysis, affecting enzyme loading (*99*). The use of xylanase causes the degradation of hemicelluloses, increasing the surface of cellulose in contact with the enzyme, which increases the efficiency of cellulose hydrolysis.

The use of surfactants (surface-active substances such as polyethylene glycol and Tween (*63*)), can improve the efficiency of enzymatic hydrolysis, since surfactants are adsorbed onto the surface of lignin instead of the enzymes, as a consequence of which the enzymes are not inactivated.

Due to the fact that hydrolysates contain the highest concentrations of glucose and xylose, genetic engineering has been used to construct xylose-fermenting microorganisms, with the aim of using the raw material to its full potential (*63,100*). Advanced microorganisms used for the fermentation of hexose and pentose into ethanol and carbon dioxide have been investigated in terms of the efficiency of the process, their rates of growth and the inhibiting effects of ethanol (*101*). During simultaneous saccharification and co-fermentation glucose is used more quickly than xylose. This may be explained by the fact that glucose has a stronger affinity for transporters in the cell than xylose. Sugars (xylose and glucose) can be fermented simultaneously when the concentration of glucose during fermentation is kept low (*63*).

The use of SSF or SSCF for the production of bioethanol reduces enzyme loading and the time required for processing polysaccharides during enzymatic hydrolysis. Reducing sugars obtained by enzymatic hydrolysis are fermented immediately by the yeast, counteracting enzyme inhibition. The efficiency of SSF and ethanol production is influenced by many factors, including the efficiency of pretreatment, the parameters of the fermenting microorganisms and the use of highly active cellulase and hemicellulase preparations.

CONCLUSIONS

Shifting the transport sector from petroleum and gasoline towards more sustainable, renewable and environmentally friendly energy sources such as second generation bioethanol is one of the grand challenges in engineering. The production of lignocellulosic bioethanol requires improvements related to the pretreatment, enzymatic hydrolysis and fermentation stages, in order to increase the cost-effectiveness of ethanol production and to make the transition from the laboratory to the industrial/commercial scale. One of the most important goals is to increase the efficiency of the fermentation process to the point where all sugars (pentoses and hexoses) released during the pretreatment and hydrolysis steps are fermented into ethanol. Technical barriers to second generation biofuel production include the variable composition of biomass, generation of inhibitors during presaccharification treatment, end-product inhibition, osmotic and oxidative stress, and ethanol accumulation. However, progress is being made and these technical barriers can be expected to be overcome in the near future, optimizing the biochemical pathway for second generation liquid bioethanol production.

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