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Production of 2nd Generation of Liquid Biofuels

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1. Introduction

Fluctuations in the price of oil and projections on depletion of accessible oil deposits have led to national and international efforts to enhance the proportion of energy derived from renewable sources (bioenergy) with special emphasis on the transport sector (e.g. according to Directive 2009/28 EC, by 2020, 20% of energy in EU-27 should be met from renewable sources and 10% should be used in transportation). To fulfil the legal requirements, wider exploitation of biofuels made from renewable feedstocks, as a substitute for traditional liquid fuels, will be inevitable; e.g. the demand for bioethanol in the EU is expected to reach 28.5 billion litres by 2020 [1], while in America 36 billion gallons of ethanol must be produced by 2022 [2]. Bioethanol, which has a higher octane level than petrol but only contains 66% of the energy yield of petrol, can be used as blend or burned in its pure form in modified spark-ignition engines [2]. This will improve fuel combustion, and will contribute to a reduction in atmospheric carbon monoxide, unburned hydrocarbons, carcinogenic emissions and reduce emissions of oxides of nitrogen and sulphur, the main cause of acid rain [2]. Butanol-gasoline blends might outcompete ethanol-gasoline ones because they have better phase stability in the presence of water, better low-temperature properties, higher oxidation stability during long term storage, more favourable distillation characteristics and lower volatility with respect to possible air pollution. Recently performed ECE 83.03 emission tests [3] have shown negligible or no adverse effects on air pollution by burning butanol-gasoline blends (containing up to 30% v/v of butanol) in spark ignition engines of Skoda passenger cars.

Although most of the world's bioethanol is currently produced from starch or sugar raw materials, attention is increasingly turning to 2nd generation biofuels made from lignocellulose, e.g. agriculture and forest wastes, fast growing trees, herbaceous plants, industrial

wastes or wastes from wood and paper processing. The concept of ethanol production from lignocellulose sugars is not new. Probably the first technical attempt to degrade polysaccharides in wood was carried out by the French scientist Henri Braconnot in 1819 using 90% sulfuric acid [4]. His findings were exploited much later, in 1898, with the opening of the first cellulosic ethanol plant in Germany, followed by another one in 1910 in the US [5, 6]. During World War II, several industrial plants were built to produce fuel ethanol from cellulose (e.g. in Germany, Russia, China, Korea, Switzerland, US), but since the end of the war, most of these have been closed due to their non-competitiveness with synthetically produced ethanol [7]. In spite of all the advantages of lignocellulosic as a raw material (e.g. low and stable price, renewability, versatility, local availability, high sugar content, noncompetitiveness with food chain, waste revaluation) and extensive efforts of many research groups to reduce bottlenecks in technology of lignocellulosic ethanol production (e.g. energy intensive pretreatment, costly enzymatic treatment, need for utilization of pentose/hexose mixtures, low sugar concentration, low ethanol concentration), large scale commercial production of 2nd generation bioethanol has not been reopened yet [8], although many pilot and demonstration plants operate worldwide [9]. Identically, only first generation biobutanol is produced in China (approx. annual amount 100 000 t) and Brazil (approx. annual amount 8 000 t) [10]. At the 2012 London Olympic Games, British Petrol introduced its three most advanced biofuels i.e. cellulosic ethanol, renewable diesel and biobutanol. At a demonstration plant at Hull UK, biobutanol, produced by Butamax (joint venture of BP and DuPont) was blended at 24 % v/v with standard gasoline and used in BMW-5 series hybrids without engine modifications [11]. As the final price of both ethanol and 1-butanol produced by fermentation is influenced mostly by the price of feedstock, the future success of industrial ABE fermentation is tightly linked with the cost of pre-treatment of lignocellulosic material into a fermentable substrate.

2. Characterization of 2nd generation feedstock

Plant biomass can be used as a sustainable source of organic carbon to create bioenergy, either directly in the form of heat and electricity, or as liquid biofuels produced by thermochemical or biochemical methods or their combination [12]. In contrast to fossil energy sources, which are the result of long-term transformation of organic matter, plant biomass is created via photosynthesis using carbon dioxide as a source of carbon and sunlight as a source of energy and therefore is rapidly produced. The world annual production of biomass is estimated to be 146 billion metric tons [13], which could contribute 9-13% of the global energy supply yielding 45±10 EJ per year [14, 15].

Lignocellulose, which is stored in plant cell walls makes up a significant part of biomass representing 60-80% of woody tissue of stems, 15-30% of leaves or 30-60% of herbal stems [16]. Since it is not digestible for human beings, its use as a feedstock for bioprocesses does not compete with food production as in the case of sugar or starch raw materials.

All lignocellulose consist of three main polymeric components – cellulose, non-cellulosic carbohydrates (predominantly represented by hemicellulose) and lignin; its proportion and

structure differs for different types of biomass (Table1) and it is also influenced by variety, climatic conditions, cultivation methods and location. Minor components of the cell wall are represented by proteoglycans, pectin, starch, minerals, terpenes, resins tannins and waxes.

Biomass	Cellulose	Hemicellulose	Lignin	Reference
Hardwood	45-47	25-40	20-55	[17, 18]
Softwood	40-45	25-29	30-60	[17, 18]
Wheat straw	30-49	20-50	8-20	[19-22]
Rye straw	30.9	21.5	25.3	[21]
Corn fibre	15	35	8	[23]
Corn cobs	35-45	35-42	5-15	[22, 23]
Corn stover	39-42	19-25	15-18	[22, 23]
Corn straw	42.6	21.3	8.2	[20]
Rice straw	32-47	15-27	5-24	[20, 22, 23]
Rice hulls	24-36	12-19	11-19	[22]
Sugarcane bagasse	40	24-30	12-25	[20, 22, 23]
Switchgrass	30-50	10-40	5-20	[17, 23, 24]
Bermuda grass	25-48	13-35	6-19	[22, 23]
Cotton seed hairs, flax	80-95	5-20	0	[18, 22]
Municipal solid waste – separated fibre	49	16	10	[25]
Primary municipal sludge	29.3	not identified	not identified	[26]
Thickened waste activated sludge	13.8	not identified	not identified	[26]
Sawdust	45.0	15.1	25.3	[22]
Waste paper from chemical pulps	50-70	12-20	6-10	[17]
Newspaper	40-55	25-40	18-20	[1, 17]
Used office paper	55.7	13.9	5.8	[1]
Magazine	34.3	27.1	14.2	[1]
Cardboard	49.6	15.9	14.9	[1]
Paper sludge	33-61	14.2	8.4-15.4	[27, 28]
Chemical pulps	60-80	20-30	2-10	[18]

Table 1. Overview and composition of lignocellulosic biomass and other lignocellulosic sources

Cellulose is a homopolymer of 500-1 000 000 D-glucose units (e.g. 10 000 units in wood, 15 000 in native cotton) linked by β -1,4-glycosidic bonds [19, 26, 29]; the cellulose chains (200-300) are grouped together to form cellulose fibres. The strong inter-chain hydrogen bonds between hydroxyl groups of glucose residues in radial orientation and the aliphatic hydrogen atoms in axial positions creates a semi-crystalline structure resistant to enzymatic hydrolysis; weaker hydrophobic interactions between cellulose sheets promote the formation of a water layer near the cellulose surface, which protects cellulose from acid hydrolysis [30]. Cellulose originating from different plants has the same chemical structure, but it differs in crystalline structure and inter-connections between other biomass components. Microfibrils made of cellulose are surrounded by covalently or non-covalently bound hemicellulose, which is a highly branched heteropolymer made from 70-300 monomers units of pentoses (xylose, arabinose), hexoses (galactose, glucose, mannose) and acetylated sugars (e.g. glucuronic, galacturonic acids). Unlike cellulose, hemicellulose is not chemically homogenous and its composition depends on the type of material - hardwood contains predominantly xylans while softwood consists mainly of glucomannans [17, 23, 29,31]. Lignin, an amorphous heteropolymer of three phenolic monomers of phenyl propionic alcohols, namely p-coumaryl, coniferyl and sinapylalcohol, creates a hydrophobic filler, which is synthesized as a matrix displacing water in the late phase of plant fibre synthesis, and forms a layer encasing the cellulose fibres. Its covalent crosslinking with hemicellulose and cellulose forms a strong matrix, which protects polysaccharides from microbial degradation, makes it resistant to oxidative stress, and prevents its extraction by neutral aqueous solvents [31]. Forest biomass has the highest content of lignin (30-60% and 30-55 % for softwoods and hardwoods, respectively), while grasses and agricultural residues contain less lignin (10-30% and 3-15% respectively) [17].

There are several groups of lignocellulosic plant biomasses that can be exploited as a feedstock for bioprocessing. Woody biomass is represented mainly by hardwoods (angiosperm trees, e.g. poplar, willow, oak, cottonwood, aspen) and softwoods (conifers and gymnosperm trees e.g. pine, cedar, spruce, cypress, fir, redwood) together with forest wastes such as sawdust, wood chips or pruning residues. Nowadays the trend in this area is to use fast growing trees (poplar, willow) with genetically changed wood structures e.g. lower lignin content [32]. The advantage of forest biomass is its flexible harvesting time, thus avoiding long storage periods, and its high density, contributing to cost-effective transportation. Agricultural residues are represented mainly by corn stover or stalks, rice and wheat straw or sugarcane bagasse. The world's annual production of rice straw, wheat straw and corn straw that can be exploited for bioethanol production is 694.1, 354.3 and 203.6 million tons, respectively [20]. In the USA, 370 million and 350-450 million tons of forest biomass and agricultural wastes respectively are produced per year [17]. Although agrowastes are partly reutilized, e.g. as animal fodder, bedding, domestic fuel, used for cogeneration of electricity or reused in agriculture, a large fraction is still disposed as waste and is left in the fields; this can be utilized as a raw material for biofuels production. Sugarcane is nowadays one of the most important feedstocks for production of 1st generation bioethanol and also one of the plants with the highest photosynthetic efficiency, yielding around 55 tons of dry matter per hectare annually (approx. 176 kg/ha/day). Sugar cane bagasse, the fibrous lignocellulosic

material remaining as waste is mostly used as a solid fuel in sugar mills or distilleries but due to its high cellulose content (Table 1) it can be reutilized as a feedstock for production of 2nd generation bioethanol. In the sugarcane season of 2010/11, the total sugar cane crop reached almost 1.627 billion tons (on 23 million hectares), which corresponds to 600 million tons of wet sugar cane bagasse [33]. Minor, but also important residues are the leaves, called sugarcane trash, amounting to 6-8 tons per hectare of sugarcane crop [34]. Another group of lignocellulosic biomass, herbaceous energy crops and grasses, which are represented predominantly by switch grass, alfalfa, sorrel or miscanthus [24], are interesting due to their low demands on soil quality, low-cost investments, fast growth, low moisture content, high yield per hectare (e.g. 20 t/ha for miscanthus) and high carbohydrate content (Table 1). Besides lignocellulosic plant materials, other low-cost large volume feedstocks such as municipal solid waste, municipal wastewater, food-processing waste or waste from the paper industry can be utilized for bioethanol production. Mixed municipal recovery solid waste (MSW) consists of approximately 55% mineral waste, 6% of metallic waste, 5% animal and vegetable waste (food residues, garden waste), 3% of paper and cardboard waste and 31% of others [35]. In the EU alone, the annual production of municipal wastes amounts 2.6 million tons, 65% of which is derived from renewable resources [35, 36]. The main challenge in its bioprocessing is its heterogeneous composition. To be used for ethanol production, degradable fractions of MSW should be separated after sterilization; cellulosic material (paper, wood or yard waste) represents approximately 60% of the dry weight of typical MSW as shown in Table 1 [25, 37]. Beside the solid wastes, lignocellulose extracted from municipal wastewater treatment processes can also be used as low-cost feedstock for biofuel production [26]. In Canada, 6.22 Mt of sugar could be annually produced using municipal sludge/biosolids and livestock manures [26]. Municipal wastewaters, which include faecal materials, scraps of toilet paper and food residues, should be pre-treated to separate solid and liquid fractions, the former of which is processed further to gain simple sugars. Primary sludge contains more cellulose compared to activated sludge (Table 1) because it is consumed in the activated sludge process and is further degraded by anaerobic digestion processes in the sewage disposal plant [26]. When talking about industrial wastes as 2nd generation raw materials for biofuels, wastes from cellulose/paper production cannot be neglected. Paper sludge is waste solid residue from wood pulping and papermaking processes and is represented by poor-quality paper fibres, which are too short to be used in paper machines. It is attractive as a raw material for bioprocessing mainly due to its low cost (it is currently disposed of in landfills or burned), its high carbohydrate content (Table 1) and its structure, which doesn't require any pretreatment [8, 27, 28]. Another waste is represented by sulphite waste liquor (SWL), a solution of monomeric sugars formed during the sulfite pulping process by dissolution of lignin and most hemicelluloses. About 1 ton of solid waste is dissolved in SWL (11-14% solids) per ton of pulp and its annual production is around 90 billion litres [38]. SWL is usually burned after its concentration and evaporation, but since its main components are sugars and lignosulfonates, its use as a raw material for bioethanol production has potential. Chemical composition of SWL (a spectrum of fermentable sugars, inhibitors, nutrients and minerals) differs significantly with the type of wood and technological procedures, e.g. concentration of the main sugars in SWL (% of dry matter) ranges for xylose from

3 to 5 % in soft wood (spruce, western hemlock) up to 21 % in eucalyptus, the highest concentration of galactose and glucose around 2.5 % is in soft wood SWL, content of mannose can reach values of almost 15 % in soft wood SWL [39-42]. SWL cannot be fermented without careful pretreatment - stripping off free sulfur dioxide and simultaneous concentration, steaming, removing inhibitors, adding nutrients, and adjusting the pH [43].

Although lignocellulose biomass is cheap and predominantly comprises waste material, the logistics, handling, storage and transportation dramatically increases its cost and therefore its use directly on site is preferred over to its processing in a central plant [8]. Further price increases occur due to the character of material - most lignocelluloses mentioned above are not fermentable by common ethanol producers and must be decomposed and hydrolysed into simple sugars before fermentation is carried out.

3. Biomass disruption in pretreatment process

A prerequisite for ethanol production from lignocellulose is to break recalcitrant structure of material by removal of lignin, and to expose cellulose, making it more accessible to cellulolytic enzymes by modifying its structure; this happens in the pretreatment process. Basically, lignocellulose processing into fermentable sugars occurs in two steps: a) pretreatment yielding a liquid fraction that is mostly derived from hemicellulose and lignin and a solid fraction rich in cellulose, b) further enzymatic or chemical hydrolysis of the solid (wet) cellulose fraction to yield fermentable sugars.

Delignification (extraction of lignin by chemicals) is an essential prerequisite for enzymatic digestion of biomass; it disrupts the lignin polymeric structure, leading to biomass swelling and increase in its surface area and enables contact of cellulolytic enzymes with cellulose fibres. Although some pretreatment methods do not lead to a significant decrease in lignin content, all of them alter its chemical structure making biomass more digestible even though it may contain the same amount of lignin as non-pretreated biomass [29]. Hemicellulose is often dissolved during pretreatment because it is thermosensitive and easily acid-hydrolysed due to its amorphous branched structure; the liquid fraction obtained after pretreatment thus contains mainly pentose sugars (D-xylose, D-arabinose) originating from hemicelluloses, and strains fermenting pentose sugars must be used for its processing into ethanol as discussed later. The solid wet fraction obtained after pretreatment contains predominantly cellulose and needs further processing to yield fermentable sugars.

The conversion of lignocellulose into fermentable sugars is more difficult to achieve than conversion of starch; starchy material is converted from a crystalline to an amorphous structure at temperatures of 60-70°C, while lignocellulose is more resistant - a temperature of 320°C and a pressure of 25 MPa is needed to achieve its amorphous structure in water [17]. Therefore complete decomposition of cellulose is rarely attainable. Although lignocellulose pretreatment is an energy-intensive process, which contributes significantly to the price of the final product (18-20% of the total cost of lignocellulosic bioethanol is attributed to pretreatment) [8], it is a necessary expense because enzymatic hydrolysis of non-pretreated ma-

terial provides less than 20% of the theoretical maximum yield of fermentable sugars for the majority of lignocellulose feedstocks [44]. The resistance of biomass to enzymatic attack is characterized by a number of physical variables such as lignin content, crystallinity index (ratio of crystalline to amorphous composition of cellulose), degree of polymerization, chain length, specific surface area, pore volume or particle size [31], which are material specific; e.g. pretreatment of woody biomass differs considerably from agriculture biomass, while paper sludge doesn't need any processing.

Efficient pretreatment of biomass is characterized by an optimum combination of variables which leads to effective disruption of the complex lignocellulosic structure, removes most of the lignin, reduces cellulose crystallinity and increases the surface area of cellulose that is accessible to enzymatic attack. At the same time, it should minimize the loss of sugars, limit the formation of toxic compounds, enable the recovery of valuable components (e.g. lignin or furfural), use high solids loading, be effective for many lignocellulosic materials, reduce energy expenses, minimize operating costs and maximize the sugar yield in the subsequent enzymatic processing [45-47]. Pretreatment efficiency is usually assessed as: a) total amount of recoverable carbohydrates analysed as concentration of sugars released in the liquid and solid fraction after pretreatment, b) conversion of cellulose, expressed as the amount of sugars released by enzymatic hydrolysis of the solid phase, c) fermentability of released sugars, expressed as the amount of ethanol produced in the subsequent fermentation or d) its toxicity (concentration of inhibitory compounds released by sugar and lignin decomposition) analysed by HPLC or measured as the ability of test strains to grow.

Although it might seem that the problem of lignocellulose pretreatment has been solved by the chemical pulping process, which has been used commercially for a long time to produce various paper products, the opposite is true; despite most lignin is removed in these processes, they have been optimized to maintain the strength and integrity of cellulose fibres that are used for papermaking or as chemical feedstock and thus they are not easily hydrolysed by enzymes. The traditional sulfite pulping process was first reported in 1857 where treatment of wood with a mixture of sulfur dioxide in hot water considerably softened the wood; in 1900 the sulfurous acid process was patented [6]. Nowadays chemical pulp production based on the sulphite method [38] use sulfurous acid and its salts (Ca^{2+} , Mg^{2+} , Na^+ and NH_4^+) in combination with SO_2 as a cooking liquor at temperatures of 120 - 150 °C. Sulfurous acid is an impregnation agent, improving the penetration of hydrolytic chemicals inside the wood structure [48], and importantly, promotes sulfonation of lignin leading to formation of lignosulfonic acid and its salts, that are soluble [49, 50]. Combinations of salts and cooking conditions produce different qualities of cellulose and different compositions of the sulfite waste liquors. Possibility to optimize old sulphite pulping process to obtain higher degree of saccharification of hard and softwoods had led to various modifications of process condition [48, 51-54]. So called SPORL technique is based on application of solution of bisulphate salts and sulfur dioxide (sulfurous acid) on biomass; sulfuric acid can also be added depending on lignin content (the higher amount of sulfuric acid is necessary for biomass with higher content of lignin, e.g. softwood, eucalyptus).

Many other processes have been investigated over the last decades in order to intensify lignocellulose pretreatment process by exploiting various physical, chemical and biological methods or their combination as reviewed elsewhere [29, 47] and summarized in Table 2.

Pretreatment	Condition	Advantages	Disadvantages	Reference
Physical pretreatment				
Mechanical (chipping, shredding, milling, grinding)	Normal temperature and pressure	Decreased cellulose crystallinity, increased surface area, decreased degree of polymerization	High energy demand, no lignin removal	[29, 47]
Biological pretreatment				
Biological pretreatment – soft, brown or white rot fungi	Normal temperature and pressure	Low cost, low energy consumption, degradation of lignin and hemicellulose	Low efficiency, loss of carbohydrates (consumed by fungi), long residence times (10-14 days), need for carefully controlled growth condition, big space	[29, 45, 47]
Chemical pretreatment				
Dilute acid pretreatment (H ₂ SO ₄ , HCl, H ₃ PO ₄ , HNO ₃)	Concentration <4%, temperature 140-215 °C, pressure 0.5 MPa, reaction time seconds to minutes	High reaction rates, lignin disruption, increased accessibility of cellulose, improved digestibility, moderate temperatures	Little lignin removed, hemicellulose dissolved, sugar decomposition (inhibitors), need for acid recycling and pH adjustment	[29, 31, 45, 55]
Concentrated acid hydrolysis (H ₂ SO ₄ , H ₃ PO ₄)	Concentration 70-77%, temperature 40-100 °C	Crystalline structure of cellulose completely destroyed, amorphous cellulose achieved, low temperature	Hemicellulose dissolved, equipment corrosion, sugar decomposition (inhibitors), need for acid regeneration, pH adjustment, environmental concerns	[45]
Alkali pretreatment (NaOH, KOH, Ca(OH) ₂)	Temperature 25-130 °C	Decreased crystallinity of cellulose, decreased polymerization, lignin removal, few inhibitors	Hemicellulose dissolved, pH adjustment	[29, 55]
Ammonia pretreatment	Temperature 25-60 °C, reaction time several days	High delignification, cellulose swelling, high volatility of ammonia, low cost, ammonia	Cellulose crystallinity not reduced, environmental concerns	[45, 47]

Pretreatment	Condition	Advantages	Disadvantages	Reference
		recycle, continuous process, short residence times		
Ozonolysis	Room temperature, normal pressure, reaction time - hours	Lignin degradation, no inhibitors, ambient temperature	Hemicellulose dissolved	[21]
Combined acid and alkali pretreatment (formic acid-aqueous ammonia, dilute sulphuric acid-sodium hydroxide)		Cellulose digestion, fractionation of lignocellulose, most of non-cellulosic components removed, high loading		[45]
Combined acid and organic solvent (concentrated H ₃ PO ₄ + acetone),	Moderate temperatures	Cellulose crystalline structure disrupted, high yield of amorphous cellulose, lignin removed, reduced enzyme loading	Hemicellulose dissolved	[45]
Ionic liquid (IL) pretreatment	Temperature <100 °C, cellulose recovered by addition of water, ethanol or acetone	Lignin extraction, low temperature, high biomass loading, high lignin solubility, cellulose dissolution, solvents recovered and reused, environmentally friendly	Cellulose recovered by addition of acetone, IL deionized water or alcohol, IL denaturates enzymes, IL must be washed before reused	[29, 44, 45]
Physicochemical pretreatment				
Steam explosion	Temperature 160-240 °C, pressure 0.7-4.8 MPa, reaction time 1-10 min followed by biomass explosion	Extensive redistribution of lignin, high cellulose digestibility, cellulose swelling, limited use of chemicals	Little lignin removed, incomplete destruction of biomass matrix, sugar decomposition (inhibitors), hemicellulose dissolved, high energy consumption	[29, 31, 45, 55]
Acid-catalyzed steam explosion	Steam explosion catalysed by addition of H ₂ SO ₄ or SO ₂	Decreased time and temperature compared to steam explosion	Inhibitors formation, hemicellulose dissolved, high temperature	[45]

Pretreatment	Condition	Advantages	Disadvantages	Reference
Liquid hot water pretreatment	Temperature 180-230 °C, elevated pressure, pH 4-7, reaction time up to 15 min	Increased accessibility of cellulose, no inhibitors, no chemicals added, no need for pH adjustment and washing	Hemicellulose dissolved, lower loading	[45, 56]
Ammonia fiber explosion (AFEX)	Anhydrous liquid ammonia, temperature 60-120° C, pressure above 3 MPa, reaction time 30-60 min, followed decompression	Decreased crystallinity of cellulose, expanded fibre structure, increased accessible surface area, lignin depolymerisation and removal, low inhibitor concentrations, low temperature	Not suitable for softwood, hemicellulose dissolved, cost of ammonia, environmental concerns	[29, 31, 45, 55]
Ammonia recycle percolation	Aqueous ammonia (5-15%), temperature 150-180 °C, reaction time 10-90 min, flow 1-5 ml/min	Lignin removed, decreased crystallinity, low inhibitor concentrations, moderate temperatures	Hemicellulose dissolved, environmental concerns	[29]
Organosolv pretreatment	Organic (ethanol, methanol, ethylene glycol, glycerol, DMSO) or organic-aqueous mixtures, with catalyst at temperature >180 °C (HCl, H ₂ SO ₄), temperature 100-250°C	Biomass fractionalization, pure cellulose, selectivity, effective for high-lignin biomass, organic solvents easily recovered (distillation) and reused, less energy	Hemicellulose dissolved, high cost of chemicals, inhibitors formation, need for containment vessels, explosion hazard, environmental concerns	[29]
Carbon dioxide explosion treatment	Supercritical CO ₂ , pressure 7-28 MPa, temperature 200 °C, time – several minutes	Increased surface area, low cost chemical, no inhibitors, high solid loading	Effectivity increased with moisture content, costly equipment	[29]
Wet oxidative pretreatment	Addition of oxidizing agent (oxygen, water, hydrogen peroxide)	Low concentration of inhibitors	High pressure and temperature, costly equipment and chemicals (oxygen)	[29]

Table 2. Overview and main characteristics of methods leading to biomass pretreatment

Acid treatments lead mainly to hydrolysis of hemicelluloses (pentose and hexose fractions) while alkaline treatments bring about lignin removal. Concentrated acids such as sulphuric or hydrochloric have been used as powerful agents to treat lignocelluloses, but

due to their toxicity, corrosivity and necessity of recovery after hydrolysis, attention has shifted to milder conditions e.g. 0.5 % (v/v) sulfuric acid [57]. To improve cellulose hydrolysis in dilute acid processes, higher temperatures are favoured [58] since at a moderate temperature, direct saccharification resulted in low yields. As demonstrated by Candido et al. [59] for bagasse, dilute acid hydrolysis is greatly influenced by reaction time; at 100°C in 10% v/v sulfuric acid, the loss of mass and hemicellulose content decreased with time while soluble lignin concentration increased. Several modifications of the dilute acid hydrolysis method have been reported, e.g. acid hydrolysis with 1 % H₂SO₄ to remove hemicellulose and lignin followed by an alkaline step to increase the yield of cellulose. Methods based on the use of organosolv, wet oxidation, steam explosion or steam enriched with various impregnating agents (SO₂, CO₂, NH₃) are also often used for lignocellulose pretreatment as summarized in Table 2. The principle of the organosolv is mild hydrolysis of lignocellulose catalysed by sulfuric acid or sodium hydroxide in the reactor followed by extraction into ethanol at temperatures around 175 °C. Taking sugar cane bagasse as an example, the solid to liquid ratio can vary from 1 to 5 kg/l or lower, and solubilized lignin and hemicellulose appear in the liquid phase [34]. Wet oxidation is widely used in research and development technologies. Martín et al. [60] compared wet oxidation of bagasse, which was mixed with water (ca. 6 % w/v dry bagasse) in a special autoclave under slightly alkaline conditions, with steam explosion. In the wet oxidation procedure, slightly lower solubilisation of lignin, higher solubilisation of hemicellulose and higher cellulose content in the solid phase (approx. 60 % w/w) was achieved in comparison with steam explosion (45 % w/w). The effect of steam enrichment with CO₂ or SO₂ proved promising results as for enzymatic hydrolysis of cellulose and the low content of inhibitors, especially 2-furalaldehyde and 5-hydroxymethyl-2-furalaldehyde.

In summary, biomass pretreatment is a key bottleneck in the bioprocessing of lignocellulose biomass and even though all methods have distinct advantages, as summarized in Table 2, the main problems are high energy consumption and low substrate loading, leading to low sugar recovery. However, increasing the biomass concentration leads to high solid slurries which are very viscous, with a pasta-like behaviour, creating a challenge for mixing, pumping and handling; this increases energy demands reflected in a higher price for the ethanol as well as concentrates toxic compounds, thus counteracting any potential benefits [61].

Although the pretreatment process disrupts the complex structure of the material and causes partial hydrolysis of cellulose, the content of fermentable sugars is still very low; further enzymatic degradation of the cellulose polymeric chain must be carried out to increase the concentration of glucose, which is utilized (optimally together with hemicellulose-derived monomers) in fermentation as shown in Figure 1.

Most commercial enzyme preparations (the largest producers are Genencor, Novozymes or Spezyme) are produced by cultivation of *Trichoderma reesei* as mixtures of enzymes with endo-1,4-β-D-glucanase (EC 3.2.1.4, hydrolysis of (1→4) glucosidic linkages inside the chain), exo-1,4-β-glucosidase (EC 3.2.1.74, hydrolysis of (1→4) linkage in (1→4)-β-D-glucans to remove successive glucose units), β-glucosidase (EC 3.2.1.21, hydrolysis of terminal non-re-

ducing β -D-glucosyl residues with release of β -D-glucose) and β -1,4-glucan cellobiohydrolase (EC 3.2.1.91, hydrolysis of (1 \rightarrow 4)- β -D-glucosidic linkages in cellulose and cellotetraose releasing cellobiose from non-reducing ends of the chains) activities working in synergy.

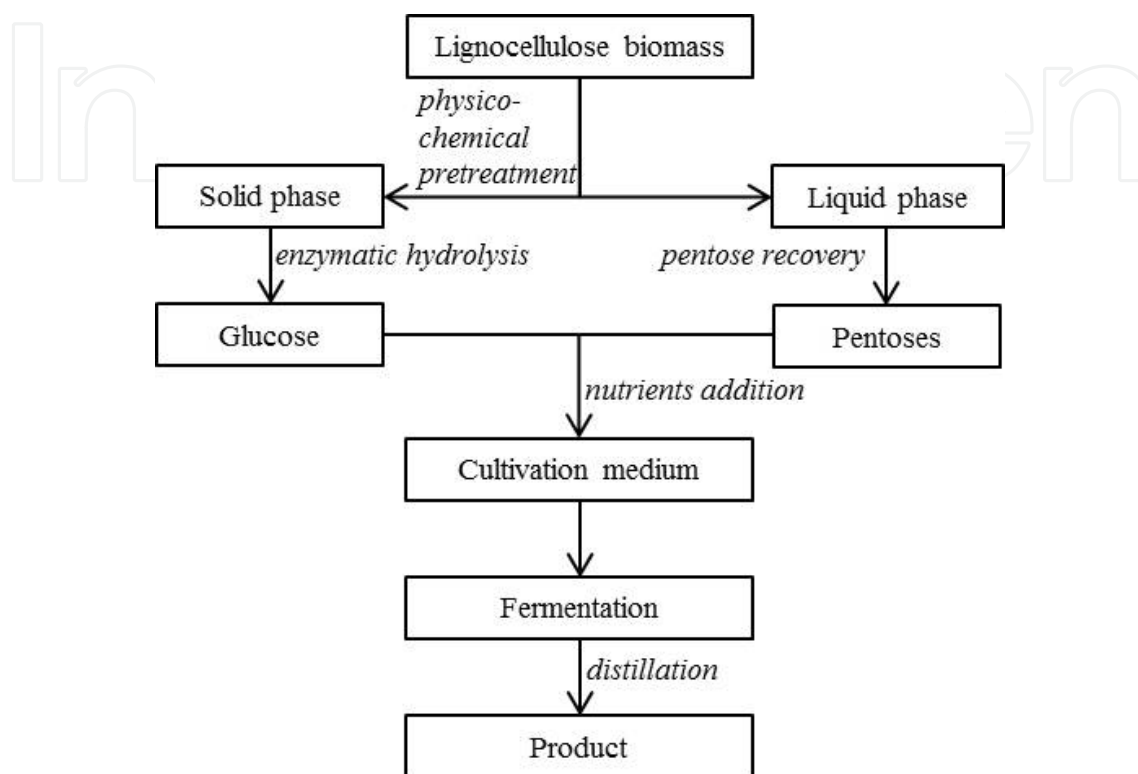


Figure 1. Simplified diagram of production of liquid biofuels from lignocellulose biomass

In recent years, the efficiency of commercial enzyme mixtures has rapidly increased and permits high conversions of cellulose to glucose; e.g. 85% and 91% yields of glucose were reported for ionic liquid pretreated poplar and switchgrass [62] and 85% and 83% yields were achieved for acid pretreated poplar and rice straws respectively [17, 63, 64]. Although the differential between the price of amylolytic and cellulolytic enzymes is currently reduced, the major difference is in dosing; about 40 -100 times more enzyme (based on protein weight) is required to breakdown cellulose compared to starch [29]. According to economic analyses, the conversion of biomass into fermentable sugars, which includes enzyme production and enzymatic hydrolysis together with indispensable pretreatment of biomass, comprises 33 % of the total cost [8, 17] and the estimated cost of cellulases is 50 cents per gallon (3.785 l) of ethanol, which is often comparable to the purchase cost of the feedstock [65]. For this reason attention has turned to further improvement of the composition and activity of enzyme cocktails, e.g. by constructing tailor-made multienzyme systems. It was shown that addition of xylanase and pectinase to alkali-pretreated biomass can reduce the negative effect of hemicellulose and pectin, which can restrict access of cellulases to the cellulose surface, while β -xylosidase can decompose xylobiose and polymerized xylooligomers

to avoid inhibition of cellulolytic enzymes [22, 45]. Unfortunately, improved enzyme cocktails are not generally applicable, e.g. an enzyme complex enriched with β -mannanase and amyloglucosidase improved digestibility of dried distillers grains, but this was not required for corn stover [22]. Furthermore, the rate and efficiency of enzymatic hydrolysis can be affected by enzyme adsorption to non-cellulolytic substrates, e.g. lignin through phenolic groups and hydrophobic interactions, which limits the accessibility of cellulose to cellulases [45, 47]. To reduce this effect, “designer cellulosomes” have been recently constructed [45]. The cellulosome is a large complex of cellulolytic enzymes, originally produced by anaerobic bacteria [66], and has been engineered to comprise a recombinant chimeric scaffolding protein and many bound protein hybrids that have low lignin binding affinity. A different approach is represented by the addition of non-catalytic additives, e.g. surfactants (e.g. Tween, polyethylene glycol), polymers or proteins (bovine serum albumin, gelatine), which compete with cellulolytic enzymes for adsorption sites of lignin and thus prevent non-productive enzyme binding and can also facilitate enzyme recycling. Addition of expansins (plant proteins), expansin-like proteins or swollenin (fungal protein) promotes enhanced enzymatic hydrolysis by disrupting hydrogen bonding between cellulose and other cell-wall polysaccharides [45]. Recycling of enzymes, e.g. by ultrafiltration, re-adsorption onto fresh substrate, enzyme immobilization onto various materials e.g. chitosan-alginate composite, chitosan-clay composite, Eupergit C, mesoporous silicates, silicagel or kaolin are other approaches to reduce pretreatment costs [45].

The activity of cellulolytic enzymes can be reduced not only by ineffective binding, but also by feedback inhibition by glucose and cellobiose released by hydrolysis of cellulose as reviewed by Andric et al. [67] and by inhibitory effects of toxic products that may be released during pretreatment (type and concentration depends on biomass and process conditions) and can affect not only the rate and yield of saccharification but also substrate fermentability.

4. Toxic compounds released in pretreatment process

Toxic products can generally be divided into three main groups – aliphatic acids, furan derivatives and phenolic compounds [68-70] released by degradation of carbohydrates, and compounds arising from lignin. In acidic solutions, cellulose and hemicellulose are broken down into hexose and pentose sugars, which are further decomposed at high temperatures into furan derivatives represented mainly by 2-furaldehyde (furfural, FF) and 5-hydroxymethyl-2-furaldehyde (hydroxymethylfurfural, HMF). Free aliphatic acids, represented mainly by acetic, formic or levulinic acids, are created by substituents cleaved from lignin and hemicelluloses within the pretreatment, or are produced by cells during fermentation, while phenolic derivatives (4-hydroxybenzoic acid, 3,4-dihydroxybenzoic acid or vanillin) arise mainly from lignin decomposition in alkaline solution [71]. About 40 lignocellulose degradation products have been identified in various hydrolysates [71], the type and amount depending on type of biomass and pretreatment conditions [68]; e.g. furfural, hydroxymethylfurfural and levulinic acid occur in higher concentrations at low pH combined

with high temperature and pressure [68, 71], while vanillin, vanilic, benzoic and 4-hydroxycoumaric acids are formed under alkaline conditions at elevated temperatures and acetic acid is produced in significant concentrations independent of the process and type of biomass [71]. Although many studies on the effect of inhibitors on cellulolytic enzymes have been published, a general conclusion is not easy to draw because it is influenced not only by the type and origin of the enzyme preparation, but also by its dosing and the concentration of inhibitors. However, in general, compounds exhibiting higher hydrophobicity tend to be more inhibitory to cellulolytic enzymes, the greatest inhibitory effect being caused by acetic and formic acids [72-74], while the activity of enzymes is not practically influenced by levulinic acid [73]. On the other hand, the presence of inhibitory compounds also affects ethanol productivity in the subsequent fermentation by influencing metabolic functions of ethanol producing strains. Inhibitory effects are described by type and concentration of toxic compounds (their effect is intensified when present in combination) and the strain used for ethanol production, but generally, fermentation is mainly influenced by the presence of furan derivatives together with phenolic compounds and weak acids (at low pH). As reviewed elsewhere [70, 75], low molecular weight compounds are able to penetrate the cell, while inhibitors with high molecular weights affect expression and activity of sugar and ion transporters. Growth and rate of ethanol production by *Saccharomyces cerevisiae*, the main ethanol producing strain, is significantly inhibited by furfural, while ethanol yield is almost not influenced [75] due to its ability to detoxify the broth by reduction of furfural to furfuryl alcohol, which is less toxic.

Surprisingly, in butanol production process, *C.beijerinckii* BA101, *C. acetobutylicum* P260, *C. acetobutylicum* ATCC 824, *Clostridium saccharobutylicum* 262 and *Clostridium butylicum* 592 were not sensitive towards sugar degradation products like furfural or hydroxymethylfurfural (up to concentrations of 2-3 g/l) but its growth and solvent production were inhibited by *o*-coumaric and ferulic acids present at a concentration of 0.3 g/l [76-78]. Solvent productivity and final solvent concentration in *C. beijerinckii* P260 were stimulated by addition of furfural or hydroxy methylfurfural (or both compounds) to the fermentation medium, at concentrations of up to 1 g/l [79]. *C. acetobutylicum* ATCC 824 metabolized furfural and hydroxymethyl furfural into furfuryl alcohol and 2,5-bis-hydroxymethylfuran, respectively and these compounds positively influenced solvent production up to a concentration of 2 g/l. It was hypothesised that this biotransformation step, independent of initial furfural and HMF concentrations, might increase solventogenesis via an increased rate of regeneration of NAD⁺ [80]. Another possible inhibitor of phenolic origin, syringaldehyde, caused inhibition of solvent production by *C. beijerinckii* NCIMB 8052 over the whole range tested (0.2-1 g/l). This inhibition was probably caused by decreased expression and activity of coenzyme A transferase, which participated in utilization of butyric and acetic acids, because these acids accumulated in the medium [81].

The inhibitory effects of toxic compounds released by sugars and lignin degradation can be reduced in several ways, e.g. optimization of pretreatment conditions to minimize the formation of inhibitors, use of specific detoxification methods, e.g. precipitation by calcium hydroxide (overliming) alone or in combination with sulphite addition, adsorption on

charcoal, evaporation of the volatile fraction, extraction with ethyl acetate or diethyl ether, ion extraction, treatment with peroxidase (E.C. 1.11.7) and laccase (EC 1.10.3.2), or use of microbial strains with increased resistance to inhibitors (achieved by adaptation or prepared by genetic modification) [75, 82, 83]. Lignin degradation products, *o*-coumaric, ferulic and vanillic acids, together with vanillin, were effectively removed from a model solution of phenolic compounds by treatment with 0.01 μ M peroxidase (E.C. 1.11.7), resulting in improved growth and butanol production by *C. beijerinckii* NCIMB 8052 [84]. Sulphuric acid-hydrolysed corn fiber was treated with XAD-4 resin, resulting in an improvement of butanol yield achieved with *C. beijerinckii* BA101 [85]. Another popular approach for detoxification of acid hydrolysates for butanol production is “overliming” i.e. addition of Ca(OH)₂ in excess to hydrolysate [78, 85]. Although this detoxification method has been known for a long time, its mode of action, especially in the case of butanol production, is not completely clear. Addition of Ca(OH)₂ to an acid hydrolysate decreases furfural and HMF concentrations [86, 87] but does not affect acid concentrations; thus it is only possible to assume a beneficial neutralization effect. Furthermore it may be useful to treat hydrolysates with activated carbon [88].

5. Fermentation of lignocellulosic substrates

5.1. Ethanol fermentation

Fermentation of lignocellulose hydrolysates is more complicated compared to fermentation of 1st generation feedstock (sugar cane juice, molasses, grains) for several reasons: a) pentose sugars (predominantly xylose) are present along with hexoses (mainly glucose, mannose, galactose) in the hydrolysate, b) toxic compounds released during pretreatment can influence metabolic activity of the fermentation strain, c) low concentrations of fermentable sugars hamper the attainment of a high ethanol concentration. Because lignocellulose hydrolysates are poor in some nutrients (phosphorus, trace elements, and vitamins) they are usually supplemented, e.g. by addition of corn steep or yeast extract before being used as a substrate for fermentation. For an efficient process it is necessary to identify a strain that utilizes both pentose and hexose sugars, produces ethanol with a high yield and productivity and is tolerant to both inhibitors and ethanol. One of the main challenges is to simultaneously co-ferment pentose and hexose sugars, but neither yeast *S. cerevisiae* nor the bacterium *Z. mobilis*, which are usually used for ethanol production, contain genes for expression of xylose reductase and xylitol dehydrogenase [89]. In order to enhance process effectiveness, co-fermentation or sequential fermentation of hexoses and pentoses has been examined by combining good ethanol producers with strains naturally utilizing pentoses e.g. *Pichia stipitis*, *Candida shehatae*, *Pachysolen tannophilus*, *Klebsiella oxytoca*. However, xylose utilization is the rate limiting step due to catabolite repression by hexoses and the low availability of oxygen, and inhibition of pentose-utilizing strains by ethanol [90, 91]. Moreover, the yield of ethanol by co-fermentation is usually lower than with separate processes, e.g. yields of 0.5 g ethanol per g glucose (98% of theoretical) and 0.15 g/g xylose (29% theoretical) were achieved by separate cultivation of *Z. mobilis* and *P. tannophilus* respectively, but in optimized

co-fermentation, the yield was just 0.33 g ethanol/g sugar. The same yield was obtained in a 5-reactor process combining *P. stipitis* and *S. cerevisiae* [92], but it was enhanced to 0.49 g/g sugars (96% theoretical) by cultivation of an adapted co-culture of *S. cerevisiae*, *P. tannophilis* and recombinant *E. coli* in dilute-acid softwood hydrolysate [93]. In a subsequent process employing *P. stipitis* and *S. cerevisiae*, which was inactivated before *Pichia* inoculation to avoid oxygen competition, 75% of theoretical ethanol yield was achieved [94]. A different approach is represented by the use of a recombinant strain prepared either by cloning genes encoding xylose utilization into good ethanol producers or to construct synthetic pathways for ethanol production in pentose-utilizing hosts. Wild type yeasts can be genetically modified to utilize xylose by introducing fungal genes encoding xylose reductase and xylitol dehydrogenase or bacterial/fungal genes for xylose isomerase [95]. Yeast *S. cerevisiae* was transformed with the *xylA* gene from *Thermus thermophilus* and *Piromyces sp.* to produce xylose isomerase, but unfortunately, this enzyme was inhibited by xylitol, favouring instead, its formation. Recently a recombinant strain of *S. cerevisiae* expressing a heterologous *xylA* gene produced 0.42 g/g of ethanol from xylose [96]. A strategy using *xyl1* and *xyl2* genes from *P. stipitis* introduced into *S. cerevisiae* produced transformants that exclusively consumed xylose, but produced significant amounts of xylitol [97]. On the other hand, with recombinant *Z. mobilis*, which carried *E. coli* genes encoding for xylose isomerase, xylulokinase, transketolase and transaldolase, 86% ethanol yield from xylose was achieved. Another strain of *Z. mobilis*, expressing genes *araABD* from *E. coli*, encoding L-arabinose isomerase, L-ribulokinase, L-ribulose-5-P-4 epimerase together with genes for transketolase and transaldolase, was able to grow on arabinose with 98% ethanol yield. *E. coli*, which naturally utilizes a wide range of substrates including pentoses, was transformed by genes encoding pyruvate decarboxylase and alcohol dehydrogenase, resulting in enhanced ethanol production [96]. Adaptation of recombinant strains to inhibitors can further increase the yield of ethanol, e.g. the ethanol yield achieved with a genetically engineered strain of *S. cerevisiae* grown on bagasse hydrolysate was increased from 0.18 g/g to 0.38 g/g after adaptation [98]. Recombinant strains that not only consume pentoses but also hydrolyse hemicelluloses by co-expressing endoxylanase, β -xylosidase and β -glucosidase activities has recently been constructed [95] and yields of 0.41 g/g of ethanol were obtained from total sugars in a rice straw hydrolysate.

In addition to the wide range of sugars, their low concentration in hydrolysates is problematic. Since ethanol recovery by distillation is only economically viable on the industrial scale for yields greater than 4% (w/w), which for most hydrolysates requires a dry mass concentration greater than 20% [45], the use of high substrate loading is needed. Effect of substrate concentration (unbleached hardwood pulp and organosolve pretreated poplar) on glucose concentration resulting from the enzyme hydrolysis was studied in [52] and [99]. In laboratory scale after 48 h of enzymatic hydrolysis 158 g/l glucose in the hydrolyzate was reached, ethanol concentration after fermentation ranged between 50.4 and 63.1 g/l. The general problem for this kind of conversions is that high load of the pulp or pretreated lignocellulosic material gives rise to high viscosity and thus also to mixing and transport problems. These extremely high yields of glucose can be attributed to a very efficient peg mixer. Problems connected with use of such high viscosity slurries can be overcome by various strat-

gies, e.g. maximizing dry matter by removing most hemicellulose and lignin, utilizing alternative bioreactors with novel mixing modes (e.g. peg mixer, shaking, gravitational tumbling, hand stirring) or gradual dosing of substrate into the bioreactor (fed-batch), which enables the use of more substrate and thus increases the yield of ethanol above values achievable in batch mode. Moreover, the actual concentration of toxic substrates is reduced and yield and/or productivity is enhanced by controlled dosing of substrate and prolonged cultivation time, thus shortening unprofitable periods between batches [45, 89]. Feed rates should reflect the type of hydrolysate and strain. Continuous cultures usually using immobilized cells (to prevent their wash out from the bioreactor at high dilution rates) is another strategy to increase process productivity [89].

Integration strategies, which replace classical separate hydrolysis and fermentation processes (SHF) by combining several process steps in one vessel represents another approach for lignocellulosic ethanol production. Simultaneous saccharification and fermentation (SSF), which combines enzymatic hydrolysis and fermentation in one step, permits an increased rate of cellulose hydrolysis by elimination of product inhibition (the released glucose is consumed by the microbial strain), an increased rate of sugar consumption, reduced contamination due to the presence of ethanol and a reduced number of reactors. However, SSF is constrained by different temperature optima for each process (the cellulase optimum is usually 40-50 °C, whereas the fermentation temperature usually cannot exceed 35 °C for most ethanol producers) and carbon source limitation in the early stages of the process. Several modifications of SSF to ease the problems and increase productivity have been published. These include the use of thermotolerant ethanol producers [100, 101], application of a pre-saccharification step [102] or the use of recombinant strains consuming both hexose and pentose sugars (a simultaneous saccharification and co-fermentation process (SSCF)) [103] in batch or fed-batch mode [104]. Consolidated bioprocessing (CBP), which combines cellulase production, cellulose hydrolysis and fermentation into a single step have been investigated as a way of reducing the cost of cellulolytic enzymes, increasing volumetric productivity and reducing capital investment [105]. Some biofuel companies (e.g. Mascoma and Qteros) have been founded based on this concept [105]. CBP microorganisms should combine high cellulase production and secretory capability, the ability to utilize a broad range of sugars, tolerance to high concentrations of salts, solvents and inhibitors, high ethanol productivity and yield, have a known genomic DNA sequence and developed recombinant technologies and ideally be usable as feed protein after fermentation [105]. There is a lack of native organisms that combine the ability to produce cellulolytic enzymes and be homoethanolic with high titres and yields. Although some thermophilic anaerobic bacteria e.g. *Clostridium thermocellum*, are high cellulase producers and utilize both pentose and hexose sugars, they have a low tolerance to ethanol ~30 g/l [106] and an insufficient yield ~0.2 g/g [107]. Therefore recombinant strains have been prepared by engineering cellulolytic microorganisms (e.g. *C. thermocellum*, *C. phytofermentans*, *C. cellulolyticum*, *T. reesei* or *F.oxysporum*) to produce ethanol. Knockout mutants of *Thermoanaerobacterium saccharolyticum* that lack lactic and acetic acid production exhibited an ethanol yield from xylose of 0.46 g/g [108], while recombinant *Geobacillus thermoglucosidasius* produced 0.42-0.47 g/g of ethanol from hexoses [65]. Another attempt, to create a recombinant cellulose-utilizing microorganism using non-cellu-

lytic strains with high ethanol production have not been very successful; although some recombinant ethanologenic strains secreting some active cellulases have been prepared [106, 109, 110], their requirement for a nutrient rich medium and often sensitivity to end-product inhibition hamper their use [105].

5.2. ABE (acetone-butanol-ethanol) fermentation

Different, so-called solventogenic species of the genus *Clostridium*, like *Clostridium acetobutylicum*, *Clostridium beijerinckii* or *Clostridium saccharoperbutylacetonicum*, can be used for 1-butanol production by ABE fermentation. The fermentation usually proceeds in two steps; at first butyric and acetic acids, along with hydrogen and carbon dioxide, are formed and then metabolic switching leads to the formation of solvents (mainly 1-butanol and acetone) and the cessation/slowdown of acid and gas production (for recent reviews see [111-114]). Industrial fermentative ABE (butanol) production, which has quite a long and impressive history connected with both World Wars, is nowadays carried out only in China and Brazil (estimated annual production of 100 000 t and 8 000 t from corn starch and sugar cane juice, respectively) [10]. However, many corporations such as BP, DuPont, Gevo, Green Biologics, Cobalt Technologies and others have declared their interest in this field. A unique example of the use of lignocellulosic hydrolysate on an industrial scale is the former Dukshukino plant (operated in the Soviet Union up to 1980s) producing acetone and butanol by fermentation. The plant was based on current "very modern" biorefinery concepts which assumed the conversion of complex feedstocks (hydrolysates of agricultural waste + molasses or corn) into many valuable products i.e. in addition to solvents (acetone, butanol and ethanol), it was possible to produce liquid CO₂, dry ice, H₂, fodder yeast, vitamin B₁₂ and biogas [115].

The most interesting approach to fermentation of any lignocellulosic substrate is probably consolidated bioprocessing (CBP) i.e. a method in which a single microorganism is used for both substrate decomposition and fermentation to produce the required metabolites. Although some clostridial species such as *Clostridium thermocellum* can utilise cellulosic substrates and produce ethanol [116, 117], the ABE fermentation pattern unfortunately cannot be produced using clostridia. However, *C. acetobutylicum* ATCC 824 possesses genes for various cellulases and a complete cellulosome [118-120]. But even if production of some cellulases by *C. acetobutylicum* ATCC 824 was induced by xylose or lichenan [118], cellulose utilization was not achieved, possibly because of insufficient or deficient synthesis of an unknown specific chaperone that could be responsible for correct secretion of cellulases [119]. Nevertheless as solventogenic *Clostridium* species are soil bacteria that differ significantly in fermentative abilities and genome sizes, it is not excluded that in the future, some solventogenic species with cellulolytic activity will be isolated from an appropriate environment. Recently, a new strain of *Clostridium saccharobutylicum* with hemicellulolytic activity and ABE fermentation pattern was found amongst 50 soil-borne, anaerobic, sporulating isolates [121].

Substrate	Pretreatment	Microbial strain	ABE concentration (g/l) /yield (%) /productivity (g/l/h)	Reference
Wheat straw	Diluted sulphuric acid+ enzyme	<i>C. beijerinckii</i> P260	13/25/0.14	[139]
Wheat bran	Diluted sulphuric acid	<i>C. beijerinckii</i> ATCC 55025	12/32/0.16	[140]
Corn fiber	Diluted sulphuric acid+ XAD-4 resin treatment + enzyme	<i>C. beijerinckii</i> BA101	8/32/0.11	[127]
Corn cobs	Steam explosion + enzyme	<i>C. acetobutylicum</i>	21/31/0.45	[131]
Rice straw	Alkali + (NH ₄) ₂ SO ₄ precipitation + activated carbon treatment + enzyme	<i>C. saccharoperbutylacetonicum</i> ATCC 27022	13/28/0.15	[88]
Sugar cane bagasse	Alkali + (NH ₄) ₂ SO ₄ precipitation + activated carbon treatment + enzyme	<i>C. saccharoperbutylacetonicum</i> ATCC 27022	14/30/0.17	[88]
Cassava bagasse	Heat + enzyme	<i>C. acetobutylicum</i> JB200	34/39/0.63	[130]
Domestic organic waste	Steam explosion, lyophilization + enzyme +4 fold concentration of released sugars	<i>C. acetobutylicum</i> DSM 792	9/26/0.08	[132]
Dried distiller`s grain and solubles	Diluted acid + overliming +enzyme hot water + overliming+ enzyme AFEX +overliming+ enzyme	<i>C. saccharobutylicum</i> 260 <i>C. butylicum</i> 592 <i>C. butylicum</i> 592	12/35/0.20 13/32/0.20 12/32/0.20	[78]
Sweet sorghum stem	Diluted acetic acid	<i>C. acetobutylicum</i> ABE 0801	19/32/0.10	[141]

All fermentations were run in SHF mode i.e. sugar release and fermentation were separate processes.

AFEX stands for ammonium fiber expansion process.

Table 3. Selection of batch ABE fermentations in laboratory scale using lignocellulosic hydrolysates as a substrate

Until now, lignocellulosic substrates must be prehydrolysed for the ABE process. In the case of fermentation of lignocellulosic hydrolysate, usually containing low concentrations of fermentable sugars, one of the main bottlenecks in the ABE process, the low final titre of buta-

nol (caused by severe butanol toxicity towards bacterial cells), is of minor importance. In fact hydrolysates are very good substrates for clostridia that express extensive fermentative abilities [122, 123] and can utilise not only cellulose-derived glucose but also hemicellulose monomers (xylose, arabinose, galactose, mannose). Co-fermentation of various sugar mixtures was described for *Clostridium beijerinckii* SA-1 (ATCC 35702) [124], *Clostridium acetobutylicum* DSM 792 [125], *C. acetobutylicum* ATCC 824 [126] and *C. beijerinckii* P260 [127] however, at the same time, catabolic repression of xylose utilization in the presence of glucose was demonstrated in *C. acetobutylicum* ATCC 824 [128, 129].

An overview of fermentation parameters achieved in batch ABE fermentations of different hydrolysates is presented in Table 3. The most promising results were obtained by Lu et al. [130] using cassava bagasse and a mutant strain, *C. acetobutylicum* JB200; the results of Marchal et al. [131] were unique at the scale used (48 m³) as shown in Table 3. A frequent problem of lignocellulosic hydrolysates is a low final concentration of fermentable sugars caused by low density of the original substrate. This can be overcome by evaporation of the hydrolysate [132] (see Table 3) or by addition of glucose and/or other carbohydrates present in the hydrolysate (this is only possible in laboratory scale experiments) [85, 133-135]. In the case of glucose supplemented corn stover and switchgrass hydrolysates, final ABE concentrations of 26 and 15 g/l were achieved [135]. With *C. beijerinckii* P260, use of diluted and Ca(OH)₂ treated barley straw hydrolysate supplemented with glucose resulted in a solvent concentration of 27 g/l, a yield of 43% and productivity of 0.39 g/l/h [133]. In addition to materials presented in Table 3, other substrates like diluted sulfite spent liquor supplemented with glucose [134], palm empty fruit bunches [136, 137] or hardwood [138] were used in the ABE process but in these cases, additional optimizations were necessary.

In addition to a batch fermentation arrangement, semi-continuous fermentation of enzymatically hydrolyzed SO₂ pretreated pine wood using *C. acetobutylicum* P262 resulted in 18 g/l of solvents, a yield of 36% and solvent productivity of 0.73 g/l/h [142]. Further, fed-batch fermentation of wheat straw hydrolysate supplemented with varying concentrations of hydrolysate sugars (glucose, xylose, arabinose and mannose) using *C. beijerinckii* P260 yielded a solvent productivity of 0.36 g/l/h if gas stripping was used [127]. In the cases shown in Table 3, enzyme hydrolysis preceded fermentation, however simultaneous saccharification and fermentation (SSF) was also tested. In SSF of acid pre-hydrolyzed wheat straw using *C. beijerinckii* P262 and solvent removal by gas stripping, 21 g/l of ABE was produced with a productivity of 0.31 g/l/h [127]. Nevertheless, the solvent yield from hardwood using SSF was rather low, at 15% [138].

6. Conclusion

Intensive research over the last decades on lignocellulose-derived ethanol have focused mainly on intensification of biomass pretreatment, production of cellulolytic enzymes, and strain and process improvements, and have eliminated some of the main technological bottlenecks. Although a number of projects on 2nd generation bioethanol ended with

the opening of pilot and demonstration plants around the world (production capacity in millions of gallons for the year 2012 given in brackets) e.g. the POET demonstration plant in Iowa (0.02 from corn stover and cobs), Abengoa in Kansas (0.01 from corn stover), Blue Sugarsin Wyoming (1.3 from stover and cobs), Chempolis in Finland (3.7 from paper waste), Fiberight in Iowa (6.0 MSW), Iogen in Canada (0.48 from stover), Praj MATRIX in India (0.01 from cellulose), UPM-Kymmene/Mesto in Finland (0.68 from mixed cellulose) and in spite of several proclamations, none of them is operating at the industrial scale [9]. To make this possible, further reductions in processing costs will be necessary to achieve a product that is competitive with 1st generation bioethanol. Further process integration is required, including decreased energy demand during pretreatment, increased sugar concentration, higher enzyme activity and strain recycling. By-products, e.g. lignin separated after pretreatment procedure can be used to generate energy for ethanol plant operations (lignin has higher caloric value (25.4 MJ/kg) than the biomass itself [8]) or used as a dispersant and binder in concrete admixtures, as an alternative to phenolic and epoxy resins, or as the principal component in thermoplastic blends, polyurethane foams or surfactants [143]. A combination of 1st and 2nd generation feedstocks (e.g. corn cobs together with stover) can eliminate bottlenecks and lead to product competitiveness. Higher bioethanol production costs can also be compensated for by political and economic instruments such as tax incentives (e.g. tax exemption on biofuels and higher excise taxes for fossil fuels) and legislation (mandatory blends) to enable ready access of 2nd generation biofuels to the market [30]. Butanol, as a second generation biofuel, might be produced via fermentation and used as an excellent fuel extender in addition to ethanol if the technological bottleneck of a low final concentration, yield and productivity could be overcome, and the assumption that suitable cheap waste pretreatments were possible.

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