Chapter 16
Production of Bioethanol from Biomass: An Overview

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Abstract This chapter analyzes the main research trends on production of fuel ethanol from lignocellulosic materials. The main features of different pretreatment and detoxification methods are presented. The importance of process integration to simplify the overall process and improve the conversion of biomass into ethanol is discussed. Strategies for microbial strain development are disclosed in the framework of such integrated processes like simultaneous saccharification and co-fermentation and consolidated bioprocessing. The main challenges to fully develop the biomass-to-ethanol process are highlighted. Finally, the need of integrating the research efforts on molecular techniques and process integration is recognized.

16.1 Lignocellulosic Biomass as Feedstock

The biomass is organic matter made by living organisms that contain energy stored from the sun. The radiant energy from sunlight is absorbed by plants. This energy is converted into chemical energy in the form of glucose, starch or cellulose, through photosynthesis. The energy contained in the biomass (bioenergy) can be released and used by means of its combustion. Thus, the woody biomass is employed by many rural communities all around the world for heating and cooking. The biomass can also be burned in boilers to produce heat and electricity (solid biofuels). In addition, it can be transformed into liquid biofuels that, in turn,
are used by the transport sector. Many organic materials can be converted into gaseous biofuels by anaerobic digestion. In this way, the biomass has become the fourth largest energy source after coal, oil and natural gas. It is the most important renewable energy option at present. In terms of energy, the annual global primary production of biomass is equivalent to the 4,500 EJ (1 EJ = 10^{18} J) of solar energy captured each year. The global energy consumption is 490 EJ today, but the current use of biomass for energy is only 50 EJ, mainly in the form of traditional noncommercial woody biomass (Ladanai and Vinterbäck 2009).

The lignocellulosic biomass represents a source of sugars with a great availability on Earth. Many of the materials with a high lignocellulosic content are wastes from different economic activities, in particular, agricultural residues. Thus, their economic utilization for biofuel production implies the employ of plant material not usable for human food. The lignocellulosic biomass has the potential of being a valuable feedstock for production not only of liquid and solid biofuels, but also of a relatively wide spectrum of chemicals and materials. In fact, in the framework of the future biorefineries, the biomass can become the long-term source of hydrocarbon chains and building blocks needed for the humankind to meet its huge requirements of basic organic compounds, synthetic polymers, pharmaceuticals, housing products, and among many others.

In the specific case of the liquid biofuels, the lignocellulosic biomass has acquired great relevance due to its significant content of different fermentable sugars that can be processed into ethanol or even into other fuel alcohols like butanol. These so-called second generation biofuels do not employ the fermentable sugars obtained from resources utilized for food production as sugarcane, sugar beet, corn, or other cereals. Thus, the “food vs. fuel” dilemma can be solved in an environmentally and socially sustainable way leading to the potential global utilization of the vast biomass resources available in almost each country of the world. This ideal situation contrasts with the control exercised by governments and corporations on fossil resources, which are not distributed evenly in the crust. In addition, the biomass is a renewable resource that can be used to sustainably supply bioethanol over the long term. Moreover, it is recognized that the utilization of fuel ethanol produced from lignocellulosics allows the net reduction of greenhouse gas emissions and can contribute to the diversification of rural economies in particular cases (e.g. dedicated energy crops).

However, the main drawback of the lignocellulosic biomass is its recalcitrance that imposes several challenges to the scientific and engineering global community. Due to this recalcitrance, the valuable sugars contained in the biomass are not easily accessible making necessary the employ of chemical and biochemical agents and physical chemical procedures to breakdown the complex lignocellulosic structure. In addition, the most used industrial ethanologenic microorganisms are not capable of assimilating in an efficient way all the sugars released during the processing of biomass. This decreases the conversion and product yield of the overall process and discourages high scale commercial projects for bioethanol production from lignocellulosic materials. Fortunately, the ongoing research and development as well as the existing demonstrative facilities allow being optimistic
about the possibility to use this versatile resource for fuel ethanol production on a large scale as a realistic alternative to fossil fuels.

The lignocellulosic complex is the most abundant biological material on Earth. Its production is estimated at about \(200 \times 10^9\) ton per year, only 3% of which is used in nonfood areas, such as the paper and pulp industries (Zhang 2008). An important fraction of economically significant crops corresponds to lignocellulosic materials. For instance, in European countries, 35% of the harvested over-ground biomass of the wheat crop is straw and 45% is grain (Claassen et al. 1999). The lignocellulosic biomass is made up of very complex biopolymers nonusable for food. The main components of lignocellulosic biomass are cellulose, hemicellulose, and lignin in addition to a small amount of extractives, acids, and minerals (Cardona et al. 2010b).

The cellulose, a \(\beta\)-glucan, is a polymer composed of glucose molecules linked by \(\beta\) (1,4) bonds. It contains between 7,000 and 15,000 glucose units. Due to its linear nature and to the interactions by hydrogen bonds between the OH groups of a same chain or of different chains, cellulose forms very stable crystalline microfibers difficult to break. In general, the cellulose composes 40–60% of dry matter of lignocellulosic biomass (Hamelinck et al. 2003). The hemicellulose composes 20–40% of lignocellulosic biomass and consists of very short branched chains of monosaccharide units (200 in average). In their order, the monosaccharides present in hemicellulose are xylose and arabinose (both pentoses), and galactose, glucose and mannose (these latter sugars are hexoses). Other carbohydrate-related compounds like glucuronic, methyl glucuronic and galacturonic acids are also present in hemicellulose structure. Furthermore, the hemicellulose contains, in a lower proportion, acetyl groups esterified to some OH groups of its different sugars. Due to the predominance of xylose, the hemicellulose can be considered as a xylan. Considering its branched structure, the hemicellulose does not form crystalline structures but amorphous ones. Thus, this biopolymer is more soluble in water and has a higher susceptibility to the hydrolysis (Hamelinck et al. 2003). The cellulose and hemicellulose are the source of fermentable sugars for different process microorganisms to convert them into ethanol.

The lignin comprises from 10 to 25% of lignocellulosic biomass. This component is a very complex phenolic polymer composed of phenyl propane units linked by C–C and C–O–C bonds forming a three-dimensional amorphous structure (Lee 1997). The structural units of lignin are based on the cinnamyl alcohols. Thus, \(p\)-hydroxyphenyl units are derived from the \(p\)-coumaryl alcohol, the guaiacyl units are derived from the coniferyl alcohol, and the syringyl units are derived from the sinapyl alcohol. The lignin has hydrophobic character and is a sort of cement between the cells. The interaction and combination between the hemicellulose and lignin provide a covering shell to the cellulose making its degradation more difficult.

The different lignocellulosic materials can be classified into the following groups according to their origin: agricultural wastes (straws, corn stover), agroindustrial residues (sugarcane bagasse, olive stone), hardwood (poplar, oak, birch, and aspen), softwood (pine, fir, cedar, and spruce), cellulosic wastes (newspaper,
waste office paper, and paper sludge), herbaceous biomass (switchgrass, alfalfa hay, and coastal Bermuda grass), and municipal solid waste (wasted paper, cardboard, fruit and vegetable peels, garden residues, and wood items). The most representative materials regarding their potential as feedstock for fuel ethanol production among the agricultural and agro-industrial residues are sugarcane bagasse, corn stover, and wheat straw.

Sugarcane bagasse is generated in sugar mills after extracting the juice from the cane stalks. It is used as a solid biofuel for production of steam and electricity required by the same sugar-production process, remaining frequently an electricity surplus to be sold to the grid (Cardona and Sánchez 2007). About 540 million tons per year of sugarcane bagasse are produced (Cardona et al. 2010a). The yield of bagasse from each ton of sugarcane ranges from 280 kg (Moreira 2000) to 312 kg (Kim and Dale 2004). In turn, the ethanol yield from bagasse reaches 140 L/ton, which implies a global potential production of ethanol from cane bagasse of 58.2 million L/year, an amount greater than all the ethanol produced worldwide in 2007 (Cardona et al. 2010b; Kim and Dale 2004). One feature of cane bagasse related to the other lignocellulosic materials is its low ash content (about 2.4 %) that implies a better performance during fermentation.

Corn stover comprises the stems, leaves, and cobs resulting from corn harvest and is considered as a very promising feedstock for ethanol production in USA, since it is the most abundant agricultural residue in that country: 196 million ton (Graham et al. 2007). A factor to be considered in the case of agricultural residues is that, unlike sugarcane bagasse, their total utilization can lead to soil erosion and reduction of its organic matter content, so the sustainable fraction of collectable crop residues should be defined. Conservation tillage practices for crop residue removal require that 30 % or more of the soil surface be covered with crop residues after planting to reduce soil erosion by water (Kim and Dale 2004). Cereals straws comprise the dry stalks of a cereal plant, crushed or not, after the grain or seed has been removed and have a high content of hemicellulose related to cellulose. Wheat straw presents a high availability in the world (about 529 million ton/year). Wheat straw is an attractive low cost feedstock for production of fuel ethanol because of abundance, renewability and low lignin content. Wheat straw contains lower amounts of lignin and higher levels of cellulose and hemicellulose compared to corn stover (Buranov and Mazza 2008).

Wood lignocellulosic biomass has been considered as a potential feedstock for bioethanol not only in the case of the wood itself, but also in the case of its derivatives (sawdust, shavings, and the collected biomass resulting from forestry activities such as branches, stalks, trunk pieces and trees from forest thinning). Softwood has a higher content of lignin making it more difficult to process into fuel ethanol compared to hardwood. Herbaceous biomass, in turn, represents the lignocellulosic materials from grasses and related plants that have neither woody stems nor woody roots. These plants present reduced lignin content, grow very fast and have reduced nutritional requirements, so they are excellent candidates for their exploitation as crops dedicated to bioenergy production. In this sense, switchgrass has been proposed repeatedly with this purpose.
16.2 Biomass-to-Ethanol Process

Conversion of lignocellulosic raw materials into fuel ethanol is a complex process. It requires several steps, some of which are not completely developed at the present day. Compared to the ethanol production from sucrose-containing materials like sugarcane and sugar beet, the biomass processing implies a greater amount of unit processes and involves a higher amount of substances to be dealt with. For instance, after the breakdown of the lignocellulosic complex, many compounds are released along with several types of carbohydrates (fermentable and not fermentable) present in the lignocellulosic matrix. In addition, some degradation products are formed that can be toxic for fermenting microorganisms. For this reason, it is necessary to include additional steps such as detoxification and neutralization of the pretreated biomass. Moreover, the main component to be transformed into ethanol, the cellulose, should be hydrolyzed prior to or simultaneously with the fermentation step. This hydrolysis step is also distinctive of the production of fuel ethanol from starchy materials like corn. The comparison of ethanol production processes from the three main types of feedstocks is presented in Table 16.1.

Regarding the possibilities to implement several ways of process integration, the biomass-to-ethanol process offers some significant opportunities, which is derived of its inherent complexity. As a mean to decrease the productions costs and improve the efficiency of the different processing steps, the accomplishment of two or more procedures in one single vessel or in a coupled way has become one of the main research directions for production of ethanol from biomass. The goal is to decrease the cost of producing one liter of ethanol from lignocellulosic materials compared to the employ of sucrose-containing feedstocks or starchy materials (see Table 16.1).

The conversion of lignocellulosic biomass into fuel ethanol requires several process steps: pretreatment, detoxification, cellulose hydrolysis, fermentation, ethanol separation and dehydration, and effluent treatment. In addition, the production of some co-products may require some additional steps in the framework of the biorefinery concept.

16.2.1 Pretreatment of Lignocellulosic Biomass

The pretreatment plays a key role in the overall process for ethanol production from biomass. If this step is not successfully accomplished, the conversion of feedstocks will become very low and the costs will be unjustifiably high. The pretreatment procedures themselves and their efficiency directly affect the amount, availability, and quality of the fermentable sugars that will be assimilated by the microorganisms during the subsequent fermentation, the step where the ethanol is formed. At the same time, this process step is energy consuming and may require the addition of chemicals that need to be accounted in the final ethanol cost and environmental performance of the global process. In fact, the pretreatment is one
<table>
<thead>
<tr>
<th>Aspect</th>
<th>Sugar to ethanol</th>
<th>Starch to ethanol</th>
<th>Biomass to ethanol</th>
</tr>
</thead>
<tbody>
<tr>
<td>Availability of raw materials</td>
<td>High</td>
<td>Medium</td>
<td>Very high</td>
</tr>
<tr>
<td>Utilization of food resources</td>
<td>Yes</td>
<td>Yes</td>
<td>No</td>
</tr>
<tr>
<td>Conditioning of feedstock</td>
<td>Milling, simple pH adjustment</td>
<td>Wet or dry milling</td>
<td>Milling</td>
</tr>
<tr>
<td>Pretreatment</td>
<td>Not required</td>
<td>Not required</td>
<td>Required; partial or total degradation of hemicellulose</td>
</tr>
<tr>
<td>Detoxification</td>
<td>Not required</td>
<td>Not required</td>
<td>Some degradation products after pretreatment should be removed</td>
</tr>
<tr>
<td>Conditioning of feedstock</td>
<td>Milling</td>
<td>Milling</td>
<td></td>
</tr>
<tr>
<td>Pretreatment</td>
<td>Required; partial or total degradation of hemicellulose</td>
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</tr>
<tr>
<td>Detoxification</td>
<td>Some degradation products after pretreatment should be removed</td>
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<td>Some degradation products after pretreatment should be removed</td>
</tr>
<tr>
<td>Hydrolysis of carbohydrate polymers</td>
<td>Not required Amylases are used</td>
<td>Cellulases or acids are used</td>
<td></td>
</tr>
<tr>
<td>Fermentation</td>
<td>Batch and continuous regimes; fermentation of glucose and fructose</td>
<td>Batch and continuous regimes; glucose fermentation</td>
<td>Mainly batch processes; hexose fermentation, pentose fermentation (optional)</td>
</tr>
<tr>
<td>Process microorganism</td>
<td>S. cerevisiae, Z. mobilis</td>
<td>S. cerevisiae, Z. mobilis, K. marxianus; amylolytic recombinant yeasts</td>
<td>S. cerevisiae, Z. mobilis, C. thermocellum; recombinant S. cerevisiae, Z. mobilis, E. coli</td>
</tr>
<tr>
<td>Possibilities of reaction–reaction integration</td>
<td>Not needed SSF, CBP</td>
<td>SSF, SSCF, CBP</td>
<td></td>
</tr>
<tr>
<td>Possibilities of reaction–separation integration</td>
<td>Yes</td>
<td>Yes</td>
<td>Yes</td>
</tr>
<tr>
<td>Possibilities of energy integration</td>
<td>Yes</td>
<td>Yes</td>
<td>Yes</td>
</tr>
<tr>
<td>Possibilities of co-generation</td>
<td>Yes; sugarcane bagasse can be burned to obtain thermal and electric energy</td>
<td>No</td>
<td>Yes; solid residue containing lignin can be employed for co-generation</td>
</tr>
<tr>
<td>Main co-products</td>
<td>Concentrated stillage for fertilization; press mud for animal feed</td>
<td>DDGS (dry milling); CCDS (wet milling)</td>
<td>Furfural, bioproducts from xylose fermentation, and lignin</td>
</tr>
<tr>
<td>Existing large-scale commercial production facilities</td>
<td>Yes, mostly in some tropical countries (Brazil, India, Colombia)</td>
<td>Yes, mostly in North America and Europe</td>
<td>No</td>
</tr>
<tr>
<td>Production costs, US$/L anhydrous ethanol (Sánchez and Cardona 2008)</td>
<td>0.198–0.215</td>
<td>0.233–0.338</td>
<td>0.396</td>
</tr>
<tr>
<td>Output/input energy ratio (Sánchez and Cardona 2008)</td>
<td>8.0 (sugarcane); 1.9 (sugar beet)</td>
<td>1.34–1.53</td>
<td>6.0</td>
</tr>
</tbody>
</table>

CBP consolidated bioprocessing, CCDS corn condensed distiller’s solubles, DDGS dried distiller’s grains with solubles, SSCF simultaneous saccharification and co-fermentation, SSF simultaneous saccharification and fermentation
of the most expensive steps: the unit costs of pretreatment can reach about US$0.08/L EtOH (Mosier et al. 2005).

As mentioned above, the lignocellulosic biomass is a complex where the lignin and hemicellulose represent a sort of seal covering the cellulose, the biopolymer with the highest potential to release the fermentable glucose. Therefore, the pretreatment of the lignocellulosic materials has the following goals:

- Breakdown of the cellulose-hemicellulose matrix.
- Reduction of the crystallinity degree of cellulose and increase of the fraction of amorphous cellulose.
- Hydrolysis of hemicellulose and formation of hexoses and pentoses derived from it.
- Release and partial degradation of lignin.
- Increase of the biomass porosity.
- Reduction of the formation of by-products inhibiting the subsequent steps of cellulose hydrolysis and fermentation.
- Elimination of the need of reducing the biomass particle size.

During the last 50 years, many methods to pretreat the lignocellulosic biomass for ethanol production have been proposed. Some of these methods have reached a certain degree of maturity (e.g., dilute-acid pretreatment), while it is expected that the research on other less aggressive methods can contribute to the improvement of the overall process in terms of both economic and environmental performance. These methods can be divided into physical, chemical, physical–chemical, and biological. In a previous review (Sánchez and Cardona 2008) and in the well-known work of Sun and Cheng (2002), the details of several of these procedures were disclosed. The main features of the pretreatment methods are presented in Table 16.2.

Most reports on ethanol production from biomass involve the use of dilute-acid pretreatment, steam explosion, ammonia fiber explosion (AFEX), alkaline pretreatment, and organosolv process. The first two methods share certain similarity related to the employ of moderate or high pressure under acidic conditions (steam explosion releases acetic acid or may be improved by adding sulfuric acid) making the cellulose more accessible to further enzymatic hydrolysis and allowing the formation of pentoses and hexoses from hemicellulose. It is considered that steam explosion is the most effective method for hardwood while dilute-acid hydrolysis shows higher recovery of hemicellulose-derived sugars, although it is most expensive due to the need of neutralizing the resulting streams from the process (Lynd 1996; Sánchez and Cardona 2008). Both methods release potential toxic substances for fermenting microorganisms. AFEX is one of the leading pretreatment technologies, which significantly enhances enzymatic digestibility without physically stripping out hemicellulose and lignin from the biomass, unlike other pretreatments (Gao et al. 2010). Although AFEX has lower levels of inhibitor generation and lignin loss, it requires the addition of xylanases to degrade the remaining hemicellulose (Lau et al. 2009). The latter two pretreatment methods mentioned above are chemical procedures that do not need high pressures and exhibit a high decrease of the crystallinity degree of cellulose and significant
Table 16.2 Main features of different pretreatment methods of the lignocellulosic biomass

<table>
<thead>
<tr>
<th>Method</th>
<th>Fundament</th>
<th>Further cellulose conversion</th>
<th>Hemicellulose degradation</th>
<th>Lignin degradation/solubilization</th>
<th>Inhibitors formation</th>
<th>Remarks</th>
<th>References</th>
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</thead>
<tbody>
<tr>
<td><strong>Physical methods:</strong></td>
<td></td>
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</tr>
<tr>
<td>Mechanical comminution</td>
<td>Size reduction of biomass particles</td>
<td>50–60%</td>
<td>–</td>
<td>No</td>
<td>No</td>
<td>Very high energy costs; reduces cellulose crystallinity</td>
<td>Alvo and Belkacemi (1997)</td>
</tr>
<tr>
<td>Pyrolysis</td>
<td>Thermal treatment without oxygen</td>
<td>–</td>
<td>Yes</td>
<td>No</td>
<td></td>
<td>Mostly phenols, benzene, furan, furfuryl derivatives, and oxygenat. compounds</td>
<td>Khiyami et al. (2005)</td>
</tr>
<tr>
<td><strong>Physical chemical methods:</strong></td>
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<tr>
<td>Steam explosion</td>
<td>Hydrothermal treatment using saturated steam with subsequent decompression</td>
<td>Up to 90%</td>
<td>80–100%</td>
<td>No</td>
<td>Yes, mostly furfural</td>
<td>160–290 °C, $p = 0.69$–4.85 MPa; high solids load (&gt;50%); acids released from biomass catalyze the process; addition of H$_2$SO$_4$, SO$_2$ or CO$_2$ improves the effic. of further enzyme hydrolysis; hardwood</td>
<td>Ballesteros et al. (2004), Hamelinck et al. (2005)</td>
</tr>
<tr>
<td>Liquid hot water (LHW)</td>
<td>Pressurized hot water</td>
<td>&gt;90%</td>
<td>80–100%</td>
<td>20–50% lignin solubiliz.</td>
<td>None or low</td>
<td>170–230 °C, $p &gt; 5$ MPa; low solids load (&lt; 20%)</td>
<td>Laser et al. (2002)</td>
</tr>
<tr>
<td>Ammonia fiber explosion (AFEX)</td>
<td>Ammonia treatment under pressure with subsequent decompression</td>
<td>50–90%</td>
<td>0–60%</td>
<td>10–20% lignin solubiliz.</td>
<td>None or low</td>
<td>90–130 °C, $p = 1.12$–4.48 MPa</td>
<td>Dale et al. (1996), Gao et al. (2010)</td>
</tr>
<tr>
<td>CO$_2$ explosion</td>
<td>CO$_2$ treatment under pressure with subsequent decompression</td>
<td>&gt;75%</td>
<td>Yes</td>
<td>No</td>
<td>No</td>
<td>Up to 200 °C, $p = 5.62$ MPa</td>
<td>Agbor et al. (2011), Menon and Rao (2012), Sun and Cheng (2002)</td>
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<thead>
<tr>
<th>Method</th>
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</thead>
<tbody>
<tr>
<td><strong>Chemical methods:</strong></td>
<td></td>
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</tr>
<tr>
<td>Ozonolysis</td>
<td>Treatment using ozone</td>
<td>&gt;57 %</td>
<td>–</td>
<td>Yes</td>
<td>No</td>
<td>20–25 °C, ( p = 0.1 ) MPa; hardwood and softwood</td>
<td>Sun and Cheng (2002)</td>
</tr>
<tr>
<td>Dilute-acid hydrolysis</td>
<td>Treatment using dilute acids under moderate pressure and high temperature</td>
<td>–</td>
<td>80–100 %</td>
<td>No</td>
<td>Yes</td>
<td>120–200 °C, ( p = 1 ) MPa; 0.75–5 % ( H_2SO_4, HCl ) or ( HNO_3 ); up to 40 % solids load; hardwood, herbaceous biomass</td>
<td>Hamelinck et al. (2005)</td>
</tr>
<tr>
<td>Concentrated-acid hydrolysis</td>
<td>Treatment by concentrated acids and high temperature</td>
<td>–</td>
<td>~100 %</td>
<td>–</td>
<td>Yes; glucose degradation products</td>
<td>170–190 °C, ( p = 0.1 ) MPa; 10–30 % ( H_2SO_4 ); high solids load; acid recovery needed; MSW</td>
<td>Cuzens and Miller (1997)</td>
</tr>
<tr>
<td>Alkaline hydrolysis</td>
<td>Treatment using alkalis at moderate temperature</td>
<td>&gt;65 %</td>
<td>&gt;50 %</td>
<td>24–55 % lignin removal</td>
<td>Low</td>
<td>60–120 °C, ( p = 0.1 ) MPa; ( NaOH, Ca(OH)_2 ); hardwood</td>
<td>Kaar and Holtzapple (2000)</td>
</tr>
<tr>
<td>Microwave/radio-frequency assisted alkaline pretreatment</td>
<td>Dielectric heating of biomass presoaked in alkaline solutions by microwave or radio frequency</td>
<td>50–60 % ((MW)/68 % ((RF))</td>
<td>68 %</td>
<td>75 % ((RF))</td>
<td>~100 %</td>
<td>~100 % ((MW)/75 % ((RF)) lignin removal</td>
<td>Hu and Wen (2008), Hu et al. (2008)</td>
</tr>
<tr>
<td>Oxidative delignification</td>
<td>Use of peroxidase and ( H_2O_2 )</td>
<td>95 %</td>
<td>~100 %</td>
<td>50 % lignin solubiliz.</td>
<td>–</td>
<td>20 °C, ( p = 0.1 ) MPa; 2 % ( H_2O_2 )</td>
<td>Sun and Cheng (2002)</td>
</tr>
<tr>
<td>Wet oxidation</td>
<td>Treatment using oxygen under pressure and water</td>
<td>–</td>
<td>–</td>
<td>Yes</td>
<td>Yes</td>
<td>195 °C, ( pO_2 = 1.2 ) MPa; small amounts of ( Na_2CO_3 ) or ( H_2SO_4 ); high solids load</td>
<td>Varga et al. (2004)</td>
</tr>
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<th>Method</th>
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</tr>
</thead>
<tbody>
<tr>
<td>Organosolv process</td>
<td>Treatment using organic solvents</td>
<td>–</td>
<td>~100 %</td>
<td>~100 % lignin solubiliz.</td>
<td>Yes</td>
<td>100–250 °C, p = 0.1 MPa; methanol, ethanol, acetone, ethylene glycol; solvent recovery needed; hardwood and softwood</td>
<td>Agbor et al. (2011), Pan et al. (2005)</td>
</tr>
<tr>
<td>SPORL</td>
<td>Treatment by dilute solutions of sodium bisulfite and sulfuric acid</td>
<td>–</td>
<td>64–100 %</td>
<td>Up to 40 % lignin removal</td>
<td>Yes; basically furfural and HMF</td>
<td>180 °C, p = 0.1 MPa; 3:1 ratio of liquor to biomass</td>
<td>Zhu et al. (2010)</td>
</tr>
<tr>
<td>Pretreatment with ionic liquids</td>
<td>Dissolution of biomass by selecting proper ionic liquids, then addition of an anti-solvent to precipitate pretreated biomass without lignin</td>
<td>75–90 %</td>
<td>Yes</td>
<td>32–94 % lignin solubiliz.</td>
<td>Low</td>
<td>120 °C, p = 0.1 MPa; cations: alkyl methylimidazolium, alkyl methyl-pyridinium; anions: acetate, alkyl phosphate</td>
<td>Tan and Lee (2012), Weerachanchai et al. (2012)</td>
</tr>
<tr>
<td>Biological methods:</td>
<td></td>
<td></td>
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</tr>
<tr>
<td>Fungal pretreatment</td>
<td>Solid-state cultivation of brown-, white- and soft-rot fungi on biomass particles</td>
<td>–</td>
<td>–</td>
<td>Yes</td>
<td>No</td>
<td>30 °C, p = 0.1 MPa; slow process (3–5 weeks); fungi produces cellulases, xylanases and ligninases</td>
<td>Tengerdy and Szakacs (2003), Wang et al. (2012)</td>
</tr>
<tr>
<td>Biorganosolv pretreatment</td>
<td>Fungi decompose the lignin network followed by ethanol treatment</td>
<td>–</td>
<td>90–100 %</td>
<td>Partial lignin degradat.</td>
<td>No</td>
<td>30 °C (cultivat.), 140–200 °C (ethanolysis), p = 0.1 MPa</td>
<td>Itoh et al. (2003)</td>
</tr>
</tbody>
</table>

HMF hydroxymethyl furfural, MSW municipal solid waste, MW microwave, RF radio frequency, SPORL Sulfite pretreatment to overcome recalcitrance of lignocellulose
efficiencies in the degradation of lignin (especially in the case of organosolv process). The main attractive feature of the alkaline pretreatment is its simplicity but with less efficiency in terms of further cellulose conversion. The employ of organic solvents is very suitable for lignocellulosic materials with high lignin content (e.g. softwood), but has the main disadvantage that they have to be recovered, which adds additional costs to the overall process. Undoubtedly, the separation, solubilization, or degradation of lignin is quite desirable, since it can alter or block the binding of cellulases to the cellulose in the following processing steps. Taking into account that one of the challenges of the biomass-to-ethanol process is the increase of the specific activity of these costly enzymes, methods that allow the fractionation of biomass (separation of cellulose and lignin, hydrolysis of hemicellulose) are very attractive, but the level of development of such procedures is still immature to justify a cost-effective operation. One alternative is the combination of two (or more) pretreatment methods in such a way that, during the first pretreatment procedure, the lignocellulosic matrix breaks down, the fraction of amorphous cellulose increases and hemicellulose degrades to recover valuable sugars, and then the lignin can be separated or degraded through the second pretreatment method. For instance, Shahbazi et al. (2005) proposed a sequential fractionation scheme involving steam explosion and alkaline delignification where several products can be obtained during the pretreatment of softwood: extractives, cellulose, lignin, and solubilized hemicellulose.

The pretreatment with liquid hot water (LHW) is one of the most promising and attainable at mid term method (10–15 years). Laser et al. (2002) point out that this method is comparable to dilute-acid pretreatment but without addition of acids or generation of neutralization wastes. LHW presents elevated recovery rates of pentoses and do not produce inhibitors under optimal conditions. However, the allowable solids load is much less than that for steam explosion that is usually greater than 50 %.

Recently, some relatively novel pretreatment methods are being developed. The utilization of ionic liquids offers some significant advantages related to their ability of solubilizing the lignocellulosic biomass at moderate temperature and under atmospheric pressure. The properties of the ionic liquids (extractive capabilities, low melting point, nonvolatility, high polarity, environmental friendliness, and high possibilities of being functionalized to act as acids, bases or ligands, among others) have made them to be very suitable to extract lignin and dissolve the carbohydrates present in the biomass (mostly cellulose) decreasing its crystallinity (Weerachanchai et al. 2012). During pretreatment with ionic liquids, they act as solvent to dissolve cellulose or lignin or both biopolymers disrupting the shield formed by lignin and hemicellulose. Ionic liquids can also alter cellulose crystalline structure when the dissolved cellulose is regenerated for subsequent reutilizations. This is accomplished by precipitating out from ionic liquids through the addition of anti-solvent such as water, methanol, ethanol and acetone (Tan and Lee 2012). Thus, the resulting pretreated biomass is essentially more susceptible to the enzymatic hydrolysis overcoming the barrier of the biomass recalcitrance.
Among the novel pretreatment methods proposed, the combination of microwave or radio frequency with alkaline pretreatment should be highlighted. In this case, the dielectric heating (microwave or radio frequency) uses the ability of some compounds to transform electromagnetic energy into heat. When the biomass is treated by dielectric heating, the more polar part will absorb more energy leading to a “hot spot” within nonhomogeneous materials. This type of heating results in an “explosion” effect among the particles and improves the disruption of the recalcitrant structures of the biomass (Hu et al. 2008). Other efforts have been directed to the application of electrolyzed water at low pH, which has a strong oxidizing effect, or at high pH, which has a reducing potential. The employ of electrolyzed water may allow further cellulose conversion of up to 95 % if combined with other methods like a mild alkaline pretreatment in order to remove the lignin and the hemicellulose with a minimum formation of inhibitors (Wang et al. 2010). Other new pretreatment methods like the sulfite pretreatment to overcome recalcitrance of lignocellulose (SPORL) have shown a promising performance as well (see Table 16.2).

The fungal pretreatment is a low-cost option to improve the digestibility and fermentability of the lignocellulosic biomass, although its main drawbacks are the length of the process and the fact that the fungi consume part of the carbohydrates that, otherwise, could be converted into ethanol during the fermentation step. In particular, the white-rot fungi like Coriolus versicolor have the ability to degrade the lignin present in the biomass favoring the subsequent cellulose hydrolysis and glucose fermentation. If the biological pretreatment is followed with other procedure like LHW, the efficiency of the overall process can be significantly enhanced. Wang et al. (2012) demonstrated this approach obtaining, in addition, a high hemicellulose removal and significant increases in glucose yield during the following hydrolysis step using commercial cellulases. If the fungal pretreatment is accomplished following other mild pretreatment method, the length of the overall process can be significantly reduced. For instance, Yu et al. (2009) combined a chemical pretreatment (2 % H$_2$O$_2$ during 48 h) with the delignification using solid-state cultures of Pleurotus ostreatus and reached a reduction of the biological pretreatment from 60 to 18 d. The authors indicate that the enhancing of the efficiency could possibly attribute to the structure disruption of the biomass during the first pretreatment step. It should be noted that white-rot fungi produces cellulases as well, along with other valuable bioproducts like bioactive polysaccharides (Montoya et al. 2011a, b).

16.2.2 Detoxification of Biomass Hydrolyzates

After the pretreatment step, the lignocellulosic complex is broken down in a high degree, the cellulose reduces its crystallinity degree, the lignin and the cellulose are separated from each other, and the hemicellulose is mainly degraded. The resulting biomass slurry is separated into a solid fraction containing cellulose and lignin, and
a liquid fraction that contains the soluble compounds including the products of the hemicellulose hydrolysis. One indicator of the pretreatment efficiency is the recovery of sugars, i.e., the amount of released xylose, arabinose, mannose and other sugars derived from the hemicellulose. The sugars contained in this hemicellulose hydrolyzate are valuable considering that there exist some yeasts and other microorganisms, which are able to assimilate them in order to synthesize ethanol. Ethanol produced in this way is added to the ethanol produced during the conventional fermentation of the glucose released during the cellulose hydrolysis in order to improve the efficiency of the overall biomass-to-ethanol process. However, some degradation products formed as consequence of the action of chemicals, high temperature or pressure during pretreatment remain in the hemicellulose hydrolyzate along with extractives from the biomass itself. For instance, the furfural is formed as a degradation product of the xylose released under temperatures higher than 120 °C. In addition, the lignin can be partially degraded in dependence on the pretreatment method. This partial degradation is responsible for the phenolic compounds present in the hemicellulose hydrolyzate. If this hydrolyzate is to be used as a culture medium for ethanologenic fermentation (during the pentose fermentation as represented in Fig. 16.1), all these compounds are potentially toxic for the pentose-assimilating microorganisms. Alternatively, the whole slurry obtained after pretreatment can be directed to the cellulose hydrolysis step in order to obtain glucose. Thus, all the sugars from the hemicellulose degradation and cellulose hydrolysis (biomass hydrolyzate) are then fermented into ethanol by special microorganisms assimilating both hexoses (mostly glucose) and pentoses (mostly xylose) as will be discussed below. Nevertheless, the toxic compounds are present in this biomass hydrolyzate as well decreasing the efficiency of the fermentation step. For this reason, a detoxification step is required in both processing alternatives.

Several detoxification methods have been proposed in the framework of fuel ethanol production from lignocellulosic biomass. As in the case of the pretreatment, these methods can be divided into physical, chemical, and biological, whose main features are presented in Table 16.3. Alkaline detoxification is the most employed detoxification method, especially when Ca(OH)₂ is used (overliming). The addition of alkali up to very high pH values leads to the formation of a significant amount of precipitate composed by calcium salts, which entrain the inhibitory compounds or causes them to settle. In addition, many inhibitors are unstable at pH higher than 9. Alkaline treatment is considered one of the best detoxification procedures since a high percentage of substances such as furan aldehydes and phenolic compounds can be removed by this method, improving the fermentability of the resulting liquid medium especially when biomass hydrolyzates pretreated by dilute acid are employed (Persson et al. 2002). However, this method implies the implementation of a neutralization step after the lime is added and a subsequent filtration procedure to remove the precipitate. This negatively affects the economy of the global process.

The fermenting microorganisms such as yeast has a natural ability to reduce the furan aldehydes to the corresponding less inhibitory alcohols. This biological conversion of inhibitors into less inhibitory compounds directly in the fermenter is
named in situ detoxification. However, reduction of furfural by *Saccharomyces cerevisiae* has been associated with effects such as increased consumption of NADH, decreased growth, decreased ethanol productivity, and accumulation of toxic levels of acetaldehyde (Alriksson et al. 2011). Thus, the biological in situ detoxification of furfural may therefore represent a partially inhibited fermentation. In particular, it has been suggested that the primary cause of inhibition by furfural was the process of its reduction rather than a direct inhibitory action for the case of ethanologenic *Escherichia coli* (Miller et al. 2009). Precisely, a novel detoxification method proposed by Alriksson et al. (2011) implies the addition of reducing agents such as sodium dithionite or sodium sulfite directly to the fermentation vessel in order to accomplish the in situ reduction of inhibitors like furfural. In this way, the efficiency of the fermentation can be significantly enhanced and the ethanol yield can reach values above the reference fermentation based on a medium containing glucose and mannose without inhibitors.

Fig. 16.1 Schematic diagram of the overall process for fuel ethanol production from lignocellulosic biomass. The possibilities for reaction–reaction integration are shown. CF co-fermentation, SSF simultaneous saccharification and fermentation, SSCF simultaneous saccharification and co-fermentation, CBP consolidated bioprocessing. Main components of the streams: C cellulose, H hemicellulose, L lignin, Cel cellulases, G glucose, P pentoses, I inhibitors, EtOH ethanol. From Cardona and Sánchez (2007)
Table 16.3: Main features of different detoxification methods of the hemicellulose hydrolyzate resulting from the pretreatment step

<table>
<thead>
<tr>
<th>Method</th>
<th>Fundament</th>
<th>Remarks</th>
<th>Relative yield(^a)</th>
<th>References</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>Physical methods</strong></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Evaporation</td>
<td>Volatile inhibitors are removed</td>
<td>Reduction of acetic acid, phenolic compounds in nonvolatile fraction</td>
<td>93 %</td>
<td>Palmqvist and Hahn-Hägerdal (2000)</td>
</tr>
<tr>
<td>Extraction</td>
<td>Removal of inhibitors by using organic solvents</td>
<td>Diethyl ether, ethyl acetate; 3:1 org. phase to aqueous phase ratio</td>
<td>93 %</td>
<td>Cantarella et al. (2004), Palmqvist and Hahn-Hägerdal (2000)</td>
</tr>
<tr>
<td>Adsorption</td>
<td>Inhibitors are retained in the adsorbent bed</td>
<td>Activated carbon, amberlite hydrophobic adsorbent; some loss of sugars</td>
<td>90 %</td>
<td>Lee et al. (2011), Wei et al. (2002)</td>
</tr>
<tr>
<td><strong>Chemical methods</strong></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Neutralization</td>
<td>Modification of solubility of toxic compounds, then removal of them by membrane filtration or adsorption</td>
<td>Ca(OH)(_2), CaO, NaOH, pH = 6</td>
<td>–</td>
<td>Cantarella et al. (2004), Yu and Zhang (2003)</td>
</tr>
<tr>
<td>Overliming</td>
<td>Removal of toxic compounds by entrainment in the precipitate of calcium salts (e.g. gypsum)</td>
<td>Ca(OH)(_2), pH = 9–10.5, 50 °C; mostly removal of furfural, HMF, acetic acid, and part of phenolic compounds; low sugar loss</td>
<td>Comparable to that of ref. fermn.</td>
<td>Lin et al. (2012), Palmqvist and Hahn-Hägerdal (2000), Saha et al. (2005)</td>
</tr>
<tr>
<td>Ionic exchange</td>
<td>Resins adsorbs the inhibitors, regeneration with ammonia</td>
<td>Amberlyst A20, Poly(4-vinyl pyridine); removal of acetic acid, furfural and phenolic compounds</td>
<td>Comparable to that of ref. fermn.</td>
<td>Wooley et al. (1999b), Xie et al. (2005)</td>
</tr>
<tr>
<td>Detoxification with reducing agents</td>
<td>Reducing agents improves the conversion of furan aldehydes by the fermenting microorganisms</td>
<td>Sodium dithionite or sulfite; implementation of in situ detoxification during fermentation</td>
<td>&gt;100 %</td>
<td>Alriksson et al. (2011)</td>
</tr>
<tr>
<td><strong>Biological methods</strong></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Enzymatic detoxification</td>
<td>Degradation (oxidation) of phenolic compounds by ligininas</td>
<td>Laccase, lignin peroxidase, 30 °C</td>
<td>–</td>
<td>Moreno et al. (2012)</td>
</tr>
<tr>
<td>Microbial detoxification</td>
<td>Bacteria degrade toxic compounds without utilizing sugars</td>
<td>50 °C, <em>Ureihacillus thermosphaericus</em></td>
<td>Comparable to that of overliming</td>
<td>Okuda et al. (2008)</td>
</tr>
</tbody>
</table>

\(^a\) Comparison of the ethanol yield for fermentation of the detoxified hydrolyzate related to that of the reference fermentation (100 %) based on a glucose-based medium without inhibitors

\(HMF\) hydroxymethyl furfural, \textit{ref. fermn} reference fermentation
16.2.3 Cellulose Hydrolysis

The cellulose is the main source of fermentable sugars present in the lignocellulosic biomass for ethanologenic fermentation. After pretreatment and detoxification steps, the cellulose has been separated from the lignocellulosic complex and increased its amorphous fraction. Thus, this biopolymer is ready for its hydrolysis into glucose units or even for its direct conversion into ethanol. Unfortunately, the two microorganisms with the greatest probability to be used in a commercial process for bioethanol production at high scale nowadays (S. cerevisiae or Zymomonas mobilis) are not able to assimilate the cellulose. For this reason, almost all the process configurations proposed for production of ethanol from lignocellulosics comprise a cellulose hydrolysis step. This process can be carried out by strong acids or cellulolytic enzymes. The former method implies the use of dilute or concentrated sulfuric or hydrochloric acids. The hydrolysis of cellulose through dilute acids is performed at 200–240 °C at 1.5 % acid concentration and entails the partial degradation of glucose into hydroxymethyl furfural (HMF) and other nondesirable products. The hydrolysis using concentrated acids (e.g. 30–70 % H2SO4) exhibits higher glucose yields (about 90 %) in relatively short times (10–12 h), but the acid recovery is required (Sánchez and Cardona 2008). This significantly increases the cost of the hydrolysis step.

The employ of cellulases has demonstrated better results for the subsequent fermentation since no degradation products are formed from glucose. However, the process is slower than the acid-catalyzed hydrolysis. Actually, complex cellulolytic preparations are used within the process for fuel ethanol production from biomass. These preparations contain several types of cellulases each one with different mechanisms of action on the cellulose. In general, the commercial cellulolytic products are obtained from Trichoderma reesei by submerged aerobic fermentation using glucose or lignocellulosic materials as feedstocks. This fungus releases a mixture of cellulases, among which at least two cellobiohydrolases, five endoglucanases, β-glucosidases and hemicellulases can be found (Zhang and Lynd 2004). Cellobiohydrolases break down β(1,4) linkages from nonreducing or reducing ends of the cellulose chain releasing cellobiose or even glucose, whereas endoglucanases hydrolyze these same linkages randomly inside the chain. The action of cellobiohydrolases causes a gradual decrease in the polymerization degree while endoglucanases cause the rupture of cellulose in smaller chains reducing rapidly the polymerization degree. Endoglucanases especially act on amorphous cellulose, whereas cellobiohydrolases are capable to act on crystalline cellulose as well (Lynd et al. 2002). The β-glucosidases are responsible of hydrolyzing cellobiose formed by the action of cellobiohydrolases into two molecules of glucose. The combined action of these enzymes is synergic leading to the conversion of cellulose into glucose. The cellulases should be adsorbed onto the surface of substrate particles before hydrolysis of insoluble cellulose takes place. Three-dimensional structure of these particles in combination with their size and shape determines if β-glycosidic bonds are or are not accessible to enzymatic
attack (Zhang and Lynd 2004). This makes hydrolysis process to be slower related to the enzymatic degradation of other biopolymers. For comparison, the hydrolysis rate of starch by amylases is 100 times faster than the hydrolysis rate of cellulose by cellulases under industrial processing conditions (Cardona et al. 2010b). The most important factors to be taken into account for hydrolysis of cellulose contained in lignocellulosic materials are the reaction time, temperature, pH, enzyme dosage, and substrate load.

The utilization of cellulases directly affects the global costs of biomass-to-ethanol process. The cellulases available for ethanol industry account for 36–45 % of the costs of bioethanol produced from lignocellulosic materials. According to different evaluations (Mielenz 2001; Reith et al. 2002; Sheehan and Himmel 1999), a 30 % reduction in capital costs and 10-fold decrease in the cost of current cellulases are required for this process to be competitive related to ethanol produced from starchy materials. These analyses evidence the need of improving the cellulase performance in the following aspects: Increase of thermal stability, improvement of the binding to cellulose, increase of specific activity, and reduction of the non-specific binding to lignin. In particular, the increase of specific activity of cellulases may reduce the costs significantly. It is estimated that a 10-fold increase in specific activity could lead to US¢15.85 savings per liter of ethanol produced. Among the strategies to enhance the specific activity are the increase in the efficiency of active sites through protein engineering or random mutagenesis, augment of thermal tolerance, improvement in the degradation of the crystalline structure of cellulose, enhancement of the synergism among the cellulases from different sources, and reduction of non-specific bindings (Cardona et al. 2010b; Sheehan and Himmel 1999).

In general, the costs of cellulases are considered high. The cost of the currently available cellulase preparations is very high that limits the commercial implementation of ethanol production from biomass. This cost can reach US$16/100,000 filter paper units (FPU, a way for measuring cellulase activity). Percival Zhang et al. (2006) point out that Genencor International and Novozymes Biotech have developed submerged fermentation processes allowing the reduction of the cellulase cost from US$5.40/gal ethanol to about US$0.20/gal ethanol. Reduction in the cost of cellulases can be achieved only by concerted efforts, which address several aspects of enzyme production from the raw material used for production to microbial strain improvement. Use of cheaper raw materials and cost-effective fermentation strategies like solid-state fermentation can improve the economics of cellulase production (Sukumaran et al. 2009). According to preliminary evaluations of the National Renewable Energy Laboratory of the United States (NREL) cited by Tengerdy and Szakacs (2003), the cost of in situ cellulase production by submerged culture is US$0.38/100,000 FPU. Hence, cellulase costs comprise 20 % of ethanol production costs assuming them in US$1.5/gallon. On the other hand, and as cited above, commercial cellulase cost is prohibitive for this process. In contrast, these authors indicate that the cost for producing cellulases by solid-state fermentation of corn stover would be US$0.15/100,000 FPU that would correspond to US$0.12/gal EtOH, i.e. near 8 % of total costs.
Reduction in the cost of fuel ethanol may also be achieved by efficient technologies for saccharification (hydrolysis), which includes the use of more effective enzyme cocktails and hydrolysis conditions. *Trichoderma* fungi currently employed for commercial cellulase production produce very less quantities of \( \beta \)-glucosidase compared to the other cellulases. As cellobiohydrolases are inhibited by cellobiose, \( \beta \)-glucosidase from other source needs to be added in order to complement the action of the cellulases from this fungus. Moreover, the product of the reaction catalyzed by the \( \beta \)-glucosidase, the glucose, inhibits it. Due to this, the efficiency of enzymatic hydrolysis cannot be improved much more by increasing enzyme loading, so most of the enzyme added for saccharification remains unutilized. In this way, commercial preparations are supplemented with \( \beta \)-glucosidases from sources like *Aspergillus niger*. In the seek of a cost-effective process, the production of *T. reesei* cellulases and *A. niger* \( \beta \)-glucosidase by solid-state fermentation is being developed (Sukumaran et al. 2009).

In general, the main research directions for cellulase engineering of noncomplexed cellulase systems (fungal cellulases) are aimed at the rational design (protein engineering) for each cellulase, based on knowledge of the cellulase structure and the catalytic mechanism. In addition, the research efforts are also aimed at the directed evolution for each cellulase, in which the improved enzymes or ones with new properties were selected or screened after random mutagenesis and/or molecular recombination. The greatest advantage of directed evolution is that it is independent of knowledge of enzyme structure and of the interactions between enzyme and substrate. Finally, the research trends should be also directed to the reconstitution of cellulase mixtures (cocktails) active on insoluble cellulosic substrates, yielding an improved hydrolysis rate or higher cellulose digestibility (Percival Zhang et al. 2006).

### 16.2.4 Fermentation Step

During the fermentation step, all the sugars derived from the pretreatment and cellulose hydrolysis steps are converted into ethanol by using one or different fermenting microorganisms. There exist several options to perform this conversion depending on the type of sugars assimilated by the process microorganisms and on the technological configuration of the process.

#### 16.2.4.1 Fermentation of Cellulose Hydrolyzates

Once the cellulose has been hydrolyzed, the resulting glucose-containing stream should be processed into ethanol. For this, conventional fermentation using yeast is mostly used. In this sense, the process is similar to that employed for fermenting the glucose solutions obtained after the enzymatic hydrolysis of the starch when starchy materials are used as feedstock. The ethanolic fermentation is one of the most
studied biological processes. Nevertheless, the need of increasing the efficiency of ethanol production including the usage of alternative feedstocks has led to the development of new fermentation methods with better techno-economic and environmental indicators. Traditionally, the most used microorganism for ethanolic fermentation is the yeast *S. cerevisiae*. However, for ethanol production from lignocellulosic biomass, there is a wider variety of process microorganisms employed (e.g. *Zymomonas* bacteria, xylose-assimilating yeasts, or thermophilic clostridia). *S. cerevisiae* converts hexoses in pyruvate through glycolysis, which is decarboxylated to obtain acetaldehyde that is finally reduced to ethanol generating two moles of ATP by each mol of consumed hexose under anaerobic conditions. In addition, this microorganism also has the ability to convert hexoses in CO2 by aerobic respiration favoring the production of yeast cells. Therefore, aeration is an important factor for both cell growth and ethanol production. Although these yeasts have the ability to grow under anaerobic conditions, small amounts of oxygen are needed for synthesis of such substances like fatty acids and sterols. Inhibition of cell growth by ethanol decreases at microaerobic conditions related to fully anaerobic cultivation. *S. cerevisiae* has demonstrated its elevated resistance to the presence of inhibitors in the lignocellulosic hydrolyzate. In the case of the more productive continuous regime, one way to enhance this resistance is the increase in the cell retention to prevent wash-out and maintain high yeast cell density.

Other yeast species have been proposed for fermentation of cellulose hydrolyzates. In particular, the thermotolerant yeast *Kluyveromyces marxianus* has demonstrated their capability for fermenting glucose at relatively high temperatures of 40–45 °C (Singh et al. 1998). The bacterium *Z. mobilis* is one promising ethanologenic microorganism considering its ethanol yield higher than that of *S. cerevisiae* and its growth rate. The spectrum of assimilable substrate by this bacterium is quite similar to the yeast: glucose, fructose, sucrose and maltose.

The fermentation of cellulose hydrolyzates obtained after the enzymatic hydrolysis of the washed solid fraction of the pretreated biomass generally does not imply special difficulties since the inhibitor concentrations are very low. Nevertheless, compared to starch and sugarcane fermentations, the sugar concentrations after hydrolysis are often low with values approaching typically not more than 70 g/L (Brethauer and Wyman 2010). This is explained by the difficulties associated to the handling of suspensions with solids concentrations greater than 10 % by weight to the bioreactors for enzymatic hydrolysis of cellulose and to the inhibition of cellulases with the glucose formed. Thus, a concentration of the hydrolyzate stream might be needed to achieve higher concentrations, but the operating costs would be increased as well.

The fermentation of the hydrolyzates can carried out batchwise or continuously. In the latter case, the recycling of part of the effluent stream could be required due to the need of maintaining a high cell concentration. However, the recycling leads to the accumulation of inhibitors within the fermenter. Thus, a detoxification process is required if the whole slurry from pretreatment step enters the cellulose hydrolysis and fermentation steps.
16.2.4.2 Pentose Fermentation

The detoxified hemicellulose hydrolyzate resulting from the pretreatment step contains an important amount of pentoses and hexoses. The xylose, a pentose, is the most relevant sugar of this hydrolyzate. To take advantage of this stream, xylose-assimilating yeasts can be employed to produce ethanol, but in this case, the biomass utilization rates are lower related to microorganisms that only assimilate hexoses. Ogier et al. (1999) have compiled information about the main fermentative indexes for the most promising pentose-assimilating yeasts: *Candida shehatae*, *Pichia stipitis* and *Pachysolen tannophilus*. Most pentose-fermenting yeasts are mesophiles. One of the main challenges in pentose fermentation lies in the fact that the productivities of pentose utilizing microorganisms are less than those of hexose-fermenting ones. On the other hand, there are a few cases where the immobilization of these yeasts increases the ethanol productivity (Chandrakant and Bisaria 1998), unlike the case of hexose-fermenting yeasts or *Z. mobilis*. Generally, the assimilation rate of pentoses by natural pentose-fermenting microorganisms is slower than that for hexoses. For example, although *P. stipitis* yeast can naturally ferment pentose sugars to ethanol, hexose sugars are used preferentially, and pentose uptake is competitively inhibited by hexoses. Thus, pentose fermentation is only possible at very low glucose concentrations. In addition, microaerophilic conditions are required, which are difficult to maintain in large-scale systems, and even then yields are low (Brethauer and Wyman 2010).

The dimorphic filamentous fungus *Mucor indicus* is a promising alternative to *S. cerevisiae* as it is capable of xylose fermentation, is safe for humans, and produces ethanol from hexoses with comparable yields and productivities. Brethauer and Wyman (2010) have indicated the possibility of implementing continuous processes for ethanol production from biomass using this microorganism. Other microorganisms are able to assimilate both hexoses and pentoses. In particular, thermophilic clostridia have the ability to utilize xylose and synthesize ethanol as well. Ogier et al. (1999) also have compiled information about the fermentative indexes for the xylose-assimilating thermophilic bacteria *Clostridium thermosaccharolyticum*, *Thermoanaerobacter ethanolicus* and *Bacillus steatothermophilus*. Lynd et al. (2001) report low concentrations (in the order of 25 g/L) of ethanol obtained by *C. thermosaccharolyticum* cultivated in xylose-based media during batch and continuous cultures. These authors studied the influence of different factors limiting the substrate utilization for continuous cultures at progressively higher feed xylose concentrations.

16.2.4.3 Co-fermentation of Lignocellulosic Hydrolyzates

The co-fermentation process is aimed at the complete assimilation by the microbial cells of all the sugars resulting from lignocellulosic degradation and consists in the employ of a mixture of two or more compatible microorganisms that assimilate both the hexoses and pentoses present in the medium. This means that
the fermentation is carried out by a mixed culture. However, the use of mixed cultures faces the problem consisting in that microorganisms utilizing only hexoses grow faster than pentose-utilizing microorganisms leading to a more elevated conversion of hexoses into ethanol (Cardona and Sánchez 2007). To solve this problem, the employ of respiratory deficient mutants of the hexose-fermenting microorganisms has been proposed. In this way, the fermentation and growth activities of the pentose-fermenting microorganisms are increased considering that they grow very low when are cultivated along with rapid hexose-fermenting yeasts. Considering the indicators for the process using only the glucose-assimilating bacterium *Z. mobilis* grown on the biomass hydrolyzate, the productivities of the mixed culture are less than those of the bacterium, but the yields are comparable, which offers a space for further research (Delgenes et al. 1996). One of the additional problems arisen in this kind of configurations is that pentose-fermenting yeasts present a greater inhibition by ethanol that limits the use of concentrated substrates in the system (Cardona et al. 2010b).

Other variant of co-fermentation consists in the utilization of a single microorganism capable of assimilating both hexoses and pentoses in an optimal way allowing high conversion and ethanol yield. Although in the nature these microorganisms exist, their efficiency and ethanol conversion rates are reduced for implementation of an industrial process. Hence, the addition of an enzyme transforming the xylose into xylulose (xylose-isomerase) to the culture medium has been proposed. In this way, microorganisms exhibiting high rates of conversion to ethanol and elevated yields (like *S. cerevisiae*) can assimilate the xylulose involving it in the metabolic pathways leading to the ethanol. On the other hand, a high efficiency in the conversion to ethanol can be reached through the genetic modification of yeasts or bacteria already adapted to the ethanolic fermentation. The microorganisms most commonly modified for this purpose are *S. cerevisiae* and *Z. mobilis*, to which genes encoding the assimilation of pentoses have been introduced. The other approach for genetic modification is the introduction of genes encoding the metabolic pathways for ethanol production to microorganisms that are capable of fermenting both hexoses and pentoses in their native form. The “design” of ethanologenic bacteria like *E. coli* or *Klebsiella oxytoca* is an example of such type of approach. The employ of these recombinant microorganisms allows the implementation of the co-fermentation process intended to the more complete utilization of the sugars contained in the hydrolyzates of lignocellulosic biomass (Cardona and Sánchez 2007).

### 16.2.5 Ethanol Recovery and Dehydration

The recovery of ethanol produced by different technological configurations and from diverse types of feedstocks is accomplished in a very similar way. The ethanol content in the culture broth resulting from fermentation processes oscillates between 2.5 and 10 % (by weight). The utilization of fuel ethanol as a
gasoline oxygenate requires a high-purity ethanol, so it is necessary to concentrate the ethyl alcohol up to 99.5 % obtaining the anhydrous ethanol, which is the suitable form used for ethanol-gasoline blends. The first step of the ethanol recovery scheme is the concentration of ethanol contained in culture broths. This process is carried out in a distillation (concentration) column achieving ethanol content about 50 %. This product is removed from the column by a side stream. The overhead vapors contain mostly CO₂ (about 84 %), a significant amount of ethanol (12 %), and a small amount of water. The following step is the rectification of this concentrated stream in order to obtain a product with 90–92 % ethanol composition, which is near to the azeotropic mixture of ethanol and water (95.6 %). To achieve 99.5 % or more of ethanol purity from streams containing 90–92 % ethanol, it is necessary to employ nonconventional separation operations like pressure-swing distillation, azeotropic distillation, extractive distillation, adsorption, and pervaporation. All these operations have found industrial application in the fuel ethanol industry (Cardona et al. 2010b).

The adsorption is one of the most important unit operations currently employed in the biofuel industry for ethanol dehydration. In this operation, the ethanol–water mixture passes through an apparatus usually cylindrical that contains a bed with an adsorbent material. Due to the difference in the affinity of molecules of water and ethanol with respect to the adsorbent, the former remains entrapped in the bed while the ethyl alcohol passes through this same bed increasing its concentration in the stream leaving the apparatus. Adsorption of water employing the so-called molecular sieves to dehydrate ethanol has been the technology that has acquired more development in the last years in the fuel ethanol industry. In fact, this technology has been replacing the azeotropic distillation (Cardona et al. 2009).

The adsorption operation requires that, once the adsorbent bed is saturated with the water that needs to be removed, its desorption should be accomplished to make possible the reutilization of the adsorbent material (regeneration cycle). For regeneration of the sieves, hot gas is needed. This rapidly deteriorates them especially if the bed is fed with a liquid stream during the previous water adsorption cycle. To counter this deterioration, the pressure-swing adsorption (PSA) technology was developed. This technology involves the use of two adsorption beds. While one bed produces vapors of anhydrous ethanol superheated under pressure, the other one is regenerated under vacuum conditions by recirculating a small portion of superheated ethanol vapors through the saturated sieves. The system feeding is carried out using the overhead vapors from the rectification column. The ethanolic vapors obtained in the regeneration cycle and that can contain 28 % water are recirculated to the rectification column (Montoya et al. 2005; Wooley et al. 1999b). In this way, the molecular sieves life can be prolonged for several years that, in turn implies very low costs related to the replacement of adsorbent material, and therefore reduced operating costs (Guan and Hu 2003; Madson and Monceaux 1995). The process flowsheet for ethanol recovery and dehydration in the case of the adsorption with molecular sieves is depicted in Fig. 16.2.
16.2.6 Effluent Treatment

Fuel ethanol production from lignocellulosic biomass generates solid wastes, atmospheric emissions, and liquid effluents. The atmospheric emissions mostly correspond to the gas outlet stream from fermenters or integrated bioreactors that is washed with water in the scrubbers in order to recover the volatilized ethanol (see Fig. 16.2). The gases exiting from the scrubbers contain mainly carbon dioxide that is released into the atmosphere. The CO$_2$ can be employed for production of dry ice or beverages. However, if these gases are not utilized, they should be considered in the calculation of the environmental impact of ethanol producing facilities (Cardona et al. 2010b). In this regard, it should be emphasized that the bioethanol practically presents net emissions of CO$_2$ near to zero considering that the plant biomass already fixed the CO$_2$ during its growth and only this carbon dioxide is released during the combustion of fuel ethanol in the engines. In contrast, the burning of fossil fuels releases into the atmosphere additional amounts of carbon dioxide that was fixed by the plant biomass millions years ago.

The solid wastes formed during production of fuel ethanol are strongly linked to the raw material from which it is produced. The production of lignocellulosic ethanol generates lignin as the most important solid residue. This polymer can be isolated during the pretreatment step if some pretreatment methods like the pretreatment with solvents (organosolv process) or oxidative delignification are
employed (this fact is considered in Fig. 16.3 et seq.). Nevertheless, most pre-treatment methods allow that the lignin remains in the solid fraction resulting from this processing step along with the cellulose. After enzymatic hydrolysis using cellulases, the lignin remains in the liquid suspension until the end of the process where it can be recovered from the stillage stream as shown in Fig. 16.2. The lignin has a high-energy value (25.4 MJ/kg), and therefore it is used as a solid biofuel for feeding boilers or cogeneration.

The stillage is the major effluent of all flowsheets for ethanol production involving the submerged fermentation of streams containing sugars or carbohydrate polymers. The stillage represents the residual liquid material obtained after distillation of ethanol from the fermented wort (wine) and contains both solid and soluble matter. Precisely, the elevated organic load of the stillage is responsible of the high polluting properties of this burden, so this stream should undergo treatment to reduce this load and minimize the environmental impact during its discharge to the water streams. Due to the elevated organic matter content of stillage, methods for its treatment and economic utilization should be implemented in the industry. Among the most used methods for stillage treatment, the irrigation, recycling, evaporation, incineration, anaerobic digestion, and composting should be highlighted. Some of the new treatment methods allow the production of value-added products as discussed elsewhere (Cardona et al. 2010b). By applying process systems engineering tools like process simulation, Cardona et al. (2006) performed the evaluation of several option for treatment of the stillage generated during ethanol production from biomass. The results obtained show that the best option comprises the centrifugation of stillage, incineration of the solids obtained in a cogeneration unit for production of power and steam, and anaerobic digestion of the thin stillage resulting from centrifugation followed by aerobic biological treatment. This configuration is schematically depicted in Fig. 16.2. The proposed treatment of the thin stillage coincides with the treatment scheme suggested by Merrick and Company (1998).

16.3 Process Integration for Ethanol Production from Biomass

The process integration offers a great potential to process industry through the improvement of chemical and biotechnological processes. This potential is especially recognized if considering the many ways in which process integration contributes to the enhancement of energy efficiency of many industrial processes as detailed in a previous work (Cardona et al. 2008). Process integration, as a mean for process intensification, is a successful approach for designing improved technological configurations for fuel ethanol production in which production costs can be reduced. This fact is remarkably important taking into account that the main objective of using liquid biofuels like bioethanol is the progressive displacement of
Fig. 16.3 Technological options for fuel ethanol production from lignocellulosic biomass by separate hydrolysis and fermentation (SHF). a SHF without utilization of hemicellulose-derived pentoses, b SHF with hexose and pentose fermentation performed in parallel, c SHF with pentose fermentation performed after hexose fermentation; LF liquid fraction, SF solid fraction, Slr slurry, the gray-shaded boxes represent enzymatic or microbial processes; the dashed lines represent an optional source of lignin when a pretreatment method allowing the fractionation of biomass is used (e.g. organosolv process).
fossil fuels that implies the sustainable exploitation of the huge biomass resources of our planet and the utilization of clean and renewable energy sources.

### 16.3.1 Reaction–Reaction Integration

Process integration is gaining more and more interest due to the advantages related to its application in the case of bioethanol production: reduction of energy costs, decrease in the size and number of process units, intensification of the biological and downstream processes, improvement of environmental performance of the overall process, and among others. For instance, the combination in a same single unit of the enzymatic hydrolysis and the microbial transformation leads to the reduction of the negative effect due to the inhibition of the enzymes caused by the products of the reaction catalyzed by them. This corresponds to an integration of the reaction–reaction type. There exist different possibilities for reaction–reaction integration during production of ethanol from lignocellulosic biomass (see Fig. 16.1). This type of integration mainly includes the combination of the enzymatic reactions for hydrolysis of cellulose with the microbial conversion of formed sugars into ethyl alcohol.

To expose the possibilities of process integration in the framework of biomass-to-ethanol process, it is necessary to indicate the main features of the basic non-integrated configuration, the separate hydrolysis and fermentation. Then, different integrations options are briefly described.

#### 16.3.1.1 Separate Hydrolysis and Fermentation

The initial configuration proposed for bioethanol production comprises the cellulose hydrolysis followed by the fermentation of the glucose released during the enzymatic process. This configuration is called separate hydrolysis and fermentation (SHF) and has a sequential character. The main feature of SHF is that each step can be performed at its optimal operating conditions (for instance, 50 °C and pH of 4.5 for cellulose hydrolysis and 32 °C and pH of 4–5 for yeast fermentation). SHF is the technology with most possibilities to be implemented at demonstration semi-industrial facilities or commercial scale. In fact, most of the existing demonstration plant for bioethanol production from lignocellulosics employs this technology. For instance, Abengoa Bioenergy has developed a SHF technology on a pilot scale in York (Nebraska, USA), which has been tested in the demonstration plant located in Salamanca (Spain). This plant has operated over 5,000 h continuously, achieving a production of 5 million liters of ethanol per year from cereal straw and herbaceous biomass. It is considered the largest demonstration plant in the existence to produce second-generation bioethanol in the world. This company has announced the beginning of construction of the first
commercial plant that will produce 100 million L/year of bioethanol from corn stover, wheat straw, and switchgrass in Hugoton (Kansas, USA) (Abengoa 2011).

The main options for producing ethanol from biomass by SHF are depicted in Fig. 16.3. It should be noted that detoxification is not needed if the pretreated biomass is thoroughly washed with water in order to obtain a solid stream containing cellulose (and lignin) to be converted into glucose by the cellulases. Although several companies are developing new and more effective cellulases for ethanol industry, their cost is still elevated so most designs proposed involve the production of the cellulase required by the process in the same ethanol production facilities (in situ cellulase production). For this, part of the pretreated biomass is diverted to a submerged aerobic fermentation where fungal cells utilize it to synthesize extracellular cellulases. However, this scheme does not make use of the sugar released during the pretreatment that undoubtedly reduce the overall conversion of the process and final ethanol yields.

In order to increase the conversion of biomass into ethanol, the sugars derived from hemicellulose degradation should be utilized. For this, xylose-fermenting microorganisms like \textit{C. shehatae} or \textit{P. stipitis} are employed to produce ethanol from the hemicellulose hydrolyzate (this process is indicated as pentose fermentation in Fig. 16.3) along with the conventional yeast used for fermentation of the glucose derived from cellulose hydrolysis (hexose fermentation). In this case, the pentose fermentation can be carried out in a separate unit in parallel or after the glucose fermentation. In the latter scheme, ethanol should be removed from the broth resulting from hexose fermentation to decrease the end-product inhibition effect of the growth rate for xylose-assimilating yeasts, which are more sensitive to ethanol than glucose-assimilating yeasts or bacteria. In addition, a detoxification step is required as the whole slurry from pretreatment enters the cellulose hydrolysis and the two fermentation steps. This slurry contains toxic compounds that can inhibit not only the growth of fermenting microorganisms, but also the enzymes employed for glucose production from cellulose even if it is diluted with water.

Depending on the pretreatment method, the lignin can be recovered in this step (e.g. by the organosolv process) or remain in the stillage of the distillation column used for the preliminary ethanol recovery. Thus, the lignin can be separated from the stillage by centrifugation and burnt in boiler for generating the steam required by the overall process. Alternatively, the lignin may be employed as a feedstock for production of valuable chemicals or adsorbents like activated charcoal.

16.3.1.2 Separate Hydrolysis and Co-fermentation

The first integration approach is to perform the transformation of both pentoses and hexoses in one single fermenting unit (see Fig. 16.4). This process can be called separate hydrolysis and co-fermentation (SHCF), and has the advantage of reduced capital costs since no additional vessel is required for pentose fermentation. SHCF can be considered an example of reaction–reaction integration since
two biochemical processes (fermentation of glucose and fermentation of xylose) are combined and simultaneously performed in the same single unit. Although xylose-utilizing yeasts can carry out the co-fermentation of glucose and xylose, their ethanol yields are low. As mentioned above, mixed cultures can be used but problems associated with the optimal conditions for fermentation of two different microorganisms are arisen. For this reason, the current research trends are aimed at developing microorganisms with the ability of assimilating both types of sugars with increased ethanol yields by using recombinant DNA technology. There are many works reporting the development of specific strains of ethanol producing microorganisms with these desired traits. Thus, recombinant *S. cerevisiae* (Hong et al. 2003; Zaldivar et al. 2005) or *Zymomonas mobilis* (Leksawasdi et al. 2001) strains have been developed. Alternatively, bacteria naturally assimilating these two sugars like *E. coli* are transformed in order to obtain an ethanologenic microorganism (Dien et al. 1998; Ingram et al. 1999; Vinuselvi and Lee 2012).

16.3.1.3 Simultaneous Saccharification and Fermentation

One of the most important advances in bioethanol industry is the development and implementation of processes in which the hydrolysis of the glucan (starch, cellulose) and the conversion of glucose into ethanol are carried out in a simultaneous way in the same single unit. This process is known as simultaneous saccharification and fermentation (SSF) and has been successfully implemented in the production of ethanol from corn, especially in wet-milling plants. The concept of SSF process was firstly described by Takagi et al. (1977). Takagi, Suzuki and Gauss had previously patented the SSF technology for bioethanol production (Gauss et al. 1976) by which the yeasts metabolize simultaneously the glucose into ethanol in situ during the enzymatic saccharification of the cellulose. This patent expired in 1993 and it has been utilized for small-scale demonstrations (Ingram and Doran 1995), but until now, no commercial plants have been built at industrial level.
Conversion of cellulose into ethanol by SSF implies that several enzymes with cellulolytic activity (basically endoglucanases, cellobiohydrolases and β-glucosidase) are added to the suspension obtained by mixing water with the solid fraction resulting from the pretreatment step and that contains cellulose and lignin (Fig. 16.5). In the same way, glucose-fermenting microorganisms (yeasts) are added to this mixture in the bioreactor where SSF is accomplished for immediately converting the glucose formed into ethanol. Taking into account that sugars (glucose, cellobiose) are much more inhibitory for conversion process than ethanol is, SSF can reach higher rates, yields and ethanol concentrations compared to SHF (Wyman et al. 1992). The increased ethanol concentration in the culture broth allows the reduction of energy costs during distillation. In addition, SSF offers an easier operation and a lower equipment requirement than the sequential process since no hydrolysis reactors are needed. Moreover, the presence of ethanol in the broth makes it less vulnerable to the action of undesired microorganisms (Wyman 1994). Nevertheless, SSF has the inconvenient that the optimal conditions for hydrolysis and fermentation are different, which implies a difficult control and optimization of process parameters. In addition, larger amounts of exogenous enzymes are required (Cardona and Sánchez 2007).

A significant amount of reports on batch SSF has been published recognizing, in this way, that this integrated process is one of the most promising. Nevertheless, only limited information has been published about continuous SSF. The continuous regime for SSF entails experimental challenges related to the homogenous delivery of solid substrate, the extended run times needed, and the complexities of the continuous systems as stated by Brethauer and Wyman (2010). These authors point out that batch systems are hampered by mixing problems at high solid substrate loadings, which can be avoided by operation of a CSTR (continuous
stirred-tank reactor) with high conversion of insolubles to ethanol. Furthermore, in a batch reactor, the high amounts of \( \beta \)-glucosidase added are only required at the beginning of the reaction when cellobiose production is highest. They suggest that \( \beta \)-glucosidase loading can be reduced in a continuous system because cellobiose production slows with conversion.

As mentioned above, one of the main disadvantages of SSF processes using lignocellulosic biomass lies in the different optimum conditions of enzymatic hydrolysis of cellulose and fermentation. Varga et al. (2004) proposed a non-isothermal regime for batch SSF process applied to wet oxidized corn stover; In the first step of SSF, small amounts of cellulases were added at 50 °C in order to obtain better mixing conditions. In the second step, more cellulases were added along with the yeast \( S. \) cerevisiae at 30 °C. In this way, the final solid concentration in the hydrolyzate could be increased up to 17 % dry matter concentration achieving 78 % ethanol yield. In general, increased cultivation temperature accelerates metabolic processes and lowers the refrigeration requirements. Yeasts as \( K. \) marxianus have been tested as potential ethanol producers at temperatures higher than 40 °C. Ballesteros et al. (2001) carried out several fed-batch SSF tests at 42 °C during 72 h using \( K. \) marxianus in the case of by-products of olive oil extraction achieving 76 % ethanol yields of theoretical for olive pulp. If, when thermotolerant yeasts are used, the microbial cells can also assimilate pentoses, the SSF process can become more promising. Yeasts as \( C. \) acidothermophilum, \( C. \) brassicae, \( S. \) uvarum and \( H. \) polymorpha can be used for these purposes. In this case, the addition of a larger amount of nutrients to the medium is required. The difficulty lies in the fact that higher temperatures enhance the inhibitory effect of ethanol. Therefore, the isolation and selection of microorganisms that could be adapted in a better way to these hard conditions should be continued.

One of the most relevant factors in the alcoholic fermentation is the possibility of infection by acidolactic bacteria. This problem has been reported for both batch (Stenberg et al. 2000) and continuous regimes (Schell et al. 2004) of SSF processes. These bacteria can consume the sugars not utilized by the main ethanologenic microorganisms used during SSF and represent a challenge for the design of effective biomass-to-ethanol processes. Therefore, the utilization of microorganisms capable of assimilating all the sugars present in the feed stream of the SSF is crucial to avoid the proliferation of undesired contaminating microbes. This suggests the need of a higher degree of reaction–reaction integration.

### 16.3.1.4 Simultaneous Saccharification and Co-fermentation

A higher degree of integration can be achieved by including the fermentation of hemicellulose-derived pentoses in the same single unit where SSF is carried out. This process is called simultaneous saccharification and co-fermentation (SSCF). In this way, only two vessels (one for enzyme production and the other for the SSCF process) are employed for conversion of the pretreated lignocellulosic
biomass into ethanol in one integrated process as that depicted in Fig. 16.6. During the SSCF, the hydrolysis of cellulose by using cellulases added to the bioreactor, the fermentation of the glucose released from the enzymatic process, and the fermentation of pentoses present in the feed stream are simultaneously accomplished in the same single unit. Besides the effectiveness of the employed cellulases, the key factor in SSCF is the utilization of an efficient ethanol-producing microorganism with the ability of assimilating not only hexoses (mainly glucose), but also the pentoses (mainly xylose) released during the pretreatment step as a result of hemicellulose degradation. For this, genetically engineered microorganisms like *S. cerevisiae* (Jin et al. 2010), *Z. mobilis* (McMillan et al. 1999) and *E. coli* (Kang et al. 2010) has been developed and favorably proven in SSCF processes for ethanol production from lignocellulosic materials.

In an initial stage, the co-fermentation of mixed cultures was studied (Cardona and Sánchez 2007). For example, the co-culture of *P. stipitis* and *Brettanomyces clausennii* has been employed for the SSCF of aspen at a trade-off temperature of 38 °C yielding 369 L EtOH per ton of aspen during batch process, as reported by Olsson and Hahn-Hägerdal (1996). However, actual SSCF process using one process microorganism has been demonstrated in the case of ethanol production from yellow poplar through a bench-scale integrated process that included the dilute-acid pretreatment of feedstock, conditioning of hydrolyzate for fermentation, and a batch SSCF (McMillan et al. 1999). In this case, the recombinant bacterium *Z. mobilis* assimilating xylose was used. SSCF is the process on which is based on the technology designed as a model process by the NREL for production of fuel ethanol from aspen wood chips (Wooley et al. 1999b) and corn stover (Aden et al. 2002). It is projected that SSCF can be carried out in continuous regime with a residence time for the whole system of cascade fermenters of 7 d at

![Schematic diagram of fuel ethanol production from lignocellulosic biomass by simultaneous saccharification and co-fermentation (SSCF), LF liquid fraction, SF solid fraction, the gray-shaded boxes represent enzymatic or microbial processes; the dashed lines represent an optional source of lignin when a pretreatment method allowing the fractionation of biomass is used (e.g. organosolv process)](imageURL)
As in the case of SSF of biomass, the development of microbial strains able to grow at elevated temperatures can improve the techno-economic indicators of the process. Thus, ethanol-producing microorganisms capable to assimilate both types of sugars at temperatures higher than 50 °C could reduce the cellulase costs by a half taking into account that a 20 °C increase during saccharification can lead to double cellulose hydrolysis rate (Wooley et al. 1999a).

The configuration shown in Fig. 16.6 implies the separation of the slurry of pretreated biomass, washing of the solid fraction, and detoxification of the liquid hemicellulose hydrolyzate in order to prepare and condition the feed stream to the SSCF bioreactor. All these operations add costs and complexity to the overall process. For this reason, new trends in the development of SSCF processes are aimed at the development of microorganisms resistant to the toxic compounds formed during the pretreatment step. In this way, the whole slurry could be directly fed to the SSCF bioreactor without the need of any separation or washing step and avoiding the costly detoxification. Geddes et al. (2011) point out that newly developed strains such E. coli MM160 and S. cerevisiae 424A can be used for these purposes. However, the final ethanol concentrations and yields are still low for a large-scale commercial operation, although the results obtained are better than those for most SSF processes. These problems may be explained by the high solids content of the stream entering the SSCF bioreactor, which can achieve 10–20 %. One way to tackle this challenge is to implement a two-step process where a pre-hydrolysis is carried out before the actual SSCF. In this way, a sort of liquefaction analogous to the corn-to-ethanol process is carried out to reduce the solids level and enhance the efficiency of the enzymatic hydrolysis. After this step, the stream containing about 10–15 % solids undergoes SSCF (see Fig. 16.7). It is envisioned that such pre-hydrolysis can be effectively performed in a CSTR with a residence time between 1 and 6 h (Geddes et al. 2011). Other difficulty that can be overcome by a two-step SSCF process is the low xylose uptake rate related to
glucose. For instance, Jin et al. (2010) proposed a two-step SSCF process on AFEX-treated switchgrass using commercial enzymes and the inhibitor-resistant *S. cerevisiae* 424A, which gives higher ethanol yield with improved xylose consumption compared to the corresponding SSCF process. The process included the hydrolysis of the hemicellulose remaining in the pretreated biomass with hemicellulases first to release xylose, which was then fermented by recombinant *S. cerevisiae* 424A, followed by adding cellulases to hydrolyze the cellulose into glucose and continue the fermentation.

**16.3.1.5 Consolidated Bioprocessing**

The highest degree of integration is represented by a configuration where the fermentable sugars and polysaccharides (mostly cellulose) resulting from the pretreatment of lignocellulosic biomass are directly converted into ethanol by a microorganism (or consortium of microorganisms) in one single unit (see Fig. 16.8). This process is known as consolidated bioprocessing (CBP) and leads to the maximum reduction of process units during the biomass-to-ethanol process. Schematically, CBP consists in the unification of all the enzymatic and microbial processes represented by gray-shaded boxes in Figs. 16.3–16.7. This configuration implies that no capital or operation expenditures are required for enzyme production within the process. In this way, there is no need of adding the costly cellulolytic enzymes to the biomass to obtain significant amounts of glucose. In addition, the deviation of part of the feedstock to the production of cellulases is not further required since the CBP concept entails that a single microorganism produces the needed enzymatic complexes to degrade the cellulose and even the hemicellulose.

It is considered that the costs of ethanol production from biomass can be significantly reduced by improving the conversion of lignocellulosics. Lynd (1996)
had projected that the reduction of production costs due to an advanced configuration involving the CBP is three times greater than the reduction related to the scale economy of the process and ten times greater than the reduction associated with a lower cost of the feedstock. This decrease would be accomplished thanks to the reduction of more than eight times in the costs of biological conversion (Lynd et al. 1996). Afterwards, Lynd et al. (2005) reported the comparative simulation of SSCF and CBP processes assuming aggressive performance parameters intended to be representative of mature technologies. Their results indicate that production costs of ethanol for SSCF can reach US¢ 4.99 per liter including the costs of dedicated cellulase production, whereas CBP can give total costs of only US¢ 1.11 per liter demonstrating the future effectiveness of this process configuration.

To develop an effective CBP process to produce fuel ethanol from biomass, a suitable microorganism has to be selected. The candidate CBP microorganism should exhibit the following features:

- Be resistant to the inhibitors generated during the pretreatment.
- Produce cellulases or cellulosolytic complexes to convert the cellulose into glucose.
- Produce hemicellulases to obtain a higher amount of pentoses and other hemicellulose-derived sugars.
- Convert the hexoses (glucose, mannose and galactose) into ethanol.
- Convert the pentoses (xylose and arabinose) into ethanol.
- Show reduced end-product inhibition by the ethanol formed.
- Reduce the amount of fermentation by-products like lactate or acetic acid.
- Show high growth rates.
- Produce high ethanol titres.
- Be stable under industrial conditions.

As can be inferred from previous sections, such a microorganism capable of transforming biomass into ethanol with high yield and efficiency does not exist in the nature. However, some naturally occurring microorganisms exhibit most of the above-mentioned traits but with low ethanol yields. The thermophilic bacterium Clostridium thermocellum is one of these microorganisms, since it is able to degrade cellulose and convert the glucose obtained into ethanol. C. thermosaccharolyticum, in turn, has the ability to utilize pentoses. As both bacteria can be cultured in the same vessel under the same fermentation conditions, their mixed cultivation represents a CBP process to produce ethanol from biomass. Other thermophilic clostridia can be cultured along with C. thermocellum in order to depolymerize lignocellulosic carbohydrate polymers and utilize pentoses as C. thermolacticum obtaining significant ethanol yields (Xu and Tschirner 2011). In fact, the production of ethanol from biomass by using C. thermocellum is a first step toward CBP as has been demonstrated experimentally (Shao et al. 2011; South et al. 1993). Nevertheless, the employ of clostridia has some important drawbacks as their low ethanol tolerance and reduced ethanol yields due to the formation of acetic acid and salts of other organic acids like lactates (Baskaran et al. 1995; McMillan 1997; Wyman 1994). This makes that the final ethanol
concentrations be low (0.8–60 g/L) compared to the traditionally used yeasts with long cultivation times of 3–12 days (Szczodrak and Fiedurek 1996).

Unlike fungi employed for cellulase production in the framework of SHF, SSF, and SSCF processes, thermophilic bacteria like \textit{C. thermocellum} do not secrete individual cellulolytic enzymes to the culture medium to degrade the cellulose. These microbes have a complexed cellulase system named cellulosome that represents a multi-enzyme consortium that efficiently binds to heterogeneous insoluble cellulose-containing substrates being anchored to the bacterial cells, i.e. the cellulose hydrolysis implies the formation of a cellulose-enzyme-microbe complex. The cellulosome shows a higher efficiency in cellulose hydrolysis compared to the non-complexed cellulase systems from fungi like \textit{T. reesei}. Lynd et al. (1999, 2002) point out that most research efforts on cellulose hydrolysis are being carried out within the context of the enzymatically oriented intellectual paradigm, which focuses on cellulose hydrolysis as primarily an enzymatic rather than microbial phenomenon. In this context, the development of SHF, SSF, and SSCF processes that utilizes fungal cellulases is in the framework of this paradigm. In contrast, the development of CBP processes for bioethanol production corresponds to the microbial oriented paradigm, which considers the cellulose hydrolysis as a microbial phenomenon. As there exists no natural microorganism exhibiting the whole combination of features required for efficient CBP-based ethanol production, intensive research on genetic modification of different microbial strains is being carried out in past two decades. In the framework of the CBP, two strategies have been employed: native cellulolytic strategy and recombinant cellulolytic strategy. The main aspects of these strategies are presented in Table 16.4. Undoubtedly, ongoing research efforts on genetic and metabolic engineering will make possible the development of effective and stable strains of microorganisms to convert lignocellulosic biomass into ethanol. This fact will surely imply a qualitative improvement in the industrial production of fuel ethanol in the future.

16.3.2 Reaction–Separation Integration

Reaction–reaction integration allows the increase of process efficiency through the improvement of reaction processes. However, separation is the step where major costs are generated in process industry. Therefore, reaction–separation integration could have a significant impact on the overall biomass-to-ethanol process. The reaction–separation integration is particularly an attractive alternative for the intensification of alcoholic fermentation processes. When ethanol is removed from the culture broth, its inhibition effect on growth rate is diminished or neutralized that leads to a substantial improvement in the performance of ethanol-producing microorganisms. This improved performance may permit the increase of substrate conversion into ethanol. In particular, higher conversions make possible the utilization of concentrated culture media (with sugars content greater than 150 g/L) resulting in increased process productivities. From energy viewpoint, this type of
integration leads to the increase of ethanol concentration in the culture broth. This fact has a direct effect on distillation costs since more concentrated streams feeding the columns imply lower steam demands for the reboilers and therefore, lower energy costs.

This type of integration can be accomplished by coupling the separation unit to the fermenter (conjugated process) or combining the cultivation and the separation in the same unit (simultaneous process). For instance, vacuum chamber, stripping columns or pervaporation modules can be coupled to fermentation vessels. Alternatively, the membrane modules can be submerged in the culture broth or an extractive agent can be directly added to the cultivation medium during the fermentation process. These options for reaction–separation integration are presented in Table 16.5. Furthermore, one unit where reaction–reaction integration is applied (e.g. SSF) may be coupled to a separation unit. This represents a process with an integration of the reaction–reaction–separation type allowing a higher degree of integration.

### 16.3.3 Separation–Separation Integration

The separation–separation integration is particularly relevant for ethanol recovery and dehydration. This type of integration is accomplished by coupling two or more separation units with different mass-transfer foundations. For instance, the extractive distillation employed for ethanol dehydration combines the distillation
with the liquid–liquid extraction in order to “break” the ethanol–water azeotrope in multi-column systems. Variations to this process have been proposed like the utilization of salts (NaCl, KCl, KI, or CaCl₂) as extractive agents in a process called saline extractive distillation that involves conventional distillation and crystallization by vacuum evaporation and spray drying for salt recovery (Llano-Restrepo and Aguilar-Arias 2003; Pinto et al. 2000). On the other hand, the utilization of pervaporation modules for ethanol dehydration through their coupling to the previous distillation step is other example of separation–separation

<table>
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<th>Option</th>
<th>Fundament</th>
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<tbody>
<tr>
<td>Vacuum</td>
<td>Coupling the fermentation tank to a vacuum chamber allows ethanol removal due to its higher volatility</td>
<td>50 mm Hg; cell recycling; productivity 23–82 g/(L × h); high energy costs</td>
<td>Costa et al. (2001), Cysewski and Wilke (1977)</td>
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<tr>
<td>Gas stripping</td>
<td>Ethanol removal by absorption employing a stripping gas</td>
<td>Stripping gas: CO₂; fermenter coupled to a stripping column; productivity 8–16 g/(L × h)</td>
<td>Dale and Moelhman (2001), Taylor et al. (1996, 2000)</td>
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<tr>
<td>Membrane</td>
<td>Broth is passed across selective membrane to remove ethanol from water</td>
<td>Different types of membranes (e.g. silicate or polypropylene membranes); membrane distillation or pervaporation modules coupled to fermenter; productivity 2–48 g/(L × h); membrane fouling</td>
<td>Brandberg et al. (2005), Lee et al. (2000), (Nakamura et al. 2001), Sánchez et al. (2005)</td>
</tr>
<tr>
<td>Extractive fermentation</td>
<td>Addition of selective solvent to the broth for ethanol removal followed by decantation and recycling of aqueous phase to fermenter</td>
<td>Solvents: fatty alcohols like n-dodecanol, mixture of biocompatible solvents; in situ extraction or separate extractor coupled to fermenter; possibility of SSEF and HFMEF; productivity 1–55 g/(L × h); solvent regeneration is required</td>
<td>Kang et al. (1990), Moritz and Duff (1996), Sánchez et al. (2006)</td>
</tr>
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</table>

HFMEF hollow-fiber membrane extractive fermenter, SSEF simultaneous saccharification and extractive fermentation
introduction (Tsuyomoto et al. 1997). Different evaluations indicate that this dehydration scheme has lower operation costs than azeotropic or extractive distillation (Szikai et al. 2002; Tsuyomoto et al. 1997) but higher than adsorption using molecular sieves (Sánchez and Cardona 2012).

16.4 Conclusion

The advantages of the biofuels in general, and bioethanol in particular, are evident considering the progressive depletion of the fossil fuel reserves and the changes of the global climate generated by their use. Bioethanol is an environmentally clean source of energy with a high output-input energy ratio and represents an important commodity in the world energy market. Its production from sucrose-containing materials has attained a considerable degree of maturity but can directly affect the food safety. Ethanol from starchy materials has already affected the food safety and its environmental benefits are not clear. In contrast, biomass ethanol (a second generation biofuel) does not compete for food resources, its energy balance is favorable and the worldwide availability of lignocellulosic materials allows that almost all the countries can produce it. However, the production costs of ethanol from lignocellulosic biomass are still high. Many research groups and centers in different countries are making progress in the task of lowering production costs of lignocellulosic ethanol. Nevertheless, the challenges to overcome are still formidable.

The current pretreatment methods should be optimized considering the future commercial operation of ethanol production facilities employing biomass as feedstock. Some pretreatment technologies seem to have reached their best indicators (e.g., dilute acid pretreatment) and others show incremental improvements. New promising methods have to be tested at pilot scale and demonstration facilities (SPORL, ionic liquids) but they represent disruptive innovation techniques. The main goal is oriented to the reduction of inhibitor generation, biomass fractionation, improved sugars recovery, and enhancement of cellulose digestibility.

The cost of cellulolytic enzymes remains high despite the great progress of the enzyme-producing companies. Although the technologies developed for in situ production of enzymes have achieved some cost reductions, the relatively low specific activity of those enzymes and the binding of cellulases to other polymers like lignin represent serious challenges to be undertaken through genetic and metabolic engineering. The change of paradigm oriented to consider the cellulose hydrolysis as a microbial process will entail significant improvements toward the simplification of the overall process by highly integrated technologies such as CBP. However, the newly developed microbial strains for CBP are far from their industrial cost-effective utilization. In this way, such integrated processes like SSF and SSCF will be the preferred option in the mid-term. One important issue in the strain development is the utilization of microorganisms resistant to inhibitor. This will allow an important process simplification since the utilization of the whole
slurry of pretreated lignocellulosic biomass and the elimination of the detoxification procedures will have beneficial economic and environmental effects on the overall biomass-to-ethanol process. On the other hand, the application of reaction–separation integration has the potential to improve the fermentation performance by diminishing the end-product inhibition effect on growth rate. In this sense, new membranes technologies are being developed and other upgraded techniques for ethanol removal from culture broth as extractive fermentation could potentially represent important advances.

Undoubtedly, the multidisciplinary research efforts that combine new molecular approaches for strain development and process integration in the framework of process systems engineering, will allow expansion and commercial implementation of innovative technologies to exploit the vast resources of lignocellulosic materials available to mankind in a cost-effective and environmentally sustainable way.

References


Montoya MI, Quintero JA, Sánchez ÓJ, Cardona CA (2005) Efecto del esquema de separación de producto en la producción biotecnológica de alcohol carburante. In: II Simposio sobre Biofábricas, Medellín

Xu L, Tschirner U (2011) Improved ethanol production from various carbohydrates through anaerobic thermophilic co-culture. Bioreour Technol 102(21):10065–10071


